Role of NFAT (Nuclear Factor of Activated T Cells) Transcription Factors in Hematopoiesis

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To my grandpa,

though no longer with us, remains the compass of my life…

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1 Abstract

Understanding the transcriptional mechanisms that control hematopoiesis and the interaction between hematopoietic stem cells and the bone marrow (BM) microenvironment *in vivo* is of considerable interest.

The calcineurin-dependent transcription factor NFAT (Nuclear Factor of Activated T cells) is known as master regulator of cytokine production in T lymphocytes and therefore central for T cell-dependent immune reactions, but has also been shown to regulate a process of differentiation and tissue adaptation in various cell types.

The activation of NFAT is dependent on the calcium level within the cell. In resting cells, calcium levels are low and NFAT is cytoplasmic and inactive. A sustained increase in the internal calcium concentration within an external stimuli leads to activation of the calcium-dependent calcineurin, followed by dephosphorylation and nuclear translocation of NFAT.

We have previously shown that NFATc2, a member of the NFAT family, is expressed in $CD34^+$ hematopoietic stem cells (HSC). A mouse model harboring NFATc2 deficiency provides the opportunity for *in vivo* investigation of the role of NFATc2 in hematopoiesis.

Our recent observations showed that aged mice lacking the transcription factor NFATc2 develop peripheral blood anemia and thrombocytopenia, BM hypoplasia and extramedullary hematopoiesis in spleen and liver. The proliferation and differentiation of NFATc2-deficient hematopoietic stem cells *ex vivo*, however, was found to be intact. It remained therefore unclear whether the disturbed hematopoiesis in NFATc2-deficient mice was caused by the hematopoietic or the stroma component of the BM hematopoietic niche.

In the current study we dissected the relative contribution of hematopoietic and stroma cells to the phenotype of the NFATc2-deficent mice by transplanting immuno-magnetically purified NFATc2-deficient (KO) HSCs to lethally irradiated wild type (WT) mice, and vice versa. After a post-transplantation period of 6-8 months, peripheral blood, BM as well as spleen and liver of the transplanted animals were analyzed and compared to WT and KO mice transplanted with control cells.

Transplantation of NFATc2-deficient HSCs into WT recipients (KO→WT) induced similar hematological abnormalities as those occurring in non-transplanted KO mice or in KO mice transplanted with KO cells (KO→KO). Compared to WT mice transplanted with WT cells (WT→WT), KO→WT mice showed evidence of anemia, thrombocytopenia and a significantly reduced number of hematopoietic cells in their BM. Likewise, KO→WT mice developed clear signs of extramedullary hematopoiesis in spleen and liver, which was not the case in WT → WT control animals.

In addition to the hematopoietic abnormalities, transplantation of NFATc2-deficient HSC also induced osteogenic abnormalities such as BM sclerosis and fibrosis in WT mice. This phenomenon was rather subtle and of incomplete penetrance, but never seen in mice transplanted with WT cells.

These data demonstrate for the first time, that the NFATc2 transcription factor directly regulates the intrinsic function of hematopoietic stem cells *in vivo*. However, the transcriptional targets for NFAT in these cells are yet unknown.

In addition to hematopoietic stem cells, NFATc2 has been shown to be expressed in a lineage-specific manner during myeloid differentiation and, notably, is maintained during megakaryopoiesis while it is suppressed during the differentiation of neutrophils. Bone marrow megakaryocytes are the precursors of peripheral blood platelets and therefore constitute an integral part of primary hemostasis, thrombosis and wound healing. The biological role of NFAT in megakaryocytes is unknown.

We have recently shown that NFATc2 is not necessary for megakaryocytic differentiation. On the other hand, recent evidence suggests that NFATc2 is required for the transcription of specific megakaryocytic genes.

In this study, we showed that activation of the calcineurin/NFAT pathway in either primary megakaryocytes or CMK megakaryocytic cells forces the cells to go into apoptosis. Cell death in megakaryocytes is induced by treating the cells with the calcium ionophore ionomycin and suppressed by either the pan-caspase inhibitor zVAD or the calcineurin inhibitor cyclosporin A (CsA). Ionomycin stimulation of megakaryocytes leads to the expression of Fas Ligand (FASLG), a pro-apoptotic member of the tumor necrosis factor superfamily. Expression of FASLG was detectable as early as four hours after stimulation on the membrane of ionomycin-treated megakaryocytes, was augmented in cells stably overexpressing NFATc2, and was suppressed in cells either

pretreated with CsA or expressing the specific peptide inhibitor of NFAT, VIVIT. To investigate the physiological relevance of FASLG expression on megakaryocytes, we performed co-cultures of megakaryocytes with Fas-expressing T-lymphocytes, in which CMK cells were left either unstimulated or pre-stimulated with ionomycin and then added to Jurkat cells. The presence of ionomycin-stimulated CMK cells, but not of unstimulated cells or cells stimulated in the presence of CsA, significantly induced apoptosis in Jurkat cells. Overexpression of NFATc2 in CMK cells enhanced their potency to induce apoptosis in Jurkat cells, while cells expressing VIVIT were less effective. Apoptosis induction of Jurkat cells by stimulated CMK cells was partially blocked by the presence of either a neutralizing antibody against FASLG or an antagonistic antibody to Fas during the co-culture period, indicating involvement of the FASLG/Fas apoptosis pathway.

These results represent the first clear evidence for a biological function of the calcineurin/NFAT pathway in megakaryocytes, namely the regulation of Fas/FASLG-dependent apoptosis. Second, they underline that the biological role of megakaryocytes is not restricted to the production of proteins and other cellular structures for platelet assembly, but that this population of cells fulfills an independent regulatory function in the context of the surrounding tissue.

Finally, we have identified by RNA sequencing analysis of NFATc2-expressing and -deficient cells, the entire set of genes which is induced by NFATc2 in stimulated megakaryocytes. Functional pathway analysis suggests an involvement of NFATc2 in pro-inflammatory pathways in these cells. The significance of these findings has to be addressed in further studies.

2 Introduction

2.1 Nuclear Factor of Activated T cells

2.1.1 NFAT transcription factors

Nuclear factor of activated T cells (NFAT) is primarily known as an inducible transcription factor in activated T cells.[1] However, isolation and molecular characterization of NFAT family members revealed that, despite their names, NFAT expression is not restricted to T cells. In fact, NFAT proteins have been identified in cells of non-immune system as well as the other cells of the immune system.[2-5] Therefore, the role of NFAT proteins was investigated in the regulation of other cells and tissues outside of the immune system, including skeletal muscle and cardiac hypertrophy,[6-8] cardiac valve morphogenesis,[9-11] angiogenesis,[10, 12, 13] bone tissue including osteoblasts and osteoclasts,[14-16] chondrocytes,[17, 18] keratinocytes,[19, 20] adipocytes,[21, 22] axonal outgrowth of neurons.[23]

The family of NFAT transcription factors consists of five proteins: NFAT1 (also known as NFATc2 or NFATp), NFAT2 (also known as NFATc1 or NFATc), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATc3 or NFATx) and NFAT5 (also known as enhancer binding protein TonEBP). NFAT family members evolutionarily belong to the REL-nuclear factor-κB (REL–NF-κB) family of transcription factors.[3, 24, 25] NFAT proteins contain an amino-terminal transactivation domain (TAD), a regulatory domain (also known as the NFAT homology region, NHR), a highly conserved DNA-binding domain (also known as the Rel-homology domain, RHD) and a carboxy terminal domain (figure 2.1).[4, 24, 26] NFAT1–NFAT4 are regulated by intracellular $Ca2^{+}$ signaling,[3, 4] whereas NFAT5 is activated in response to osmotic stress.[24, 27]

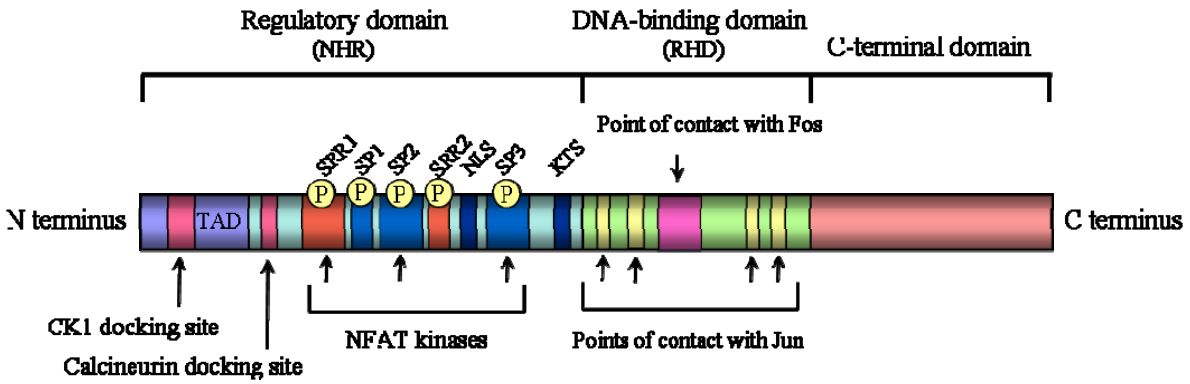


Figure 2.1. General structure of Nuclear Factor of Activated T cells (NFAT). NFAT consists of an amino-terminal regulatory domain (also NFAT homology region, NHR), a DNA-binding domain (also REL-homology domain, RHD), and a carboxy-terminal domain (C-terminal domain). The regulatory domain contains an N-terminal transactivation domain (TAD) and a docking site for casein kinase 1 (CK1). It also includes multiple serine-rich motifs (SRR1, SP1, SP2, SRR2, SP3 and KTS) and a nuclear localization sequence (NLS). The RHD also contains points of contact with Fos and Jun. (Adapted from [4, 24]).

2.1.2 Calcium-calcineurin-NFAT signaling

In the cytoplasm of resting cells, NFAT is highly phosphorylated. The activation of NFAT is initiated when cell surface receptors are coupled to Ca^{2+} release which triggers a process that leads to the activation of Ca^{2+}/calmodulin-dependent serine phosphatase calcineurin.[2, 3] Calcineurin is essential for NFAT activation by dephosphorylating multiple phosphoserines in the regulatory domain of NFAT, which contributes to the translocation of NFAT to the nucleus where it mediates specific transcriptional programs.

NFAT binds to the promoter of a diversity of inducible genes including those encoding surface receptors such as CD40L, FASLG as well as cytokines such as IL-2, IL-4, IL-5,

TNF-α and IFN-γ, which are essential for immune responses.[5, 28-30] In addition, a number of evidences suggest that NFAT proteins are involved in a wide range of biological responses,[16] however, the specific role of each NFAT family member in mediating gene transcription during cell activation and differentiation is not completely understood.[31, 32]

2.1.3 Transcriptional partners of NFAT proteins

Calcineurin-NFAT signaling has been shown to interact with many other signaling pathways in the cell which indicates that NFAT proteins cooperate with different transcription factors.

Once inside the nucleus, NFAT binds to the promoter of its target genes. Since it can also function as dimeric transcription factor (except for NFAT5),[33] NFAT binds to the DNA either with another NFAT molecule (homodimer) or most commonly with other transcription factors (heterodimer).[34] There are several transcription factor families that interact with NFAT, including activator protein 1 (AP1), forkhead box P-family proteins (such as FOXP2 and FOXP3) and proteins of MAF and GATA families.[4]

In principle, this ability to interact with different transcriptional partners allows many signaling pathways to be integrated leading to the activation of cell-type specific responses depending on which combination of transcription factors is involved.[3, 4, 25]

2.1.3.1 AP-1

Activator protein 1 (AP1) transcription factors are the major transcriptional partners of NFAT during T cell activation.[35] NFAT-AP1 cooperation integrates several signaling pathways and processes, namely, calcium signaling, which is responsible for the activation of NFAT proteins; and the RAS-MAP kinase pathway, which influences the activation of Fos and Jun.[36-38] In the absence of AP1, NFAT proteins have been found to activate a different set of genes that in T cells, for instance, leads to a completely different gene expression program.[39]

2.1.4 Inhibition of NFAT activation

2.1.4.1 Endogenous inhibitors

In recent years, a number of endogenous inhibitors have been identified that control the activity of calcineurin by binding to calcineurin and blocking its phosphatase activity. These inhibitors include calcineurin-binding protein 1 (CABIN1 or CAIN),[40] A-kinase anchor protein 79 (AKAP79),[41, 42] the members of the calcipressin family (CSP, also known as modulatory calcineurin-interacting proteins, MCIP), Down syndrome critical region (DSCR) proteins or regulator of calcineurin (RCAN)[43] and Calcineurin homologous protein (CHP).[44-46] As specific NFAT inhibition is considered to be of importance, considerable effort has been undertaken to design new inhibitors which would act with higher selectivity than those calcineurin inhibitors, such as VIVIT (see section 2.1.4.3).[47]

2.1.4.2 Immunosuppressive drugs

The interest in NFAT proteins increased by the observation that their activation was inhibited by immunosuppressive drugs such as cyclosporin A (CsA) and tacrolimus (FK506). These pharmacological agents are widely used in the treatment of patients undergoing transplantation in order to block NFAT activation in T cells.[24, 48, 49] CsA and FK506 inactivate the canonical NFAT pathway by targeting the endogenous cytosolic proteins, cyclophilin (Cyp) and FK506 binding proteins (FKBP) (collectively known as the immunophilins), respectively.[50-52] The CsA/FK506 immunophilin complexes then bind to the subunits of calcineurin (CnA and CnB) which ultimately leads to the disruption of calcineurin activity.[53, 54]

A major disadvantage of these agents, however, is that by inhibiting calcineurin they affect the entire downstream signaling pathways of calcineurin which may lead to undesired side effects and toxicity.[54] Thus, an alternative approach to selectively inhibit NFAT is crucial.[55]

2.1.4.3 Specific peptide inhibitor VIVIT

Due to the challenges following CsA/FK506 administration, there was an attempt to develop an NFAT-specific inhibitor of calcineurin signaling that would not affect other calcineurin substrates.

Efficient dephosphorylation of NFAT in activated cells depends on its interaction with calcineurin. The calcineurin docking site in the regulatory domain has the consensus sequence PxIxIT (where X can be any amino acid), which is highly conserved between different NFAT family members. Peptides binding to this sequence are able to block NFAT-calcineurin interaction as well as NFAT activation. A high-affinity peptide, VIVIT, was selected from a randomized peptide library and shown to effectively compete with NFAT for binding to calcineurin. Thus, VIVIT was found to be a suitable inhibitor that specifically blocks the activation of NFAT.[56-58]

2.2 Hematopoiesis

Hematopoiesis represents one of the well-defined systems of adult stem cell development where multipotent hematopoietic stem cells (HSCs) give rise to a range of morphologically distinct mature cell types, all characterized by their unique gene expression profiles.[59-61] Throughout adult life, normal hematopoiesis takes place in the bone marrow (BM). Schofield demonstrated for the first time that the stem cell fate is determined by the surrounding microenvironment.[62] At present, these stem cell microenvironments are referred to as stem cell niches.[63, 64] Consequently, in addition to the cell intrinsic mechanisms, HSC behavior is in part controlled by exogenous factors provided by niche cells.[65] This crosstalk between HSCs and the niche has been found to be triggered by a variety of potential regulators including chemokines, cytokines and growth factors originating from the cells itself or other type of cells, as well as interaction with the neighboring cells and the extracellular matrix (ECM), notably, CXCL12 (SDF-1)/CXCR4 and jagged-1/notch signaling.[66-68]

Gene expression induced by extrinsic signals works together with the intrinsic main differentiation program.[65] A large repertoire of molecules ranging from cell surface receptors to signal transduction molecules and transcription factors are recognized as intrinsic regulators. Because of their essential roles in the development, expansion and maintenance of HSCs, transcription factors have attracted major attention among intrinsic factors.[69] Identification and functional characterization of specific transcription factors will not only advance our understanding of normal regulation of hematopoiesis but also widen our prospect of how transcriptional regulation leads to diseases and cancer.

To date, a number of transcription factors have been identified which specify whether HSCs remain in an undifferentiated state or differentiate along myeloid, lymphoid, or erythro-megakaryocytic lineages.[70] For example, the transcription factors Runx1 and Scl (Tal1) were found to be important to retain repopulating activity and multipotency of HSC, whereas transcription factors like Sfpi1 (Pu.1) and GATA-1 may determine the development and lineage specification of HSCs.[64, 71]

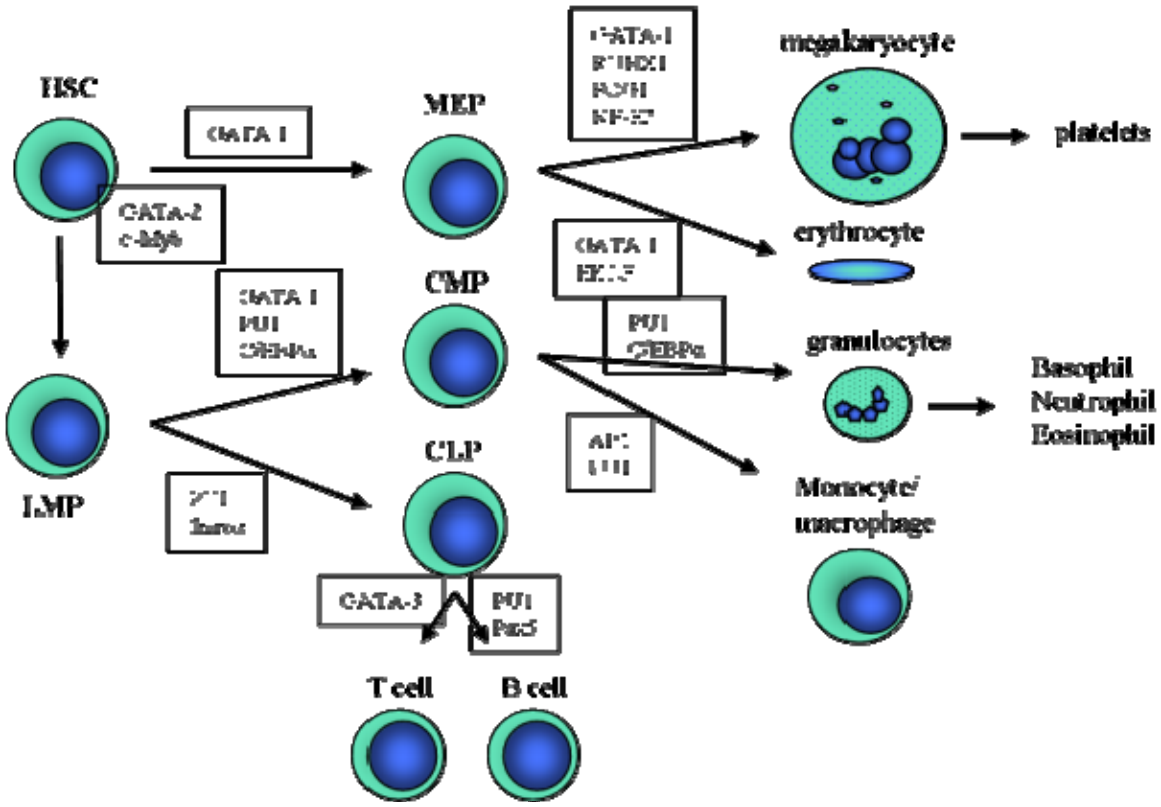


Figure 2.2. A model representing transcriptional regulation of hematopoiesis and hematopoietic lineage commitment. HSC, hematopoietic stem cell; LMP, lymphoid-myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor.[72-75]

2.2.1 NFAT in hematopoiesis

The regulated expression of lineage-specific genes during hematopoiesis is essential for the proliferation and differentiation of hematopoietic cells.[76] A wide variety of transcription factors (as mentioned above) has been identified as being critical for HSC regulation. Boer et al. suggested that NFAT is the regulator of various cytokines, which are involved in the regulation of hematopoietic cells, e.g. IL-4, IL-3, IL-5, IL-13 and granulocyte-macrophage colony stimulating factor (GM-CSF).[77]

In a study of Kiani et al, the gene expression pattern of each NFAT family member in $CD34^+$ hematopoietic stem cells and during their lineage commitment to various hematologic cell types (i.e., eosinophil, neutrophil, erythroid, or megakaryocytic lineages) was established. Subsequent results showed that NFAT expression follows a lineage- and partly also family member-specific pattern during myeloid differentiation,[78, 79] showing that NFATc2 protein is not expressed in mature neutrophils and monocytes. Eosinophils express NFATc1, c2 and c3,[80-82] whereas mature megakaryocytes only strongly express NFATc2 and NFATc4.[79] In contrast, monocytes are found to be devoid of NFAT expression.[74]

Furthermore, it is known from recent studies that while the calcineurin/NFAT signaling pathway is crucial for many aspects of vertebrate development, such as the regulation of lymphocyte development, it is not essential for the development of myeloid lineages.[83] In a study by Bauer et al, in which the role of NFATc2 in hematopoiesis was investigated, it has been shown that the differentiation potential of HSCs is preserved in the absence of NFATc2. Furthermore, it was shown in the same study that NFAT activation was not required for megakaryocytic differentiation in *ex vivo* cultures of normal HSCs.[84]

Since the expression of selected NFAT family members (including NFATc2) is found to be maintained or even upregulated during megakaryopoiesis, a major part of this study was assigned to study the role of NFAT in megakaryocytes.

2.2.2 HSC transplantation: A tool to investigate the regulation of hematopoiesis

Hematopoiesis occurs in the BM and in close contact with BM stroma cells.[63, 85, 86] A number of interactions and signaling pathways maintain the intimate association of HSCs and marrow stroma cells. An increasing number of studies is focused on the influence of the niche on viability, proliferation and differentiation of HSCs. Yet, there is only little evidence that HSCs participate in the development of the niche.[87] Because HSCs are pluripotent they have the capability of self-renewal as well as giving rise to all hematopoietic lineages.[88]

Hematopoietic stem cell transplantation (HSCT) is a medical approach used in conjugation with high dose chemotherapy and/or irradiation for the treatment of aberrant hematopoiesis resulting from intrinsic marrow defects.[89, 90] This may emerge from a lack or malfunction of either extrinsic or intrinsic factors involved in hematopoiesis (for details see section 2.2). Therefore, HSC transplantation experiments are an adequate approach to fully explore HSC functions and interactions *in vivo*.[91, 92] The standard approach in both patients and experimental animals is to inject isolated HSCs intravenously.[93] In this process, HSCs find their way to the host's BM niche via a mechanism referred to as 'homing'.[94] Transplantation chimera studies in transgenic models, in which either the host or the donor harbor a genetic defect, may facilitate the characterization of the differential contribution of HSCs and stroma cells to a given phenotype.[84]

2.3 Mouse model of NFATc2 deficiency

Recent investigations on NFAT-deficient mice suggest that the involvement of NFAT in the regulation of cellular physiology such as proliferation, cell growth and apoptosis is much broader than previously thought.[95-97] Although, NFAT proteins are considered to have redundant functions in the cell, recent studies on mice lacking NFAT family members suggest that they play distinct roles in the regulation of cell activities.[31, 32, 98]
NFATc2$^{-/-}$ mice display a lymphocyte hyperproliferative phenotype with an increase in the size of peripheral lymphoid organs, typically accompanied by a consistently enhanced T helper type 2 immune response, a reduction in programmed cell death and an increased cell cycle rate of lymphocytes. Also, they exhibit distinct defects in

controlling the promoter of cytokines and cell surface receptors, including CD25, CD40L and FASLG.[30, 99-102] However, NFATc2$^{-/-}$ mice develop normally and do not exhibit any deficiencies in their behavioral phenotype.[31, 103]

An increasing number of evidence suggests that NFAT transcription factors are associated with gene expression in a variety of cell types. In addition to lymphocyte hyperproliferation, the absence of NFATc2 *in vivo* leads to alterations in the regulation of cell cycle, most likely due to an increase in the expression of cyclins.[31, 97] Moreover, lack of NFATc2 causes an uncontrolled growth of cartilage and skeletal muscle cells implying a suppressor role for NFATc2 in these cells.[18, 104] Others also demonstrated that NFATc2 deficiency leads to articular cartilage degradation probably due to the loss of type-II collagen (collagen-2) in articular chondrocytes.[105]

In our previous study on aged NFATc2-deficient mice (>12 months old) we reported that these mice develop anemia, thrombocytopenia, and lymphocytosis. Lack of NFATc2 in these mice leads to BM hypoplasia with a remarkable loss of blood cells of erythroid, granulocytic, and megakaryocytic lineages, while the differentiation potential of their progenitors remains intact. Moreover, as reflected by the presence of bone trabeculae as well as reticular fibers in the marrow cavity, these mice display osteomyelosclerosis (in some cases osteomyelofibrosis or myelofibrosis). Also, the spleen of NFATc2$^{-/-}$ mice is enlarged and harbors extramedullary hematopoiesis. Consequently, mice with a deficiency in NFATc2 are found to be an appropriate model to study the role of NFATc2 in hematopoiesis and bone remodeling in adult organisms.[84, 106]

2.4 Megakaryocytes and megakaryopoiesis

Megakaryocytes (MKs), a small population (<0.5%) of BM cells, arise from hematopoietic stem cells and are well-known to fulfil the major function of producing

blood platelets. They therefore represent an integral part of primary hemostasis, thrombosis and wound healing.[107, 108] MKs have been characterized as the largest cell of the BM with a multilobed and polyploid nucleus.

MKs undergo a differentiation process, known as "megakaryopoiesis", in which multipotent HSCs convert to committed megakaryocyte-erythroid precursors (MEP in figure 2.2),[75] colony forming unit megakaryocytes (CFU-MK) and immature MKs or megakaryoblasts, respectively. CFU-MK are considered the first stage of megakaryopoiesis that shows distinct megakaryocyte-lineage surface markers.[109] Further maturation is followed by cell enlargement, membrane demarcation and endomitosis which results in polyploid MKs that no longer proliferate. Polyploidization is a unique hallmark of MKs as a prerequisite for proplatelet formation and ultimately platelet production.[110-112] Among a number of cytokines which mediate the process of megakaryopoiesis, thrombopoietin (TPO) has been demonstrated as the major physiological factor for megakaryocyte lineage development.[113-118] In spite of TPO which is essential for the whole process of megakaryopoiesis, other cytokines including interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF) and GM-CSF act only on early stages of megakaryopoiesis.[119, 120]

Increasing evidence suggests that MKs may serve an additional, cell-intrinsic function that extends beyond their role as platelet producers. In addition to their main location (BM), MKs can also be found in spleen, lung, liver, cord blood (CB) and mobilized peripheral blood (MPB) under certain conditions of stress, inflammation and organ damage.[121-124] MKs dysfunction has been implicated in the induction of several myeloid disorders such as myelodysplastic syndrome (MDS) and chronic myelogenous leukemia in which the number of small hypolobulated MKs is increased in the BM. Excess production of cytokines and growth factors (namely TGF-ß) by MKs has been shown to contribute to diseases such as idiopathic myelofibrosis (IMF).[125] Therefore, understanding the mechanisms regulating the physiology and pathophysiology aspects of MKs is of importance.

MKs are also known to express a number of genes with established immunomodulatory properties (e.g. TNF-α, TRAIL, CD154, FASLG), which they pass on to platelets.[126, 127] Whether expression of these membrane proteins is of intrinsic biological significance for these cells, however, is still under investigation.

2.4.1 Transcriptional regulation of megakaryopoiesis

Development and differentiation of MKs and production of platelets –megakaryopoiesis and thrombopoiesis, respectively– are controlled by molecular pathways. Like other blood cells, MKs develop from a common BM hematopoietic stem cell. A wide variety of growth factors and cytokines were identified to regulate megakaryopoiesis. In addition, a number of transcription factors are enriched in MKs.[128] The Ets-gene family including Fli-1 and Ets-1 has been reported as a positive regulator of megakaryopoiesis.[129] The activation of Fli-1 results in increased expression of megakaryocyte-specific genes, namely genes related to megakaryocytic phenotype markers such as CD41 and CD61.[112] Fli-1 deficiency in mice results in impaired megakaryopoiesis and thrombocytopenia.[128, 130] Fli-1 is involved in the interaction of GATA-1 and friend of GATA-1 (FOG-1), two transcription factors which are important for many stages of megakaryopoiesis.[131-133] GATA-1 knockout mice harbor thrombocytopenia and a delay in megakaryocytic development.[134] In other studies, GATA-1 has been introduced as a negative regulator of cell proliferation in early megakaryopoiesis.[135, 136] Mice lacking FOG-1 also show an absence of MK progenitors.[137] Humans with GATA-1 mutations that prevent interaction with its cofactor FOG-1 are severely thrombocytopenic.[138] TEL, also belonging to the Ets family of transcription factors, is a regulator of hematopoiesis. Because it can act as an inhibitor of Fli-1, its absence leads to impairment of MK maturation.[139] The transcription factor nuclear factor-erythroid 2 (NF-E2) has been widely studied and shown to regulate the expression of genes mediating the terminal stage of megakaryopoiesis as well as of thrombopoiesis including ß-tubulin and thromboxane synthase. NF-E2 knockout mice exhibit profound thrombocytopenia due to dysfunctional MKs (reviewed in Schulze and Shivdazani 2005).[128, 140] Runt-related transcription factor 1 (RUNX-1) also functions as a regulator of the later stages of megakaryopoiesis as its absence results in the reduction of polyploidization and platelet number, however, RUNX-1 target genes in MKs still remain to be identified.[141]

2.4.2 Transcriptional regulation by NFAT in megakaryocytes

As described in section 2.3, aged mice lacking NFATc2 harbor thrombocytopenia. In addition, MKs have been implicated to have a primary role in myelofibrosis, which is observed in aged NFATc2$^{-/-}$ mice. The investigation by Kiani *et al.* demonstrated for the first time that the expression of NFAT family member c2 is maintained or even upregulated during megakaryopoiesis.[79] Subsequent studies suggested that calcineurin/NFAT activation is dispensable for effective megakaryopoiesis, but contributes to MK function by regulating the expression of a number of megakaryocytic genes e.g. FASLG, RCAN1 and CD40LG.[79, 126, 142, 143] FASLG, a known NFATc2 target in T lymphocytes, was established as the first NFATc2 target gene in MKs at the mRNA level.[79, 143] However, the biological relevance of these findings remained unknown.

2.4.2.1 Fas Ligand

FASLG, a proapoptotic member of the tumor necrosis factor superfamily, has been established as an NFAT target in T cells.[28, 144, 145] The membrane-bound FASLG is cleaved by an external matrix metalloproteinase (namely, MMP-7) and generates the soluble version (sFASLG), which is found to be less efficient in inducing apoptosis in target cells.[146] Expression of functional FASLG by activated T cells and natural killer (NK) cells plays a key role in eliminating T cell populations following antigenic stimulation and clonal proliferation.[147, 148] On the other hand, FASLG expression by cells of immune-privileged tissues, such as testis and the eye, prevents the infiltration of inflammatory leucocytes.[149]

2.4.2.2 Fas/Fas Ligand-induced apoptosis

FASLG ligation leads to trimerization of its receptor (Fas) on target cells, which results in the activation of a death signaling cascade and the induction of apoptosis in Fas-bearing cells.[150] Fas is constitutively expressed in most cell types (notably on

hematopoietic cells and lymphocytes),[151] but the expression of its ligand (FALSG) is more restricted.

Apoptosis, an evolutionary conserved biological process, also known as type I programmed cell death, plays a critical role in physiological and pathological processes. Apoptosis is characterized by a diverse range of structural alterations in cells, including blebbing of plasma membrane, nucleus condensation, DNA fragmentation, cleavage of some cellular proteins, cytochrome c release from mitochondria and decreased caspase activation.[152-154] Disturbance of apoptosis pathways (e.g., in death receptor and mitochondrial pathways) may result in the pathogenesis of different diseases such as cancer.[155, 156]

2.5 RNA sequencing

Gene expression analysis is a general method by which mRNA levels of a certain gene are quantified. Although microarrays are nowadays routinely used for gene expression analysis purposes, the next-generation sequencing (NGS) methods are rapidly progressing. NGS technologies that measure genome-wide transcription levels are termed as mRNA sequencing (RNA-Seq) which have clear advantages over previously applied gene expression technologies.[157-160] In fact, RNA-Seq provides much greater sensitivity and a more accurate estimation of absolute expression levels than microarrays and allows studying the events which are beyond the reach of microarrays.[161-163]

In principle, isolated total RNA is copied to complementary DNA (cDNA) and cut at random positions. Fragments with a certain size are selected for amplification with polymerase chain reaction (PCR). Next, the amplified cDNA is sequenced by NGS method which generated tens of millions of digital signals (short sequence reads) from the transcripts of the genes of interest which are then aligned to a reference genome. These digital gene counts even from a transcript with low or moderate abundance are highly

reproducible and have the potential to be used for gene expression analysis.[159, 162, 164] In order to process these intensive computational steps, however, powerful servers and bioinformatics skills are required.[160]

Although novel NGS methods in terms of procedure, technique and accuracy vary considerably in comparison to microarray technologies, the principles of experimental design are similar.[159] One approach that ensures a successful gene expression study is the application of replicates. In particular, biological replicates ("true" replicates) are desirable as they indicate the variation among biological samples. When the same biological sample is measured repeatedly (normally ≥ three times), the replicates are termed "technical" replicates. Hence, a careful design is essential to distinguish biological variation from technical variation and to draw a more confident conclusion from experiments.[165]

3 Aim of the study

The aim of the present study was to characterize the role of NFAT transcription factors in hematopoiesis with a focus on megakaryocytes.

1- NFATc2 has been shown to be expressed in $CD34^+$ hematopoietic stem cells. Previous studies on mice lacking NFATc2 demonstrated hematological and osteological abnormalities. The role of NFATc2 in hematopoiesis and the respective contribution of hematopoietic and stroma cells to the phenotype of NFATc2-deficient mice, however, remained unclear. Therefore, transplantation experiments –in which purified HSCs from WT mice were transplanted to NFATc2-deficient mice and vice versa– were performed to address this question.

2- Mice lacking NFATc2 exhibit thrombocytopenia and myelofibrosis. Both phenotypes potentially are caused by megakaryocyte dysfunction. Moreover, NFATc2 has been shown to be upregulated during megakaryocyte differentiation, though megakaryopoiesis proved to occur normally in the absence of NFATc2. Therefore, we sought to investigate whether the calcineurin/NFAT pathway is involved in the regulation of gene expression in mature megakaryocytes.

a) Fas Ligand, a proapoptotic member of the TNF-superfamily, has been previously identified by our group as an NFATc2 transcriptional target in megakaryocytes on the mRNA level. In this study we aimed to extend these findings and, in particular, to advance them to a functional level.

b) In order to further explore the potential function of the NFAT signaling pathway in mature megakaryocytes, the global profile of NFATc2-regulated target genes in this cell type was analyzed.

4 Materials and methods

4.1 Materials

4.1.1 Reagents, stimulators, inhibitors and growth factors

Name	Company/location
Alizarin red	Sigma, St. Louis, MO, USA
L-Ascorbate	Sigma, St. Louis, MO, USA
ß-Glycerol phosphate	Sigma, St. Louis, MO, USA
Bovine serum albumin (BSA)	Sigma, St. Louis, MO, USA
Cellgro, serum-free medium	CellGenix, Freiburg, Germany
Cetylpyridinium chloride	Sigma, St. Louis, MO, USA
Collagen solution	StemCell Technologies, Vancouver, Canada
Collagenase type 1	Worthington, NJ, USA
Cyclosporin (CsA)	Sigma, Deisenhofen, Germany
DAPI (4',6-diamidino-2-phenylindole)	Sigma, St. Louis, MO, USA
DMEM	GIBCO, Darmstadt, Germany
Fetal calf serum (FCS)	GIBCO, Darmstadt, Germany
Hybond-ECL Nitrocellulose	Amersham Pharmacia Biotech, Little Chalfont, UK
Interleukin-3 (IL-3)	Strathmann Biotec AG, Hamburg, Germany
Interleukin-6 (IL-6)	Strathmann Biotec AG, Hamburg, Germany
Ionomycin	Calbiochem/Merck Biosciences, Bad Soden, Germany
Matrix metalloproteinase inhibitor	Sigma, Deisenhofen, Germany
Merckoglas	Merck KGaA, Darmstadt, Germany
Megacult-C serum free medium (M4960)	StemCell Technologies, Vancouver,

	Canada
Methylcellulose-based semisolid medium (MethoCult GF M3434)	StemCell Technologies, Vancouver, Canada
Nonidet P- 40	Roche, Mannheim, Germany
Osteosoft	Merck, Darmstadt, Germany
Paraformaldehyde (PFA)	Merck, Darmstadt, Germany
Penicillin/Streptomycin (pen/strep)	PAA Laboratories, Pasching, Austria
Phorbol 12-myristate 13-acetate (PMA)	Sigma, Deisenhofen, Germany
Phosphate buffered saline (PBS) powder	GIBCO, Darmstadt, Germany
Phosphate buffered saline (PBS) solution	PAA laboratories, Pasching, Austria
ProLong® gold anti-fade	Life technologies GmbH, Darmstadt, Germany
Roti-Load1 4x loading buffer	Roth, Karlsruhe, Germany
RPMI-1640	Invitrogen GmbH, Karlsruhe, Germany
Sodium azide (NaN3)	Sigma, St. Louis, MO, USA
Thrombopoietin (TPO)	Strathmann Biotec AG, Hamburg, Germany
Trizol	Invitrogen, Darmstadt, Germany
Tween20	SERVA, Heidelberg, Germany

4.1.2 Antibodies

Name	Isotype	Company/location
Anti-human		
Fas (clone ZB4)	mouse IgG_1 (antagonist)	Millipore ,Temecula, CA, USA
Fas (clone CH11)	mouse (agonist)	Millipore ,Temecula, CA, USA
CD178 (clone NOK-1)	Purified mouse IgG_1	BDBiosciences ,Palo Alto, CA, USA

CD178 (clone G247-4)	Mouse IgG_1	BDBiosciences ,Palo Alto, CA, USA
ß-actin (clone AC-40)	Mouse IgG_{2a}	Sigma, Deisenhofen, Germany
CD178- biotin	Mouse IgG_1	BDBiosciences ,Palo Alto, CA, USA
CD61-FITC	Mouse IgG_1	Beckman Coulter, Immunotech , CA, USA
Anti-mouse		
CD117(cKit) -APC	Rat $IgG_{2b,k}$	eBiosciences, San Diego, CA, USA
Ly-6A/E (Sca-1) -PE	Rat $IgG_{2b,k}$	eBiosciences, San Diego, CA, USA
Isotype controls		
IgG- FITC	Mouse $IgG_{2b,k}$	eBiosciences, San Diego, CA, USA
IgG-PE	Mouse IgG_{2a}	Beckman Coulter , Immunotech , CA, USA
IgG- APC	mouse $IgG_{1,k}$	BDPharmingen, PaloAlto, CA, USA
IgG-FITC	Rat $IgG_{2a,k}$	BDPharmingen, PaloAlto, CA, USA
IgG-PE	Rat $IgG_{2a,k}$	BDPharmingen, PaloAlto, CA, USA
Secondary antibodies / Fluorophores		
Allophycocyanin (APC)		BDBiosciences ,Palo Alto, CA, USA
Horseradish peroxidase (HRP)-conjugated IgG	anti-mouse	Dako ,Hamburg, Germany
Cy-3 conjugated IgG	anti-mouse	Sigma ,Deisenhofen, Germany
Streptavidin		DAKO, Glostrup, Denmark

4.1.3 KITs

Kit name	contents	Company/location
CD34 MicroBead	• CD34 MicroBeads, human • FcR blocking reagent	Miltenyi Biotec, Bergisch Gladbach, Germany
Apoptosis detection	• annexin V-APC • 7-AAD	BD Biosciences ,Palo Alto, CA, USA

CellTrace™ CFSE cell proliferation	• CFSE (6-carboxyfluorescein succinimidyl ester) • DMSO (Dimethyl sulfoxide)	Molecular probes, Eugene, OR, USA
Cell proliferation (MTT)	• MTT; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	Roche Applied Science, Mannheim, Germany
Enhanced chemiluminescence detection (ECL and ECL plus)		Amersham GE Healthcare, Buckinhamshire, UK
Lineage cell depletion (mouse)	• biotinylated lineage Ab cocktail (CD5, CD45R, CD11b, Gr-1, Ter-119) • anti-biotin MicroBeads	Miltenyi Biotec, Bergisch Gladbach, Germany
µMACS mRNA isolation		Miltenyi Biotec, Bergisch Gladbach, Germany

4.1.4 Primers

Primer pairs for DSCR1	
Forward: CCCGACAAACAGTTCCTCAT	Reverse: CACTGGGAGTGGTGTCTGTC

4.2 Equipment

4.2.1 Instruments

Name	Company/location
AB 7500 Real-time PCR system	Applied Biosystems, Foster city, CA, USA
Bioanalyzer 2100	Agilent Technologies, Santa Clara, CA, USA

FACScalibur (fluorescence activated cell sorting)	BD Biosciences, San Jose, CA, USA
FACS Aria II	BD Biosciences, San Jose, CA, USA
Hoefer SE Migthy Small Mini vertical unit (Electrophoresis device)	Hoefer, San Francisco, CA, USA
Hoefer TE 22 Series Transpher unit (Western Blotting device)	Hoefer, San Francisco, CA, USA
Illumina HiSeq 2000	Illumina Inc, San Diego, CA, USA
IMAC S30 Video CCD camera	Nikon GmbH,Düsseldorf, Germany
NanoDrop 2000 spectrophotometer	Thermo Fisher scientific Inc., Wilmington, DE, USA
Nikon digital sight camera DS-5M	Nikon GmbH, Düsseldorf, Germany
Nikon Eclipse E600 light microscope	Nikon GmbH, Düsseldorf, Germany
Nikon Eclipse E800 fluorescence microscope	Nikon GmbH, Düsseldorf, Germany
Sysmex hematology analyzer	Sysmex, Norderstedt, Germany

4.2.2 Software

Name	Company/location
Cellquest	BD Biosciences, San Jose, CA, USA
FACSDiva (version 6.1.3)	BD Biosciences, San Jose, CA, USA
GraphPad Prism (version 4.03)	GraphPad Software, La Jolla, CA, USA
Isis software (version 5.2.20)	Metasystems GmbH, Altlussheim, Germany
NIS-Elements D Imaging (version 2.30)	Nikon GmbH, Düsseldorf, Germany
'R' software environment and programming language	The R Foundation for Statistical Computing, Vienna, Austria

4.3 Cell culture

Cell lines

The Meg-01, CMK and Jurkat cell lines were obtained from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). All cell lines were cultured in RPMI-1640 supplemented with 1% penicillin/streptomycin (pen/strep) and either 15% (CMK) or 10% (Meg-01 and Jurkat) FCS.

Isolation and differentiation of human $CD34^+$ cells into megakaryocytic cells

$CD34^+$ cells were isolated by immunomagnetic selection from peripheral blood of granulocyte colony-stimulating factor-(G-CSF-) mobilized healthy stem cell donors, using CD34 MicroBead kit, according to instructions provided by the manufacturer. The purities of the resulting $CD34^+$ populations were assessed by fluorescence-activated cell sorting (FACS) and routinely exceeded 95%. The study was approved by the Institutional Review Board of the Medical Faculty of the Dresden University of Technology, and informed consent was obtained from the donors.
Ex vivo differentiation of $CD34^+$ cells into MKs was performed as follows. Purified $CD34^+$ cells were seeded at 0.4 x 10^6/ml in Cellgro Stem Cell Growth Medium and cultured for 10-12 days in the presence of 100 ng/ml TPO (with a change of medium every 4 days) to induce megakaryocytic differentiation. The differentiation efficacy was analyzed for each experiment by FACS analysis of the lineage-specific cell surface marker CD61.

Retroviral transduction

The generation of stably transduced CMK cell lines either overexpressing NFATc2-IRES (internal ribosome entry site)-EGFP (enhanced green fluorescent protein), or expressing VIVIT-GFP or IRES-EGFP (control) as well as the corresponding retroviral vectors have been described. [79, 143] The transduced cells were sorted for EGFP expression, and EGFP expression was confirmed by FACS at regular intervals.

Stimulation of human megakaryocytic cells

Culture-derived MKs or CMK cells were seeded at 1 x 10^6/ml in Cellgro or RPMI-1640 medium and were either left unstimulated or stimulated at 37°C for the indicated time points with ionomycin (1µM). Where indicated, a 30-minutes pretreatment with CsA (1µM) prior to agonist stimulation was used to block calcineurin activation. At the end of the stimulation, the cells were harvested and directly used for further analysis.

Ex vivo differentiation of murine megakaryocytes

Bone marrow was flushed from both femora and tibiae. Bone marrow cell suspensions were passed through a 70 µm nylon mesh cell strainer to obtain single cell suspensions. Afterwards, the cells were incubated with a biotin-labeled antibody cocktail of lineage-specific antibodies (CD5, CD45R, CD11b, Gr-1 and Ter-119), followed by anti-biotin MicroBeads, according to the manufacturer's instructions (mouse lineage depletion kit). Lineage-negative enriched cells were resuspended in DMEM supplemented with 10% FCS and 100 ng/ml hTPO and incubated for 3 days at 37°C to induce megakaryocytic differentiation.

Isolation and stimulation of murine megakaryocytes

After 3 days of culture, the cell suspension containing differentiated MKs was aspirated and overlaid on a two-step BSA gradient (1.5% and 3%) with minimal agitation and were incubated for 30-45 minutes at room temperature. The cells sedimenting through the lower BSA gradient was observed by eyes (see figure 5.15). When the pellet was formed, the upper phase (almost all liquid above the formed pellet) was removed gently. The pellet was resuspended in DMEM supplemented with 10%FCS and left either unstimulated or stimulated at 37°C for 3 hours with 1 µM ionomycin.

Preparation of cytospins and May-Grünwald Giemsa staining

0.05-0.1 x 10^6 cells suspended in medium (max. 75µl) were mixed with one volume of cytospin buffer (20% BSA and 10% FCS in PBS) and spun (500 rpm for 5 minutes) onto

a Shandon Cytospin slide (Thermo Fisher Scientific GmbH, Dreieich, Germany) according to the standard cytospin protocol. Samples were air-dried and stained with standard May-Grünwald Giemsa staining and mounted in Merckoglas. Cytospins were analyzed using a Nikon 100x/1.40 objective and photographed with a Digital Sight DS-5M camera. Images were captured with the NIS-Elements D Imaging software.

4.3.1 Co-culture experiments

Co-culture of human megakaryocytic cells and Jurkat T cells for apoptosis experiments

Co-culture experiments were performed to determine the induction of apoptosis in Jurkat T cells by CMK megakaryocytic cells. CMK cells (effectors) were first pre-labeled with CFSE and then remained either untreated or were stimulated with 1 µM ionomycin in the presence or absence of CsA. After 6 hours of stimulation, the cells were washed twice with PBS to remove the stimulus. The cells were then mixed with Jurkat cells (targets) in an effector-to-target ratio (E:T) of 10:1. In selected experiments, neutralizing antibodies against Fas (ZB4) or FASLG (NOK-1) were added at a concentration of 1µg/ml and 5µg/ml, respectively, during the co-culture. After 20 hours of co-incubation, the cells were harvested, stained with annexin V/ 7AAD, and the apoptosis rate of the Jurkat cells were analyzed by flow cytometry. CFSE was used to gate out the CMK effector cells (see figure 5.14). As positive control, Jurkat cell mono-cultures were incubated with a stimulating antibody against Fas (CH-11) at a concentration of 20ng/ml in the presence or absence of ZB4.

4.4 Mice

NFATc2$^{-/-}$ and wild type mice (C57BL/6 background) were held under pathogen-free conditions at the animal facility of the Technical University of Dresden. Before sacrifice, animals were anesthetized by an intraperitoneal injection of a combination of ketamine and xylazine. All animal protocols were approved by the governmental and institutional animal care committees.

Transplantation experiments

Bone marrow was flushed from both femora and tibiae of NFATc2$^{-/-}$ and wild-type mice by means of a 23-gauge needle. Cell suspensions were passed through a 70 µm nylon mesh cell strainer to obtain single cell suspensions. Afterwards, the cells were incubated with a biotin-labeled antibody cocktail of lineage-specific monoclonal antibodies, followed by anti-biotin MicroBeads as described above, according to the manufacturer's instructions. Lineage-negative enriched cells were then labeled with Sca-1 and cKit antibodies. Lin^{-}/Sca-1^{+}/cKit^{+} (LSK) cells were isolated using FACS sorter. Thirty thousand LSK cells were resuspended in 200µl of PBS+5%FCS and then injected into retro-orbital venous sinuses of lethally irradiated (8 Gy) mice without anesthesia.

Analysis of transplanted mice

After a period of 6-8 months post-transplantation, transplanted mice were sacrificed. The peripheral blood, BM as well as spleen and liver cells of transplanted mice were collected for different hematological analysis. The peripheral blood was collected by retro-orbital bleeding. Complete blood counts were performed using Sysmex. Cytomorphological analysis of peripheral blood, BM and spleen cells were performed by assessing blood smears as well as BM and spleen cytospins stained with May-Grünwald Giemsa and confirmed by flow cytometry. Slides were examined by a light microscope (Nikon). Images were captured using Digital Sight DS-5M camera and the NIS-Elements D Imaging software.

Colony forming assays

Single cell suspensions collected from BM, spleen and liver of transplanted mice were resuspended in RPMI medium (supplemented with 10%FCS and 1% pen/strep) and mixed with methylcellulose-based semisolid (MethoCult) medium according to the instructions provided by manufacturer. Hematopoietic progenitor cells were assessed by the number of burst-forming units-erythrocytes (BFU-E), colony-forming units-granulocyte/macrophage (CFU-GM), CFU-granulocyte (CFU-G), and CFU-macrophage (CFU-M). $0.1x10^6$ BM cells, $1x10^6$ splenocytes and $1x10^6$ liver cells were cultured for 12 days in 1 ml MethoCult GF M3434 (for CFU-G, CFU-M, CFU-GM, BFU-E). The number of CFU-megakaryocytes (CFU-MK) was determined by culturing $2.2x10^6$ BM cells or $11x10^6$ splenocytes for 10 days in 1 ml Megacult medium containing collagen solution and supplemented with cytokine cocktail (50ng/ml rhTPO, 10ng/ml rhIL-3, 20ng/ml rhIL-6).

Homing assays

Bone marrow was flushed from both femora and tibiae. Cell suspensions were passed through a 70 µm nylon mesh cell strainer to obtain single cell suspensions. Afterwards, the cells were incubated with biotin-labeled antibody cocktail of lineage-specific monoclonal antibodies (CD5, CD45R, CD11b, Gr-1 and Ter-119), followed by anti-biotin MicroBeads, according to the manufacturer's instructions (mouse lineage depletion kit). Lineage-negative enriched cells were labeled with 1 mM CFSE in accordance to the manufacturer's instructions. Cells were then injected into the retro-orbital venous sinuses of 3-months-old C57BL/6 mice that had been lethally irradiated (8 Gy). Mice were then killed 20 hours after injection and the numbers of homed cells were measured in the BM through the detection of $CFSE^+$ cells by flow cytometry. The number of $CFSE^+$ cells was also analyzed in the peripheral blood as control.

4.5 Protein analysis

Western blot analysis

40 µg of protein per sample was extracted and mixed with loading buffer, then denatured at 95°C for 5 minutes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to standard procedures. For Western blotting of FASLG, 10% acrylamide gel was used. The gel was run with 20 mA constant current. The separated proteins were transferred to Hybond-ECL Nitrocellulose Membrane (75 mA/gel, 1 h), and unspecific binding sites were blocked in 5% non-fat dry milk powder and 0.1% Tween20 in PBS for 1 hour in RT on a shaking plate. Membranes were stained overnight with antibodies to FASLG (G247-4) (diluted 1:500) and ß-actin (1:4000, as loading control), followed by HRP-conjugated anti-mouse antibody (1:2000) after 3 times washing for 5 minutes with washing buffer (0.1% Tween20 in PBS) to wash away unbound antibody. Membranes were then developed using the ECL (enhanced chemiluminescence) or the ECL Plus Western Blotting detection kit according to the instructions of the kit.

Immunofluorescence

Subcellular localization of FASLG protein was analyzed by immunofluorescence staining of cytospin preparations. Culture-derived MKs were mixed with cytospin buffer and spun on round (Ø 15 mm) coverslips according to the standard cytospin protocol. The coverslips were further processed in 12-well plates starting with a paraformaldehyde (3%) fixation step. After removing the fixative reagent, MKs were washed twice with washing solution containing 0.5% Nonidet P-40 for cell permeabilization and 0.01% sodium azide (NaN3) as a preservative in PBS. After samples were blocked by 10% FCS added to washing solution, FASLG primary antibody was diluted (1:200) in blocking solution and added to the wells. 30 minutes after staining the samples were washed

twice and a Cy3-labeled secondary antibody (1:1000 diluted) was added. After washing steps, samples were stained with an anti-CD61-FITC FACS antibody (diluted 1:500) and were counterstained with DAPI (diluted 1:2500). Cytospins were then mounted in ProLong Gold anti-fade reagent and analyzed and photographed with a Nikon fluorescence microscope attached to an IMAC S30 Video CCD camera. Images were acquired and processed with the Isis software.

Flow cytometry analysis

For detection of FASLG on the cell surface, the cells were incubated with a control or a biotin-conjugated anti-FASLG monoclonal antibody for 30 minutes on ice, washed twice with PBS, and incubated on ice for 30 minutes with streptavidin (1:100 diluted). After two final washes, the cells were resuspended in PBS and analyzed on FACSCalibur Cellquest software. In apoptosis experiments, apoptotic cells were quantified by assessing the membrane phosphatidylserine exposure of the cells using double-staining with the annexin V-APC staining kit and 7AAD according to the manufacturer's instructions. Events were collected on a FACScan and analyzed by Cellquest software.

4.6 Histological analysis

Histological analysis of bone marrow

Femora of NFATc2$^{-/-}$ and wild type mice were fixed in 4% PBS-buffered formalin for 24 h and then were decalcified in Osteosoft. Following dehydration with an ascending ethanol series and xylol, the tissues were embedded in paraffin. The sections were stained with hematoxylin-eosin (H&E) and Gomori for visualization of reticular fibers. Slides were examined and imaged using a light microscope.

Acetylcholinesterase staining of megakaryocytic cells

All procedures were performed exactly according to the protocol provided by the manufacturer (Megacult-C). Briefly, the slides containing CFU-MK were fixed in cold acetone and allowed to air dry. Acetylcholinesterase substrate solution was prepared freshly (acetylthiocholiniodide was dissolved in sodium phosphate buffer, then sodium citrate, copper sulfate and potassium ferricyanide were added). Slides were incubated in the staining solution for 4 hours and then were fixed with 95% ethanol, rinsed with lukewarm water and air dried. Harris' hematoxylin counterstaining was done for 30 minutes. Slides were evaluated by light microscopy.

4.7 RNA sequencing

4.7.1 Sample preparation

Total RNA was extracted using Trizol according to the manufacturer's instructions. RNAs from 6 biological replicates from either stimulated or unstimulated samples were assessed for purity and integrity using a NanoDrop 2000 spectrophotometer and a 2100 Bioanalyzer, respectively.

Sequencing libraries were prepared at the 'Deep sequencing facility SFB 655', Dresden, Germany. Briefly, mRNA was isolated by poly-dT-enrichment using the Oligo (dT)-based µMACS mRNA isolation kit according to the manufacturer's instructions, followed by DNase treatment and mRNA purification using Agencourt RNAclean XP-beads. After first and second strand cDNA synthesis, End-pair and A-tailing of double stranded cDNA were performed according to the manufacturer's instructions. After adaptor ligation, large scale amplification of library constructs was performed. Thereafter, cDNA was size-selected on 2% agarose (E-Gel) and the libraries were quantified using Qubit dsDNA HS assay kit. Single read 50-bp sequencing was performed on Illumina HiSeq 2000.

4.7.2 Analysis of RNA sequencing results

Mapping, normalization and sequence analysis

Reads were mapped against murine reference genome and splice junction library (mm9 transcriptome) using BWA (http://bio-bwa.sourceforge.net/bwa.shtml). Counts per gene were computed based on the Ensembl 61 annotation for mm9. The counts per gene were then normalized by DESeq R package (http://bioconductor.org/packages/release/bioc/html/DESeq), based on the library size.

Differential expression (DE) analysis

To test for differential expression comparisons among four experimental conditions R statistical package DESeq was used.

Biological pathways analysis

Biological enrichment was performed by Miltenyi Biotec, GmbH using TreeRanker. "P-values" which indicate the significance of enrichment, were computed with Fisher's exact test, followed by Benjamini-Hochberg correction for multiple testing.

4.8 Statistical analysis

Results are presented as means ± standard error of the mean (SEM). Statistical significances were calculated with a *t* test and chi-square test using the GraphPad Prism software. *P*-values <0.05 were considered statistically significant.

5 Results

5.1 Role of NFATc2 in hematopoietic progenitor cells

We have previously shown that the calcineurin-dependent transcription factor NFATc2 is highly expressed in hematopoietic stem cells and regulated during myeloid commitment in a lineage-specific manner.[79, 143]

In a further recent study we showed that aged mice lacking NFATc2 develop hematological and osteological abnormalities such as anemia and thrombocytopenia, BM hypoplasia (with megakaryocytic and erythroid lineages being most affected), ossification of the BM space, as well as an increased formation of reticular fibers and extramedullary hematopoiesis in the spleen and liver.

The phenotype of these mice suggested that the lack of NFATc2 apparently affects either hematopoietic cells or cells of their microenvironment, or the interaction of these two compartments. Whether these abnormalities result from defects in the hematopoietic stem and/or progenitor cells or from BM stroma malfunctioning in this study remained open. In order to find out the relative contribution of hematopoietic and stromal cells to the phenotype of the NFATc2-deficient mice, we designed a transplantation study in which functional HSCs were isolated based on their phenotype. HSCs are characterized by the lack of expression of a number of cell surface markers which are normally found on differentiating or mature blood cells (Lin^-, lineage negative) while displaying high levels of Sca1 and c-kit.[166, 167] This population of BM cells is referred to as the LSK compartment ($Lin^-/Sca1^+/c\text{-}kit^+$) and has been shown to sufficiently contribute to reconstitution and repopulation of lethally irradiated hosts.[168]

Thus, immunomagnetically-purified NFATc2-knockout (KO) LSK cells (HSCs) were transplanted into lethally irradiated wild type (WT) mice, and vice versa. After a post-transplantation period of 6-8 months which allowed the phenotype to develop, peripheral blood, BM as well as spleen and liver of the transplanted animals were analyzed and compared to WT and KO mice transplanted with control cells (figure 5.1).

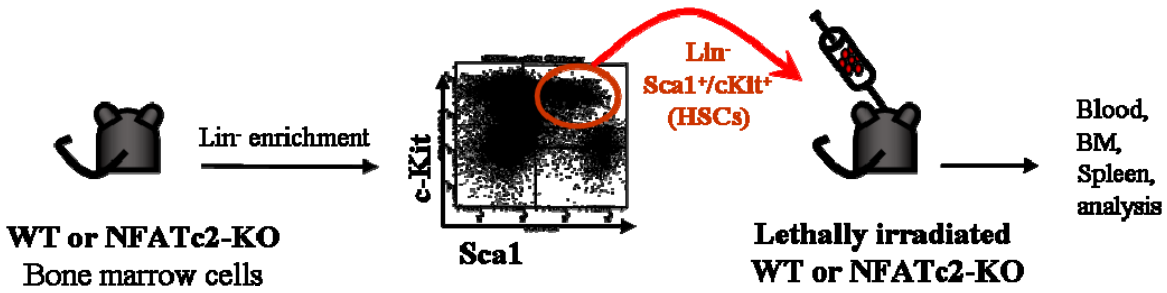


Figure 5.1. Schematic representation of the transplantation study. Bone marrow cells were flushed out of femora and tibia of WT and NFATc2$^{-/-}$ mice. Lineage positive cells were depleted with lineage depletion kit and the lineage-negative-enriched population of the cells was labelled with antibodies against hematopoietic progenitor markers (anti-Sca1-PE and anti-cKit-APC). Cells which were positive for both markers were sorted by flow cytometry and injected into lethally irradiated (8 Gy) WT or NFATc2-deficient mice. After a post-transplantation period of 6-8 months, peripheral blood, BM as well as spleen and liver of the transplanted animals were analyzed and compared to WT and KO mice transplanted with control cells.

5.1.1 Reciprocal transplantation experiments: effects on peripheral blood parameters.

In order to evaluate the impact of transplantation on hematopoiesis, we performed complete blood counts of all four groups of transplanted mice i.e., WT→WT, WT→KO (WT HSC transplanted into KO host), KO→KO and KO→WT (KO HSC transplanted into WT host). The results (shown in figure 5.2 and summarized in table 5.1) showed that WT mice transplanted by HSCs from NFATc2-deficient mice (KO→WT) were anemic with reductions in all parameters of the erythroid lineages, namely erythrocyte (RBC) number, hemoglobin (HGB) and hematocrit (HCT) when compared with those observed in non-transplanted WT mice or in WT mice transplanted with WT HSCs (WT→WT). Conversely, transplantation of WT HSCs into KO recipients (WT→KO) partially restored

the pathological blood parameters observed in KO control mice (KO→KO) (table 5.1 and figure 5.2A).

Previously, it has been reported that mice lacking NFATc2 demonstrate increased lymphoproliferative responses.[9, 101] As expected, KO→KO control mice revealed a higher number of lymphocytes in comparison to WT→WT controls. A mild lymphocytosis was also noticed in the peripheral blood of KO→WT transplanted mice (table 5.1).

Another observation in aged NFATc2-KO mice was the fact that their anemic phenotype was accompanied by reticulocytosis.[84] The pathomechanism is unclear, but may be the results of extramedullary hematopoiesis. In contrast to WT→WT mice, complete blood counts of KO→WT mice revealed an increase in the number of reticulocytes, evident by the polychromatic appearance of reticulocytes (arrows) in their peripheral blood smears, resembling KO→KO controls (figure 5.2C)

On the other hand, KO→WT mice (compared to WT→WT mice) showed a strong reduction in their platelet counts (figure 5.2 B), whereas the transplantation of WT HSCs restored the blood platelet number of NFATc2-KO recipients nearly to the WT level (table 5.1 and figure 5.2B)

Table 5.1. Hematologic parameters of transplanted mice

	WT→WT	KO→WT	KO→KO	WT→KO
RBC (10^{12}/L)	7.32±0.29	6.15±0.69	5.60±0.49	7.07±0.41
HGB (mmol/L)	6.58±0.33	5.67±0.58	4.98±0.43	6.5±0.40
HCT (%)	38.33±1.77	33.64±2.60	30.65±2.09	36.63±1.51
PLT (10^9/L)	636.42±89.51	365.42±69.24	473.62±100.11	603.33±106.24
Reticulocytes (10^9/L)	313.52±37.91	405.14±64.52	513.07±99.09	218.92±51.47
WBC (10^9/L)	2.31±0.52	2.88±0.88	3.79±0.82	2.78±0.66
Differential counts (10^8/L):				
Neutrophils	3.7	2.6	3.2	4.1
Lymphocytes	18.3	24.8	33	22.5
Monocytes	0.88	1.15	1.51	0.94
Number of mice	7	7	8	6

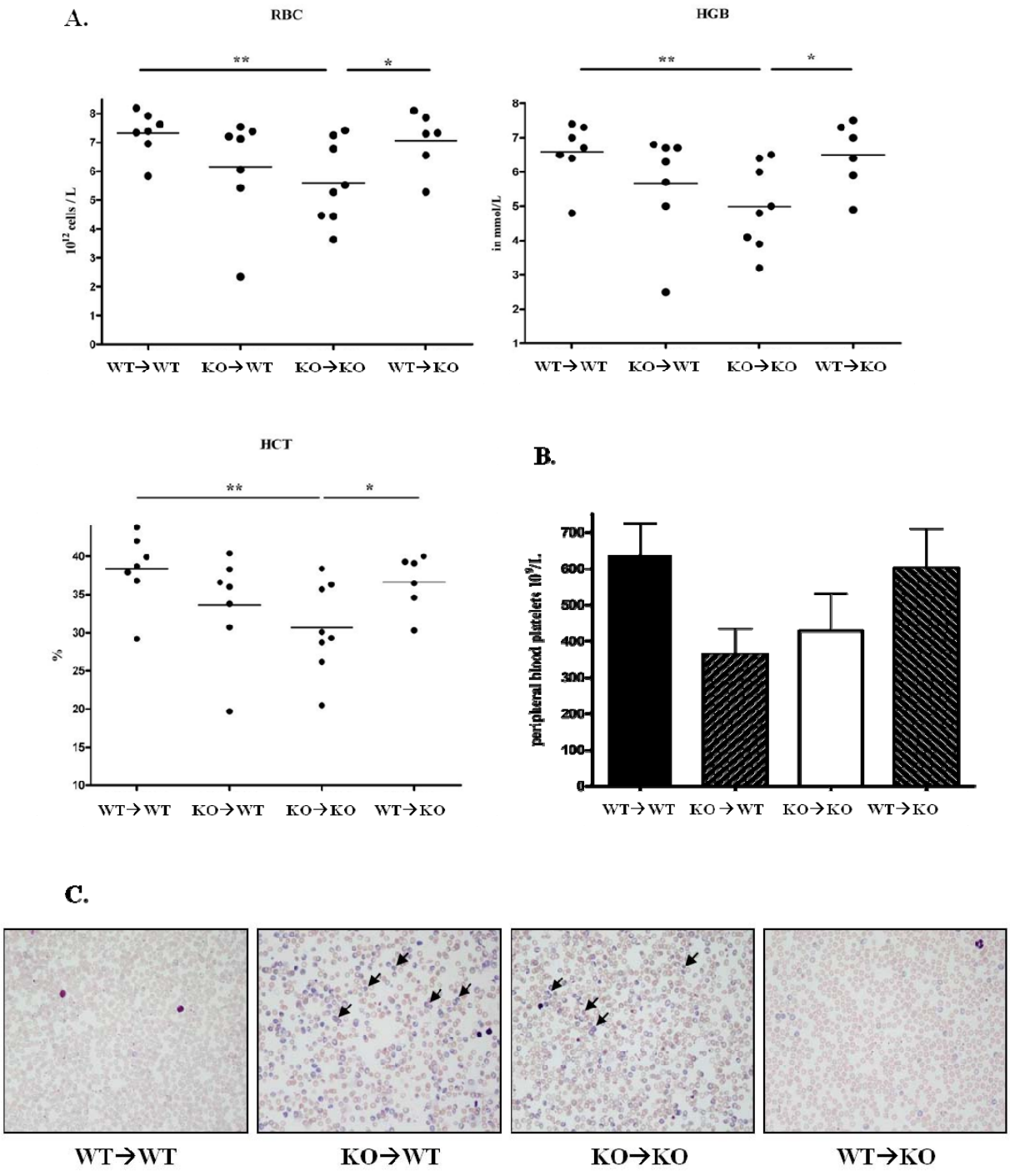


Figure 5.2. Anemia, thrombocytopenia and reticulocytosis in WT recipients transplanted with NFATc2-deficient HSCs. **(A)** Red blood cell parameters in the peripheral blood of transplanted mice. Red blood cells (RBC), hemoglobin (HGB) and hematocrit (HCT). Shown are means ±SEM. * $p<0.05$, **$p<0.01$ **(B)** Peripheral blood platelet numbers in transplanted mice. **(C)** Reticulocytes in peripheral blood smears of

transplanted mice. Arrows illustrate the polychromatic reticulocytes in the blood smears. n=7 (WT→WT), n=7 (KO→WT), n=8 (KO→KO), n=6 (WT→KO).

5.1.2 Reciprocal transplantation experiments: effects on bone marrow parameters.

Hematopoietic stem cell transplantation from NFATc2-KO donors was shown to induce anemia and thrombocytopenia in WT recipients. As the origin of hematopoiesis, we next examined the BM compartments of transplanted mice. Transplantation of NFATc2-deficient HSCs into WT recipients (KO→WT) induced BM hypoplasia (figure 5.3A) in comparison to WT→WT control animals, while in KO mice transplanted with WT HSCs (WT→KO), total BM cell counts were significantly increased compared to KO→KO control mice (figure 5.3A). Cytological differentiation of the extracted BM cells of KO→WT mice revealed that the reduction in BM cells was most pronounced in erythroid (figure 5.3B) and megakaryocytic (figure 5.3C) lineages in comparison to the control animals. In contrast, no significant effect of transplantation was detected in the absolute number of lymphocytes (figure 5.3B).

Histological analysis of the femora of WT mice transplanted by NFATc2-deficient HSCs revealed that bone trabeculae, albeit to a variable extent, had formed in the BM cavity (figure 5.3D). This observation was in sharp contrast to the BM space of all non-transplanted WT mice as well as of WT→WT control animals. To examine whether the ossification of the BM cavity in KO→WT mice was associated with further alterations in the animals' extracellular matrix, BM sections of all four types of transplanted mice were stained for the presence of reticular fibers. In addition to the presence of bone trabeculae, increased formation of reticular fibers was found in the BM space of KO→WT mice, while neither WT nor WT→ WT control mice showed any abnormalities in their BM phenotype (figure 5.3E).

In order to further characterize the BM hematopoietic compartment of the transplanted mice, we analyzed the frequency of hematopoietic progenitor cells in this organ. Colony-forming assays showed that the numbers of erythroid, granulocytic/monocytic and megakaryocytic precursors per femur were decreased in WT mice receiving NFATc2$^{-/-}$

HSCs (KO→ WT) in comparison to WT→ WT controls (figure 5.4). Thus, the reduction of hematopoietic progenitors in these mice (KO→ WT) correlated with the reduction of all myeloid cells in the BM and the hematological abnormalities in the blood.

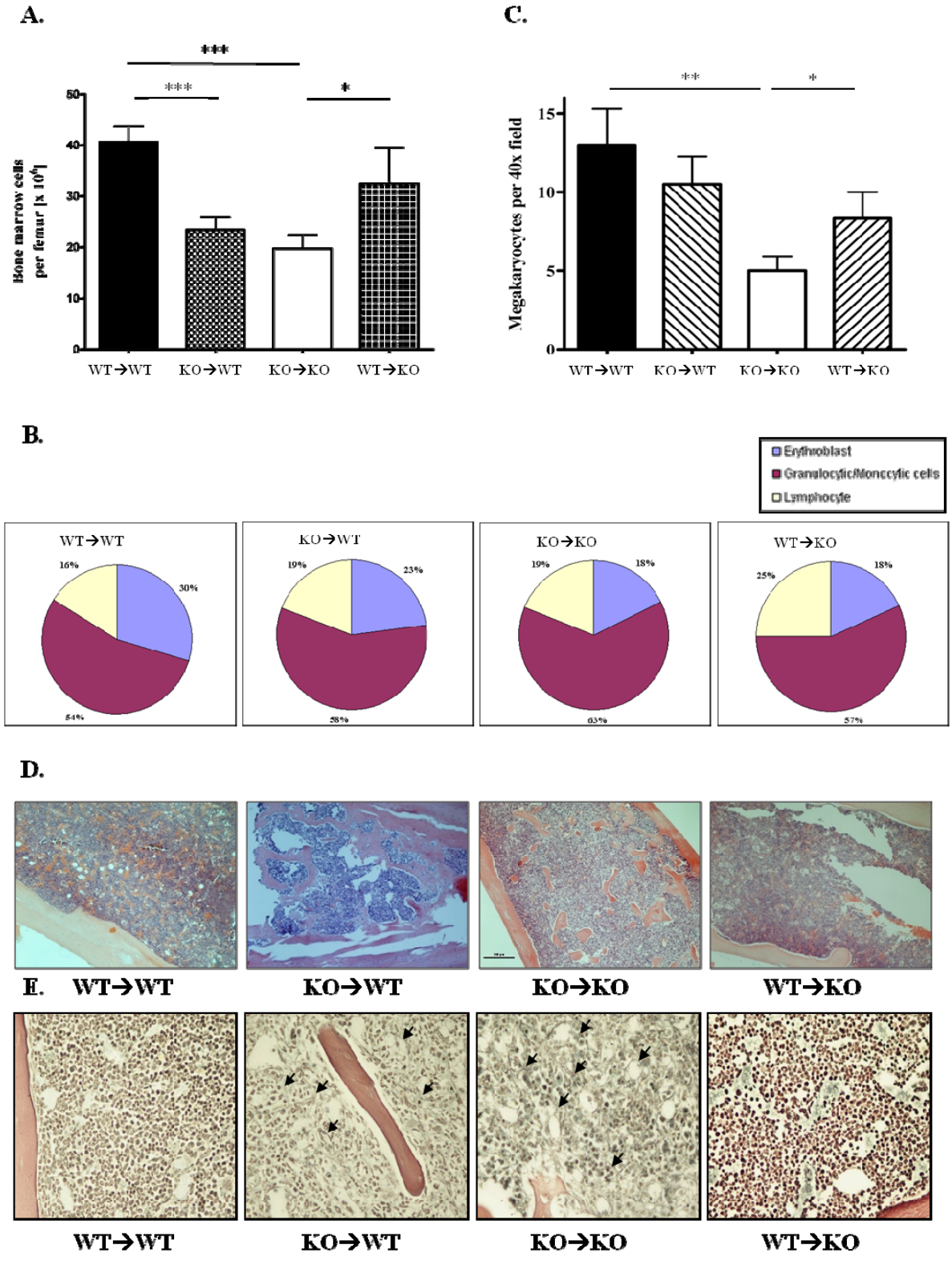


Figure 5.3. Bone marrow hypoplasia and bone abnormalities in WT mice transplanted with NFATc2-deficient HSCs. (A) Total number of BM cells per femur. Shown are means ±SEM. **(B)** Differentials of BM cells, counted on cytospin slides. Blue, purple and yellow portions of the pie chart represent erythroid, granulocytic/monocytic cells and lymphocytes, respectively. **(C)** Number of MKs per femur of transplanted mice. Shown are means ±SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. **(D, E)** Histological analysis of BM of transplanted mice. Bone marrow sections were stained with H&E (D) and Gomori (E) and analyzed by microscopy. Trabecular bone (D) as well as reticular fiber formation (stained gray in E) was observed in KO→WT mice as well as KO→KO control animals. n=7 (WT→WT), n=7 (KO→WT), n=8 (KO→KO), n=6 (WT→KO).

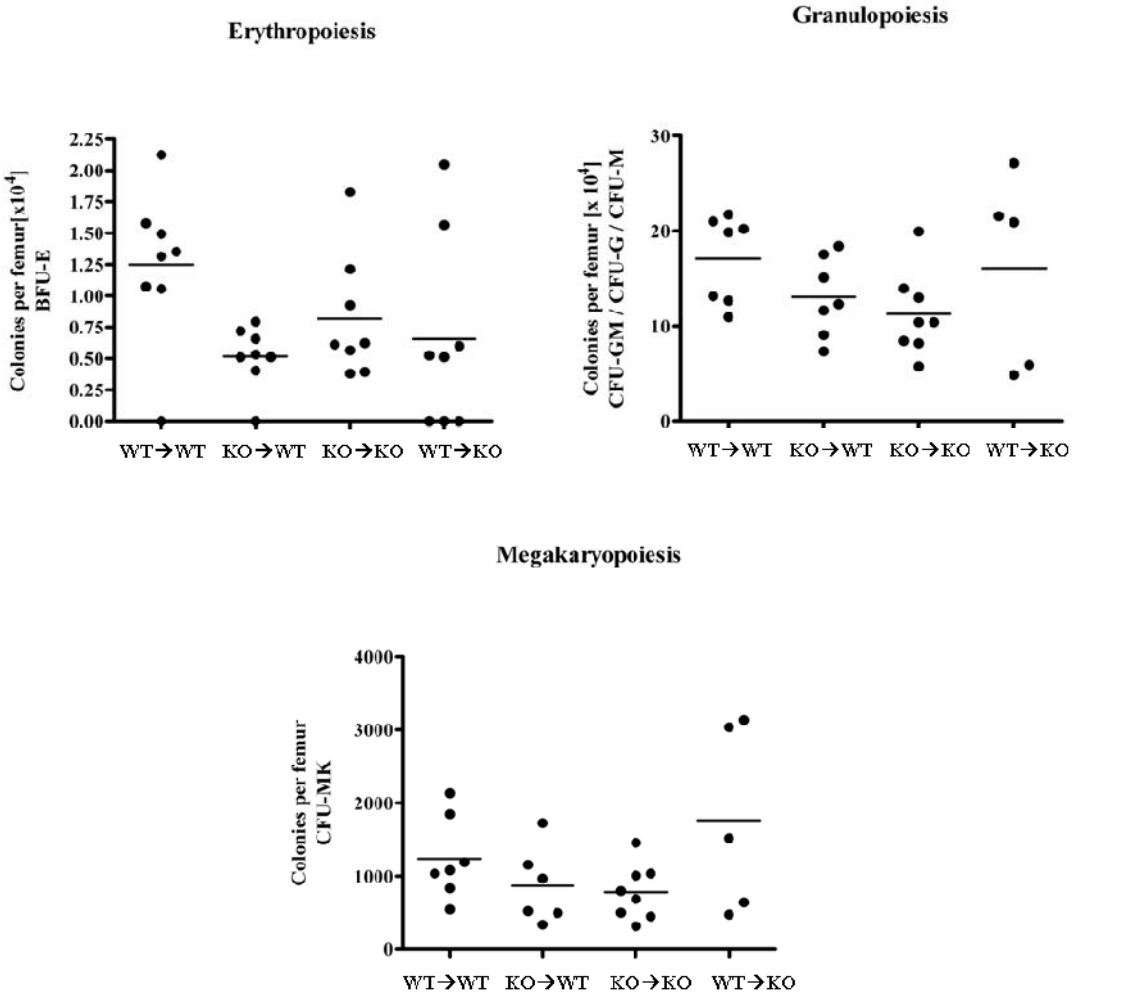


Figure 5.4. Decreased number of colony-forming cells in WT mice transplanted with NFATc2-KO HSCs. Colony-forming assays (CFU-G, CFU-M, CFU-GM, BFU-E and CFU-MK) were performed with BM cells of transplanted mice. The numbers of colonies per femur of transplanted mice are shown. n=7 (WT→WT), n=7 (KO→WT), n=8 (KO→KO), n=6 (WT→KO).

5.1.3 Reciprocal transplantation experiments: effects on extramedullary hematopoiesis.

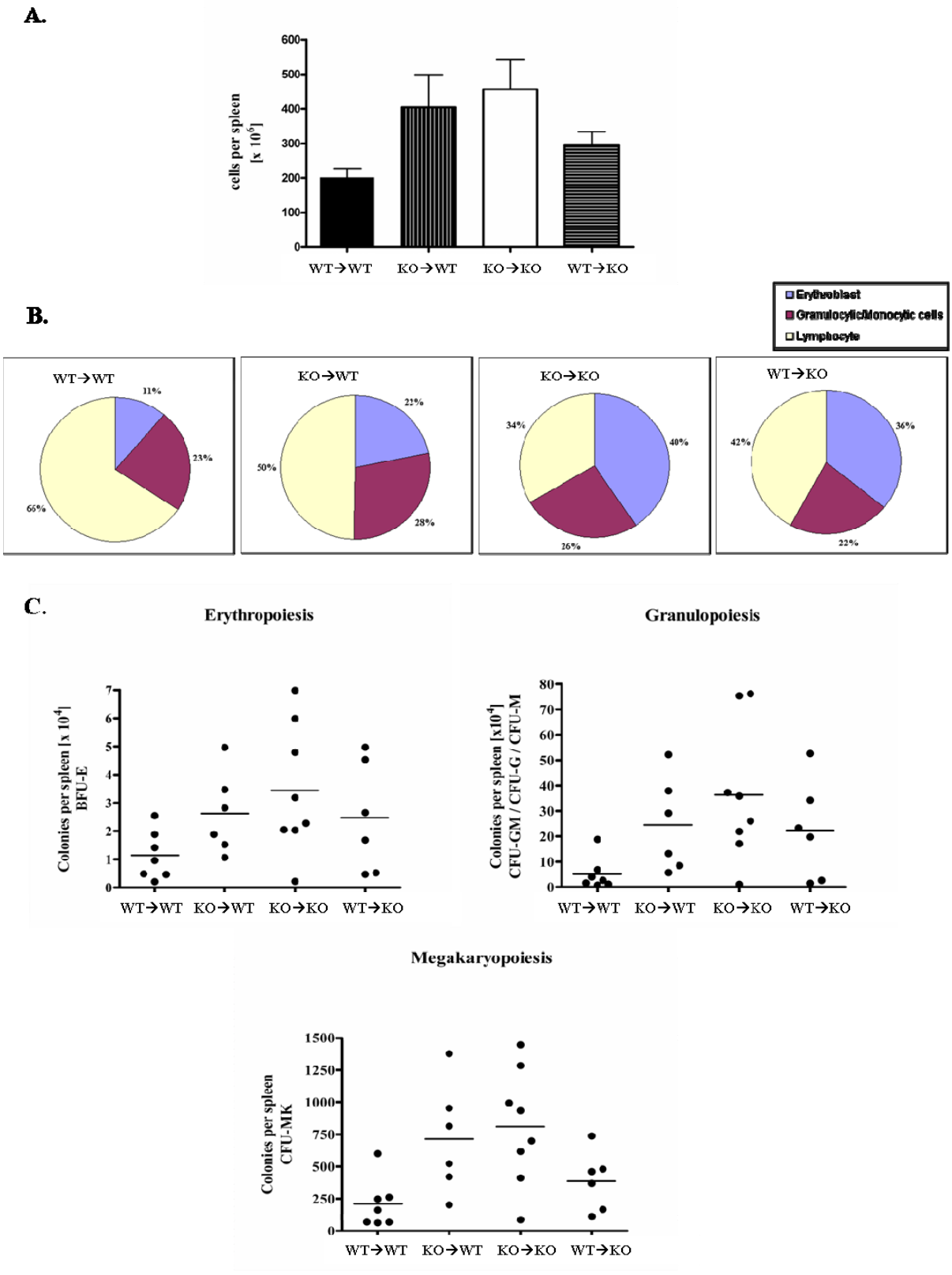
Mice lacking NFATc2 are characterized by splenomegaly and extramedullary hematopoiesis.[84] In order to analyze extramedullary hematopoiesis in our transplanted model, we next focused on the spleens and livers of WT mice transplanted with HSCs from NFATc2 deficient mice (KO→WT). Enlarged spleen as well as a drastic increase in the number of splenocytes was observed in KO→WT mice in comparison to WT→WT control animals (figure 5.5A).

Further analysis of the spleen cells of KO→WT mice showed that, while all myeloid and lymphoid had expanded numerically in comparison to control WT mice (WT→WT), the relative proportion of erythroid and megakaryocytic cells had increased over other lineages (figure 5.5B). Therefore, in KO→WT mice (as well as in KO→KO controls) the ratio of myeloid to lymphoid cells had shifted in favour of the myeloid compartment.

Our recent study on the steady-state hematopoiesis in NFATc2$^{-/-}$ mice suggested that while the frequency of hematopoietic progenitor cells is decreased in the BM of KO mice, these cells preserve their full ability to differentiate along the erythroid, granulocytic, and megakaryocytic lineages.[84] We analyzed the frequency of myeloid progenitor cells in spleen and liver of transplanted mice. Colony forming assay indicated that frequencies of granulocytic/monocytic (CFU-GM/CFU-G/CFU-M), erythroid (BFU-E) and megakaryocytic (CFU-MK) progenitors were all increased in spleens of KO→WT mice (resembling KO→KO or non-transplanted KO mice) compared to WT→WT controls (figure 5.5C). In contrast, the frequency of colonies (granulocytic/monocytic, erythroid and megakaryocytic) was reduced in WT→KO mice, which was consistent with the almost normal size of their spleens as well as the numbers of splenocytes, compared to those observed in KO→KO controls (figure 5.5C). Furthermore, colony forming assay results from the liver confirmed the presence of extramedullary hematopoiesis in KO→WT mice, as granulocytic/monocytic (CFU-GM/CFU-G/CFU-M) and erythroid (BFU-E) progenitors were increased in these mice in comparison to WT→WT control mice (figure 4.5D).

All together, these results show that most (if not all) abnormalities observed with respect to blood and BM abnormalities in aged NFATc2-deficient mice,[84] can be induced

in WT host by the transplantation of NFATc2-deficient HSCs. This includes the induction of osteomyelosclerosis/osteomyelofibrosis and extramedullary hematopoiesis.



D.

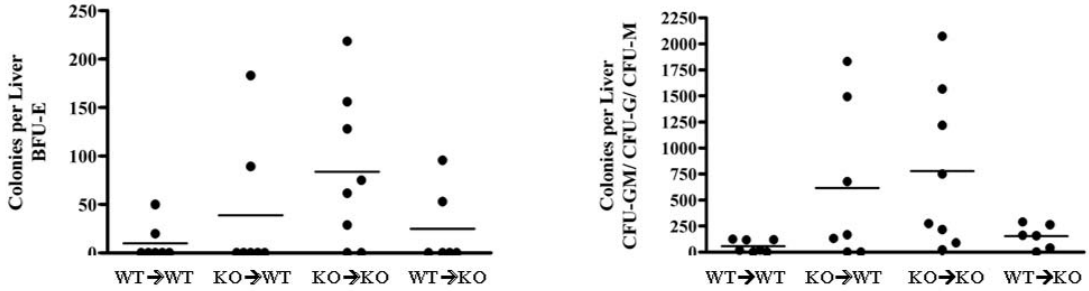


Figure 5.5. Splenomegaly and extramedullary hematopoiesis in WT recipients transplanted with NFATc2-deficient HSCs. (A) Number of splenocytes. **(B)** Differentials of spleen cells. Blue, purple and yellow portion of the pie chart represent erythroid, granulocytic/monocytic and lymphocytic cells, respectively. **(C)** Number of hematopoietic colonies (CFU-G, CFU-M, CFU-GM, BFU-E) derived from $1x10^6$ cells and CFU-MK derived from $11x10^6$ cells from spleens. **(D)** Number of colonies (CFU-G, CFU-M, CFU-GM, BFU-E) derived from $1x10^6$ cells from livers. n=7 (WT→WT), n=7 (KO→WT), n=8 (KO→KO), n=6 (WT→KO). *

5.1.4 Homing of NFATc2-deficient HSCs into the bone marrow

Homing is a process in which stem and progenitor cells migrate from the blood to BM niches.[89, 93] Next, we set out to investigate whether the reduced cellularity of the BM of KO as well as transplanted KO→KO and KO→WT mice may be the consequence of an altered homing of HSCs to the correct BM microenvironment. To examine this, purified lineage negative BM cells isolated from either WT or NFATc2$^{-/-}$ mice were pre-stained with fluorescent CFSE dye (figure 5.6 A) followed by the injection of 500,000 cells into the retro-orbital venous sinus of lethally irradiated recipient mice. Twenty hours after infusion, BM cells were collected. Fluorescent events were evaluated by high-speed FACS analysis. No difference in the number of WT and NFATc2 KO cells seeding to the

marrow space was found (figure 5.6 B). Moreover, CFSE+ cells were undetectable in the blood stream of any of the transplanted groups suggesting complete homing of the cells (figure 5.6C). As a consequence, the homing/migration capability of NFATc2-deficient hematopoietic progenitors seems to be preserved and not to be influenced by the presence or absence of NFATc2 in these cells.

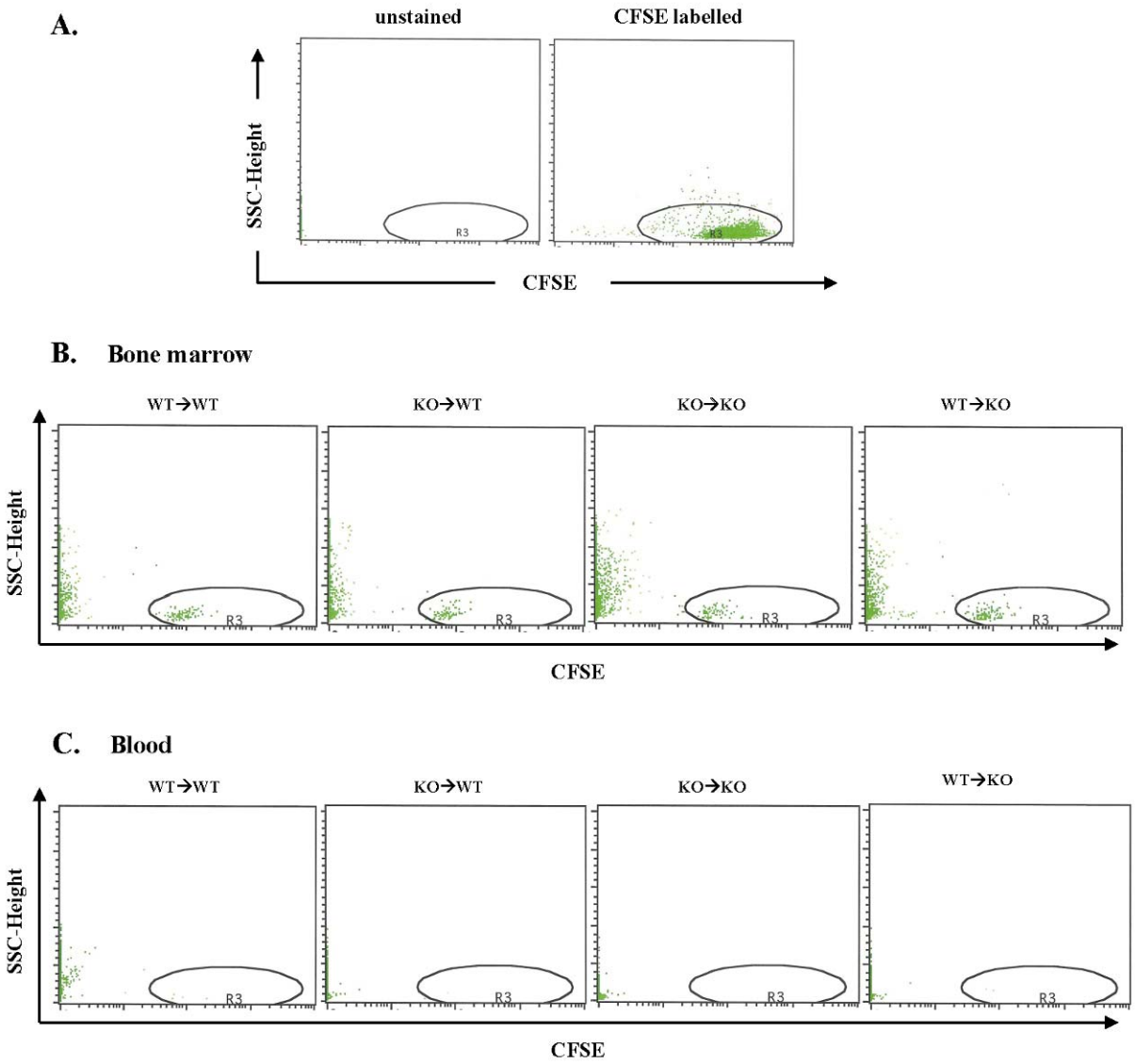


Figure 5.6. Normal homing of NFATc2-deficient hematopoietic progenitor cells to the bone marrow. (A) Lineage negative cells were isolated from the BMs of WT and KO mice. The cells were then labeled with CFSE viable dye. The efficiency of labeling was

evaluated by flow cytometry. **(B)** and **(C)** High-speed flow cytometry analysis of CFSE-positive cells in the BM (B) and peripheral blood (C) of transplanted mice 20 h after injection. The number of $CFSE^+$ cells was estimated using the same gate (here shown as R3) as shown in (A). Results are representative of at least three independent experiments.

5.2 Role of NFATc2 in mature megakaryocytes

It has been well established that, apart from T cells, NFAT is expressed in a variety of different tissues, where it regulates the expression of genes involved in cellular processes of adaptation and differentiation.[2, 3, 79]

Our group has recently established a detailed expression pattern of all NFAT genes in $CD34^+$ hematopoietic stem cells and during their differentiation into various hematological cell types.[78, 79] This phenomenon was particularly evident for NFATc2, which in megakaryocytic cells was detected throughout differentiation at a level comparable to T cells, but was rapidly downregulated during the differentiation of neutrophils.

Furthermore, NFATc2-deficient mice suffer from thrombocytopenia. However, subsequent studies showed that calcineurin/NFAT activation is dispensable for effective megakaryopoiesis. In contrast, in mature MKs, NFATc2 regulates the expression of a number of megakaryocytic genes (such as RCAN1 [DSCR1] and CD40LG [CD154]).[126, 142, 143] The biological consequence of this finding, however, remains unknown.

In the current study, therefore, we set out to define the role of NFATc2 in mature MKs further.

5.2.1 Calcium ionophore stimulation leads to megakaryocyte death

We first addressed the question of whether activation of the calcineurin/NFAT pathway affects MK viability. NFAT activation is regulated via dephosphorylation in a calcineurin-dependent manner. Since physiological agonists of NFAT signaling in MKs are unknown, we used the pharmacological agent ionomycin, a calcium ionophore which is produced by the bacterium *Streptomyces Conglobattus*.[169] Ionomycin treatment leads to a rapid rise in the intracellular levels of calcium, which activates calcineurin and subsequently leads to the activation of NFAT (i.e., its dephosphorylation and nuclear translocation).[78, 170]

To first test the viability of MKs in cell line models, megakaryocytic Meg-01 and CMK cells were left either untreated or treated for up to 48 hours with ionomycin. For cell viability measurement, the colorimetric MTT assay was used as an indirect marker. The MTT assay measures the metabolic activity of the cells which is based on the ability of active mitochondrial dehydrogenase to convert dissolved (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; MTT) to water-insoluble purple formazan crystals.
As shown in figure 5.7, treatment with ionomycin resulted in a significant decrease of the MTT signal in Meg-01 as well as in CMK cultures, suggesting reduced viability in ionomycin-treated cells.

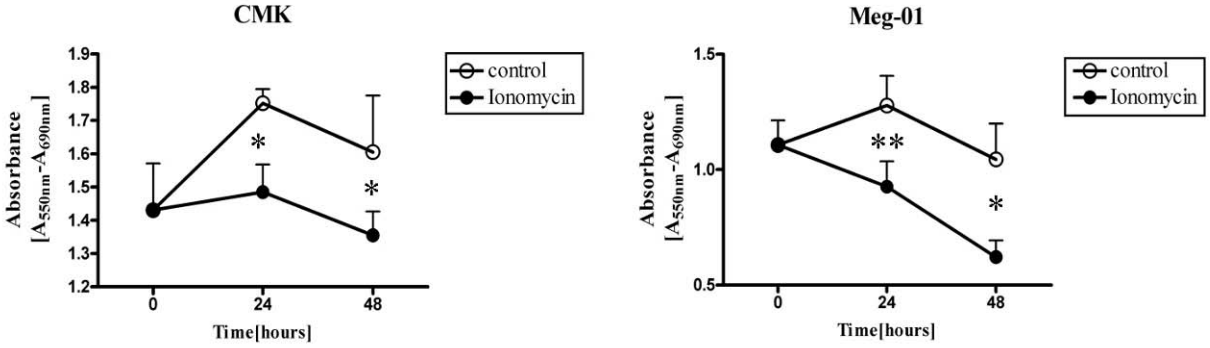


Figure 5.7. Ionomycin stimulation leads to megakaryocyte death. CMK and Meg-01 megakaryocytic cells were treated with 1 µM ionomycin for 0, 24 and 48 h, respectively. Cell viability was determined by MTT assay. Shown are means ±SEM. *p<0.05 and **p<0.01.

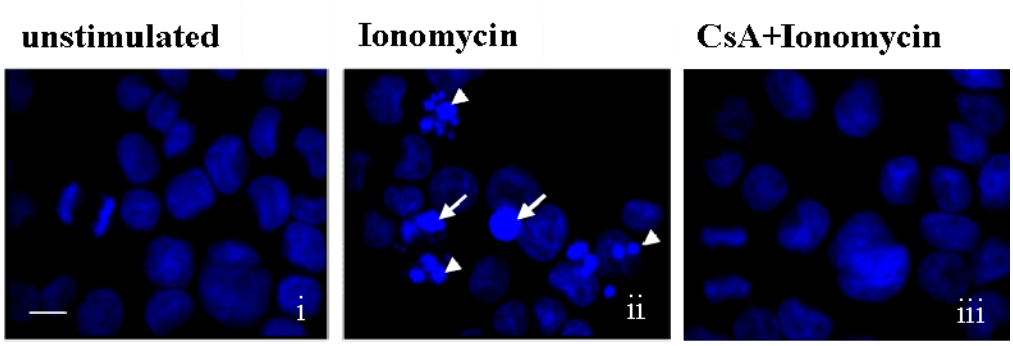
5.2.2 Activation of the calcineurin pathway induces apoptosis in megakaryocytes.

Programmed cell death –or apoptosis– is a process by which, in balance with cell proliferation, homeostasis of a cell population is maintained.[152, 171, 172]

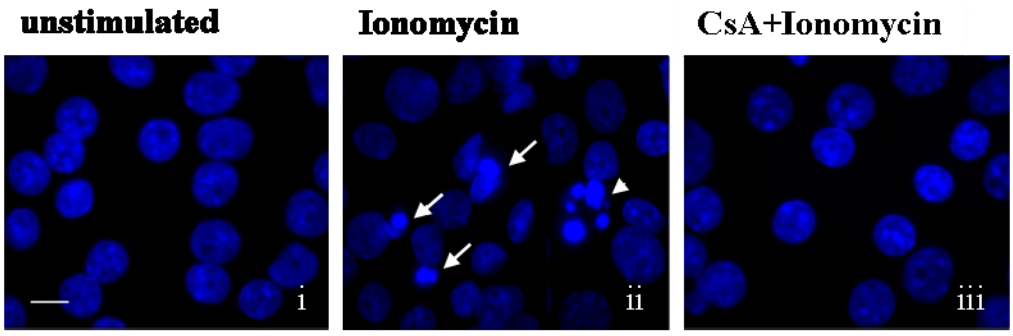
In order to assess whether the reduction of cell viability of megakaryocytic cells in response to ionomycin was due to the induction of programmed cell death, we analyzed the nuclear morphology of untreated and ionomycin-treated CMK cells by epifluorescence microscopy after nuclear staining with DAPI (figure 5.8A). The analysis was extended to primary MKs ('culture-derived megakaryocytes') (figure 5.8B), which were generated by culturing CD34+ hematopoietic progenitor cells in the presence of the megakaryocytic growth factor TPO. Ionomycin-treated nuclei of both CMK cells and culture-derived MKs presented clear features of apoptosis (figure 5.8A and B, panels ii), namely apoptotic bodies (arrow heads) as well as fragmented and condensed chromatin (arrows). In contrast, nuclei of unstimulated cells remained unremarkable (figure 5.8A and B, panels i). To better quantify the pro-apoptotic effect of ionomycin in megakaryocytic cells, we used the flow cytometric annexin V assay on untreated and ionomycin-stimulated culture-derived primary MKs. As expected, within 24 h, ionomycin treatment resulted in a significant increase in the percentage of annexin V-positive MKs, as compared to unstimulated controls (figure 5.8C, left and middle bars). These results demonstrate that ionomycin induces apoptosis in megakaryocytic cells.

To confirm that the effect of the calcium ionophore was mediated by calcineurin, the calcineurin inhibitor CsA was added to the cultures 30 min prior to the stimulation. CsA is an immunosuppressant drug used to block NFAT activation (see section 2.1.4).[46, 55, 170, 173, 174] The addition of CsA almost completely rescued the cells from ionomycin-induced apoptosis, confirming the involvement of calcineurin in this process (figure 5.8A, B, panels iii, and figure 5.8C, right bar). Together, these data demonstrate by two different methods (nuclear morphology and annexin V flow cytometry) that activation of the calcineurin pathway in MKs induces programmed cell death in these cells.

A. CMK cells



B. Culture-derived megakaryocytes



C.

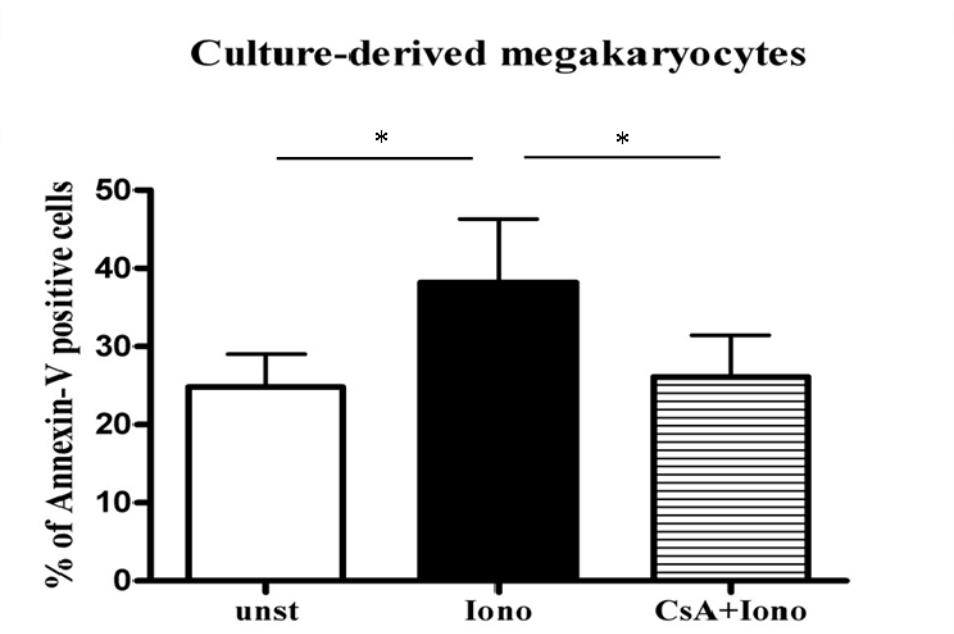


Figure 5.8. Activation of the calcineurin pathway induces apoptosis in megakaryocytes. (A) CMK cells and **(B)** culture-derived primary MKs were either left untreated or stimulated with 1μM ionomycin for 24 h in the presence or absence of the calcineurin inhibitor cyclosporin A (CsA). Morphological changes of the nuclei were analyzed by epifluorescence microscopy after staining with DAPI. Note the appearance of condensed chromatin (arrows) as well as fragmentation and apoptotic bodies (arrowheads) in the nuclei of ionomycin-treated cells indicating apoptosis of these cells. Scale bars represent 10 μm. **(C)** Culture-derived MKs were either left untreated or stimulated with ionomycin for 24 h in the presence or absence of the calcineurin inhibitor CsA, as indicated. The frequency of apoptotic cells was determined by flow cytometric analysis of annexin V-positive cells after 24 h of incubation. Statistical analysis of annexin V-positive cells of at least four independent experiments is shown. Shown are means ±SEM. * p < 0, 05.

5.2.3 Apoptosis induction in megakaryocytes is dependent on NFAT activation

Calcineurin is the key enzyme for NFAT activation, but calcineurin has also other substrates and affects various signaling pathways in addition to NFAT.[55, 57] To determine if apoptosis induced by activation of the calcineurin pathway in MKs was mediated by NFAT or by other substrates, CMK cells were retrovirally transduced with either an NFATc2-EGFP construct or with a vector encoding the peptide VIVIT-GFP, a specific inhibitor of NFAT activation.[57, 175] NFATc2 was chosen out of the NFAT family members because of its known expression in MKs.[78, 79] Cells transduced with EGFP served as control. Control cells (CMK-EGFP), cells overexpressing NFATc2 (CMK-NFATc2) and cells unable to activate any NFAT protein (CMK-VIVIT) were then either left untreated or stimulated with ionomycin in the presence or absence of CsA, and further analyzed for apoptosis induction (figure 5.9).

Microscopic examination of DAPI-stained nuclei revealed that overexpression of NFATc2 augmented, while expression of VIVIT suppressed apoptosis induction in CMK cells (figure 5.9A). The effect of NFATc2 overexpression on apoptosis was already apparent in unstimulated cells, thus mimicking the effect of ionomycin treatment (figure 5.9A, left

panel). We confirmed these results by evaluating the apoptosis rate in an annexin V assay (figure 5.9B). Ionomycin treatment significantly enhanced the apoptosis rate in both control cells and in cells overexpressing NFATc2, but remained without effect in CMK-VIVIT cells, which are unable to activate NFAT (figure 5.9B, white and black bars). In the presence of CsA, apoptosis induction by ionomycin was markedly suppressed (figure 5.9B, lined bars), re-emphasizing the role of calcineurin in this process. Altogether, these data suggest that induction of apoptosis in MKs by calcium/calcineurin is, at least partially, dependent on NFAT activation.

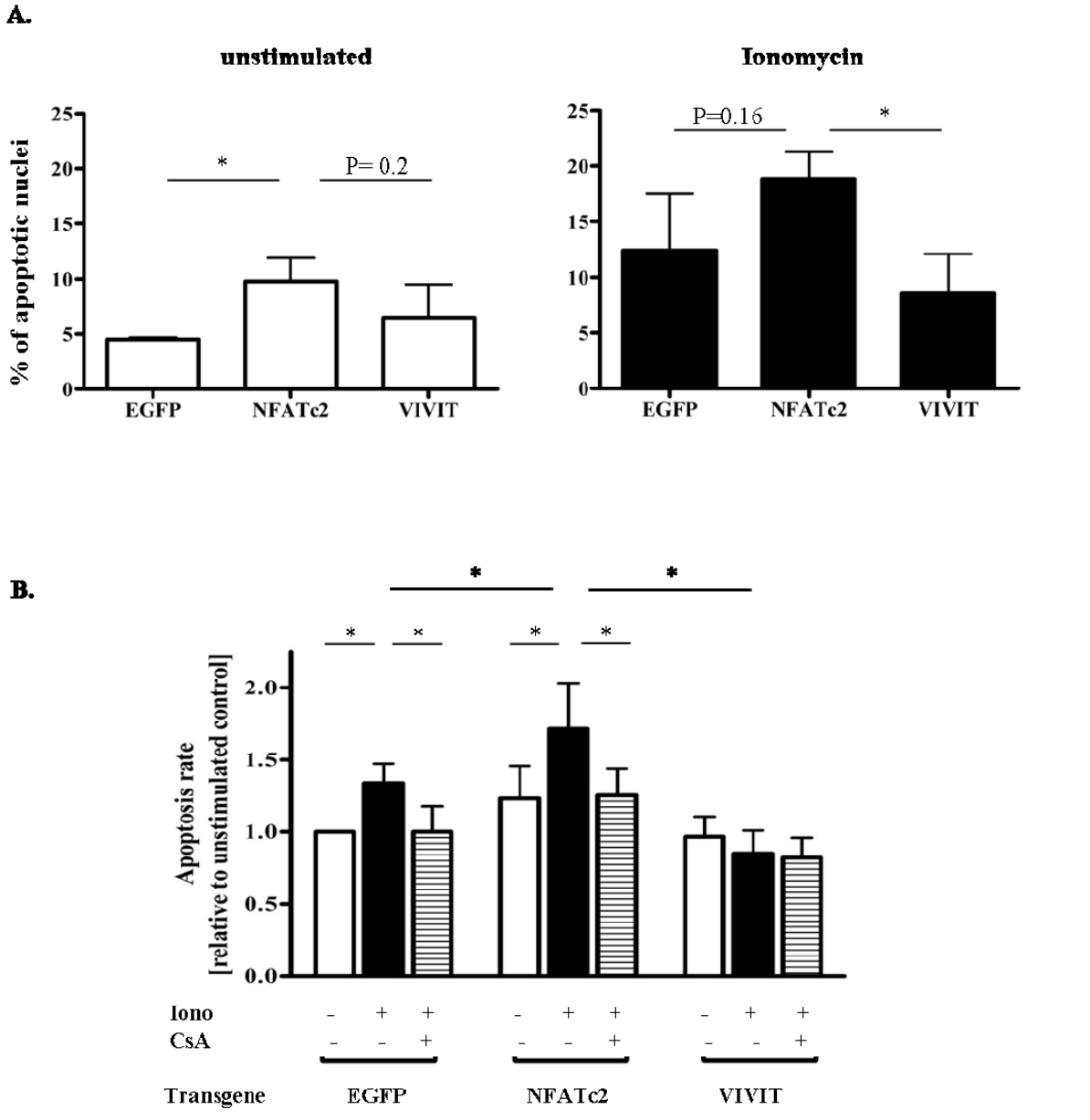


Figure 5.9. Apoptosis induction in megakaryocytes is mediated by NFAT. CMK cells were retrovirally transduced with vectors encoding either NFATc2-EGFP, the specific inhibitor of NFAT activation VIVIT, or EGFP control. The cells were sorted for uniform expression of the transgene and then either left unstimulated or stimulated with 1 µM ionomycin (Iono) for 24 h. Where indicated, 1µM cyclosporine A (CsA) was added 30 minutes prior to the stimulation. The apoptosis rate in each population was quantified by **(A)** scoring 1000 DAPI-stained nuclei for apoptotic features (compare figure 5.8A) or **(B)** by flow cytometric analysis of annexin V-APC/7AAD-labelled cells (compare figure 5.8C). Shown are means ±SEM from three independent experiments. *P<0.05.

5.2.4 NFAT induces expression of FASLG protein in megakaryocytes

Fas and its ligand (FASLG) are a pair of trans-membrane proteins critically involved in apoptosis. Fas is expressed in many types of tissues, whereas FASLG expression is more restricted.[176] FASLG expression is predominantly found in activated T cells. However, its expression is also reported in other cells inside and outside of the immune system, such as in NK cells, macrophages and Sertoli cells in the testis.[177-179] NFAT is well known to regulate gene transcription and cytokine production in T cells and various other tissues. FASLG is one of the genes established as transcriptional target of NFAT in T cells.[28, 180] Interestingly, FASLG was also established as the first NFAT target gene in MKs at the mRNA level.[79] However, the biological role of FASLG expression by NFAT in MKs, is largely unknown.

Prompted by our observation that apoptosis induction in MKs is mediated by the calcineurin/NFAT pathway (figures 5.8, 5.9), we analyzed the expression of FASLG protein in these cells. As shown in figure 5.10, FASLG was undetectable by Western blot in both untreated culture-derived primary MKs and CMK cells. Treatment with ionomycin strongly induced the expression of FASLG protein in both cell types in a time-dependent manner. Expression of FASLG protein was detectable as early as four to six h after ionomycin stimulation and followed a transient pattern, most likely because membrane-bound FASLG is shed from the cell surface by the action of matrix

metalloproteinases.[181, 182] Induction of FASLG expression by ionomycin was mediated by calcineurin, as it was completely blocked when the cells were pretreated with CsA (figure 5.10A). We then compared the expression of FASLG in CMK cells stably transduced with either EGFP (control), NFATc2 or VIVIT (figure 5.10B). After ionomycin stimulation, CMK cells overexpressing NFATc2 showed considerably higher levels of FASLG protein than control cells, while FASLG expression in CMK-VIVIT cells was reduced. These results indicate that the expression of FASLG in MKs is strongly induced by the calcium/calcineurin pathway and is dependent on the activation of NFAT.

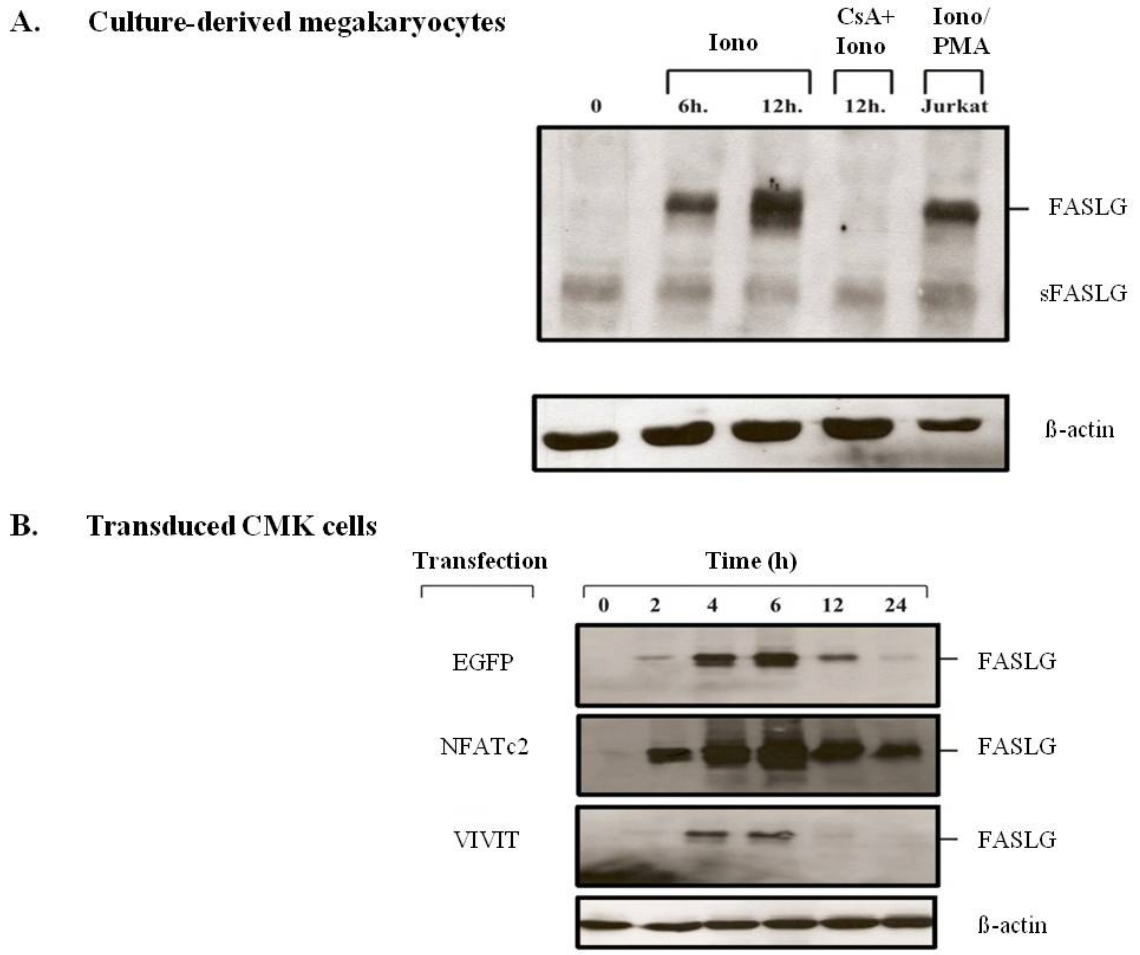


Figure 5.10. Activation of calcineurin/NFAT in megakaryocytes induces the expression of FASLG. (A) *Ex vivo* differentiated primary MKs were either left unstimulated or stimulated with 1 µM ionomycin (Iono) for 6 and 12 h in the presence

or absence of CsA. Jurkat cells stimulated with PMA/ionomycin for 4 h were used as positive control. **(B)** CMK cells stably transfected with NFATc2, VIVIT or EGFP control were either left unstimulated or stimulated for the indicated time points (hours) with 1 µM ionomycin. (A, B) Total protein isolated from the cells was analysed for FASLG expression (37 KDa) by western blotting. ß-actin was used as loading control.

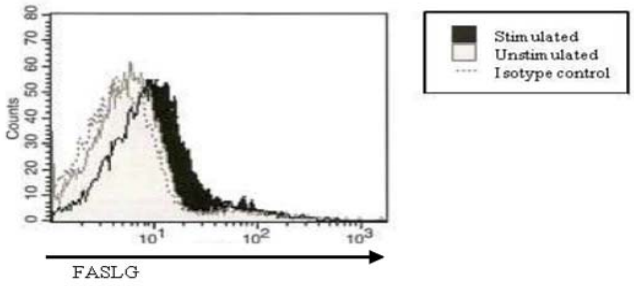
5.2.5 FASLG is located on the cell surface

Although transcription and translation of FASLG is central for its appropriate expression, increasing evidence shows that FASLG is also regulated at a post-translational level.

Cell surface FASLG cleavage by matrix metalloproteinases is one of the mechanisms that provides an efficient mechanism for limiting its apoptosis-inducing activity. Furthermore, in order to exert its pro-apoptotic effect, the FASLG protein has to be localized on the membrane of the effector cell and, for maximum Fas receptor contact and cell death-inducing potency, must be localized within membrane rafts.[182, 183] We therefore analyzed the localization of FASLG induced by ionomycin treatment in MKs by two different methods (figure 5.11). First, CMK cells were stained with an antibody against FASLG and analyzed by flow cytometry (figure 5.11A). An increase in membrane-bound FASLG was observed after stimulation with ionomycin in the presence of a metalloproteinase inhibitor (MPi), which prevents shedding of FASLG from the cell surface. Second, we investigated the cellular localization of FASLG in culture-derived primary MKs under unstimulated and stimulated conditions by fluorescence microscopy (figure 5.11B, C). An anti-CD61 antibody was used as a specific surface marker for MK differentiation. In these experiments, a preferential membrane localization of FASLG was observed in ionomycin-stimulated MKs, as opposed to diffuse staining in unstimulated cells (figure 5.11B, left panels, second and third rows). Figure 5.11C shows the distribution of the fluorescence signal in a representative indicator cell,

in which a shift of the FASLG staining (red line) from a diffuse pattern in unstimulated cells (upper panel) to a pattern localized in membrane proximity in stimulated cells (lower panel) is apparent. These results show that FASLG induced by calcineurin/NFAT activation in MKs is, at least temporarily, localized at the cell surface and therefore may be involved in the induction of apoptosis in bystander cells.

A.



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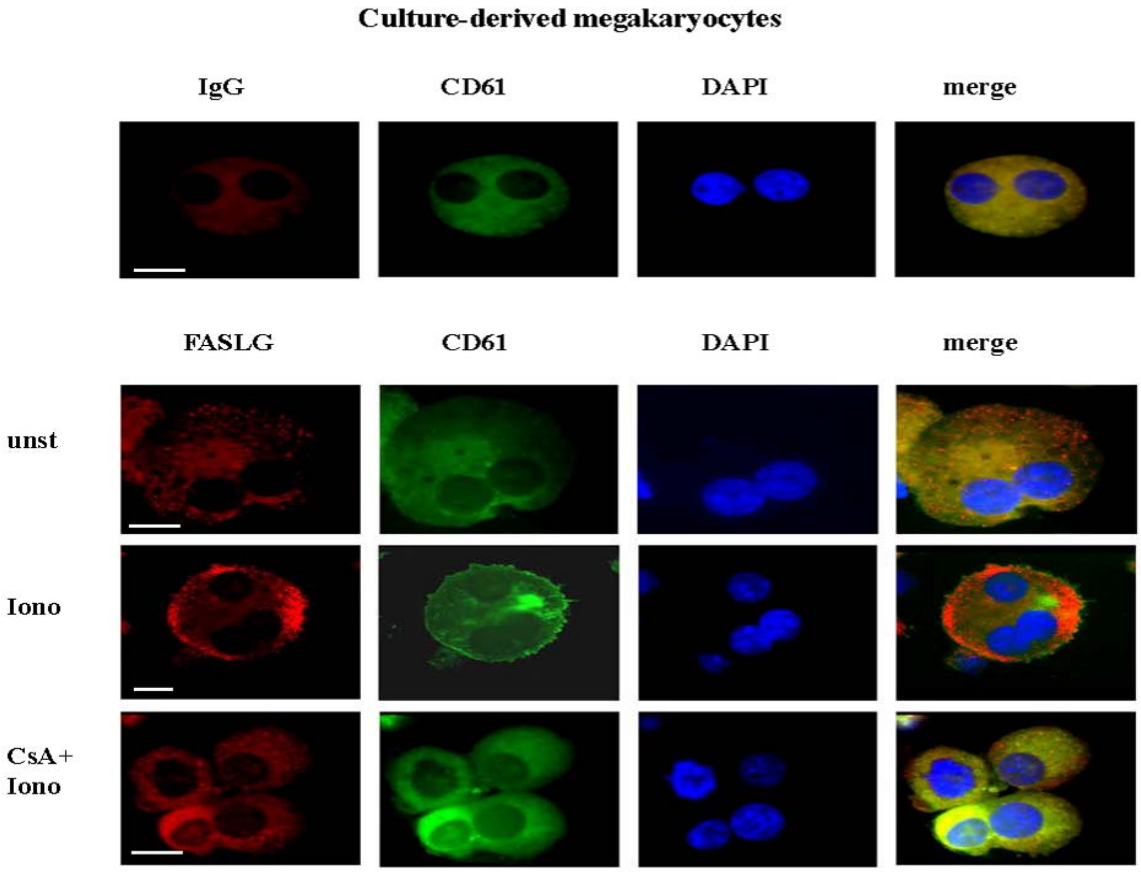


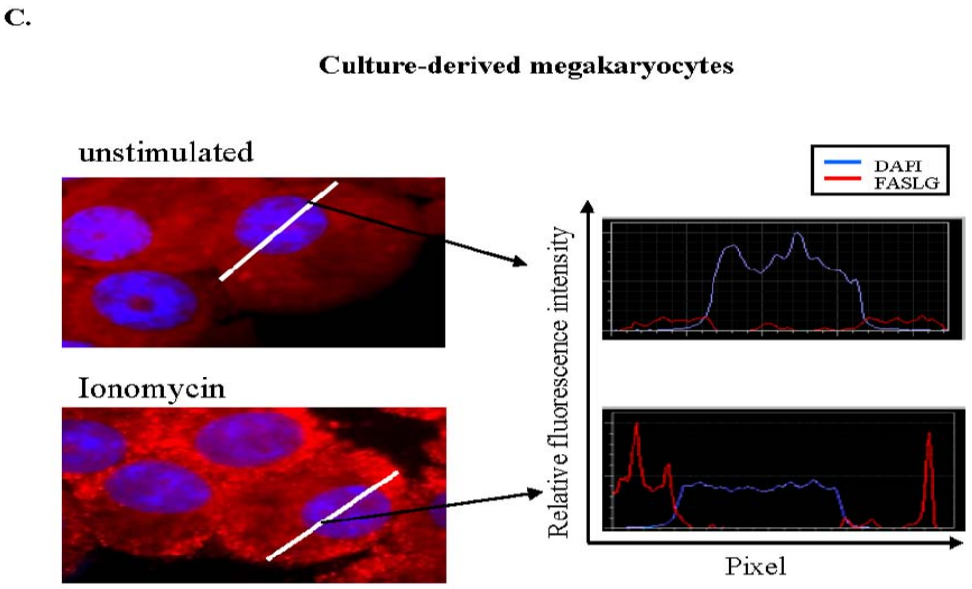


Figure 5.11. FASLG induced by calcineurin/NFAT activation in megakaryocytes is located on the cell surface. (A) CMK cells were either left unstimulated (grey solid line) or stimulated with 1 µM ionomycin (black fill) for 6 h in the presence of a metalloproteinase inhibitor (MPi) to prevent shedding of FASLG from the surface. Expression of FASLG on the surface of the cells was detected by flow cytometry using an anti-FASLG antibody. Dashed line, isotype control. **(B)** Ex vivo differentiated primary MKs were either left unstimulated or stimulated with 1 µM ionomycin (Iono) for 12 h in the presence or absence of CsA. Cytospin preparations of the cells were analyzed for FASLG subcellular localization (red) by immunofluorescence staining. DAPI counterstaining (blue) was applied to visualize the nuclear morphology of each cell, and CD61 (green) was used as megakaryocytic differentiation marker. Scale bars represent 10 µm. **(C)** Single cell fluorescence analysis of culture-derived MKs (FASLG-DAPI merge) treated as in (B). Note the diffuse staining pattern of FASLG (red) in untreated cells (upper panel), as opposed to the localized, high intensity staining in proximity to the cell membrane in stimulated cells (lower panel). Shown (A, B and C) are representatives of at least three independent experiments.

5.2.6 The Fas/FASLG pathway is involved in calcineurin/ NFAT-mediated apoptosis induction in megakaryocytes

As shown in the previous experiments, activation of the calcineurin/NFAT pathway in MKs induces apoptosis and, at the same time, cell surface expression of the pro-apoptotic FASLG protein. In addition, MKs constitutively express Fas, the FASLG receptor.[184] To determine if ionomycin-induced programmed cell death in MKs was mediated by the Fas/FASLG pathway, we tested the effect of NOK-1 and ZB4, two neutralizing antibodies directed against FASLG and Fas respectively. Both antibodies interrupt the binding of FASLG to the Fas receptor and thereby inhibit Fas/FASLG-mediated signaling. Culture-derived primary MKs (figure 5.12A, B) and CMK-NFATc2 cells (figure 5.12C) were left unstimulated or treated with 1 µM ionomycin for 24 h in the presence or absence of NOK-1 or ZB4. The rate of annexin V-positive apoptotic cells was monitored by flow cytometry. As shown in Fig 5.12, both antibodies inhibited (albeit incompletely) apoptosis induction by ionomycin treatment in these cells, suggesting that the Fas/FASLG pathway is involved in this process.

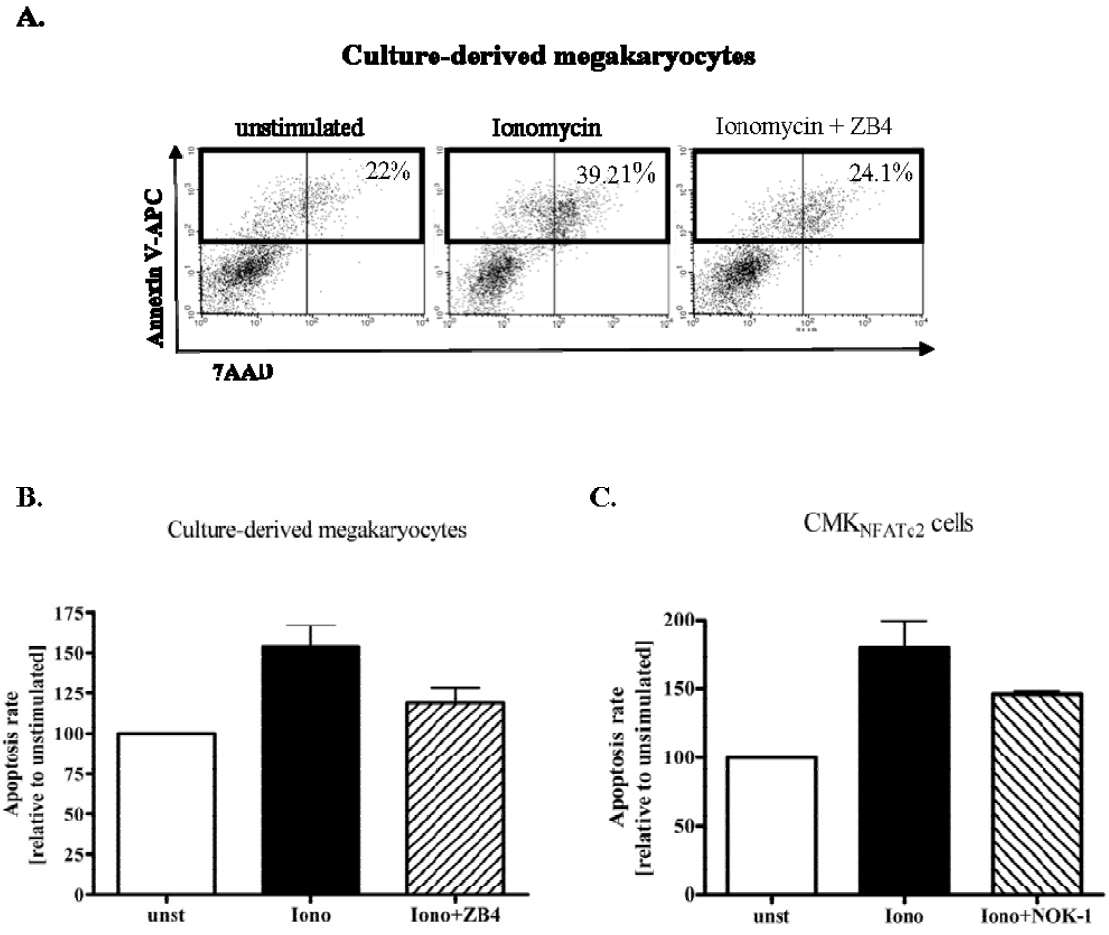


Figure 5.12. The Fas/FASLG pathway is involved in calcineurin/NFAT-mediated apoptosis induction in megakaryocytes. Ex vivo differentiated primary MKs **(A, B)** and CMK cells overexpressing NFATc2 **(C)** were either left unstimulated or stimulated with 1 µM ionomycin (Iono) for 24 h in the presence or absence of neutralizing antibodies against Fas (ZB4) or FASLG (NOK-1), as indicated. The apoptosis rate was determined by flow cytometric analysis of annexin V-positive cells. **(B, C)** Statistical analysis of at least three independent experiments as in (A). Shown are means ± SEM.

5.2.7 Calcineurin/NFATc2-dependent FASLG expression in megakaryocytes induces apoptosis in (Fas-positive) bystander cells

In addition to their role as producers of platelets, MKs are known to express various membrane-bound proteins with immunomodulatory properties (e.g., TRAIL, CD40 ligand, FASLG), but the functional significance of this phenomenon is poorly understood. To determine if MKs are able to induce apoptosis in lymphocytes, we employed a co-culture system in which CMK cells were first prelabeled with CFSE, then were either left untreated or stimulated with ionomycin for 6 h to induce maximal expression of FASLG, and finally were mixed with Fas-expressing Jurkat T cells in an effector-to-target ratio of 10:1 (figure 5.13). After a co-incubation period of 20 h, the apoptosis rate in Jurkat cells was determined on a single-cell basis by flow cytometric analysis (figure 5.14B). CFSE-positive CMK effector cells were gated out (figure 5.14, upper dot blot). We first confirmed that Jurkat cells were susceptible to Fas-mediated apoptosis induction (positive control). This was indeed the case, as incubation of Jurkat cells with CH11, an agonistic anti-Fas antibody, strongly increased the rate of apoptosis (figure 5.14A). We then tested the effect of co-incubation of CMK cells on apoptosis induction in Jurkat cells. To assess the effect of NFAT, CMK cells overexpressing NFATc2 (CMK_{NFATc2}) were compared as effector cells to CMK cells expressing VIVIT (compare left and right panels in figure 5.14B). As shown in figure 5.14B (panels i–iii) and figure 5.14C, the presence of ionomycin-prestimulated CMK_{NFATc2} cells in the co-culture induced apoptosis in Jurkat cells at a significant higher rate compared to the same cells without prestimulation or cells prestimulated with ionomycin in the presence of CsA. In contrast, ionomycin stimulation in CMK_{VIVIT} cells remained without effect (figure 5.14B, panels v–vi, and figure 5.14D), suggesting that NFAT is involved in this process. To confirm that induction of apoptosis in Jurkat target cells by stimulated CMK effectors was mediated through the Fas/FASLG pathway, CMK/Jurkat co-cultures were performed in the presence of either ZB4 (figure 5.14B, panel iv) or NOK-1 (figure 5.14E). Both antibodies completely blocked apoptosis induced in Jurkat cells by stimulated CMK cells. These data suggest that under certain conditions MKs are capable of inducing apoptosis in Fas-bearing bystander cells, for example lymphocytes. This process is dependent on the activation of

the calcineurin/NFAT pathway and the consecutive expression of FASLG on the MK membrane.

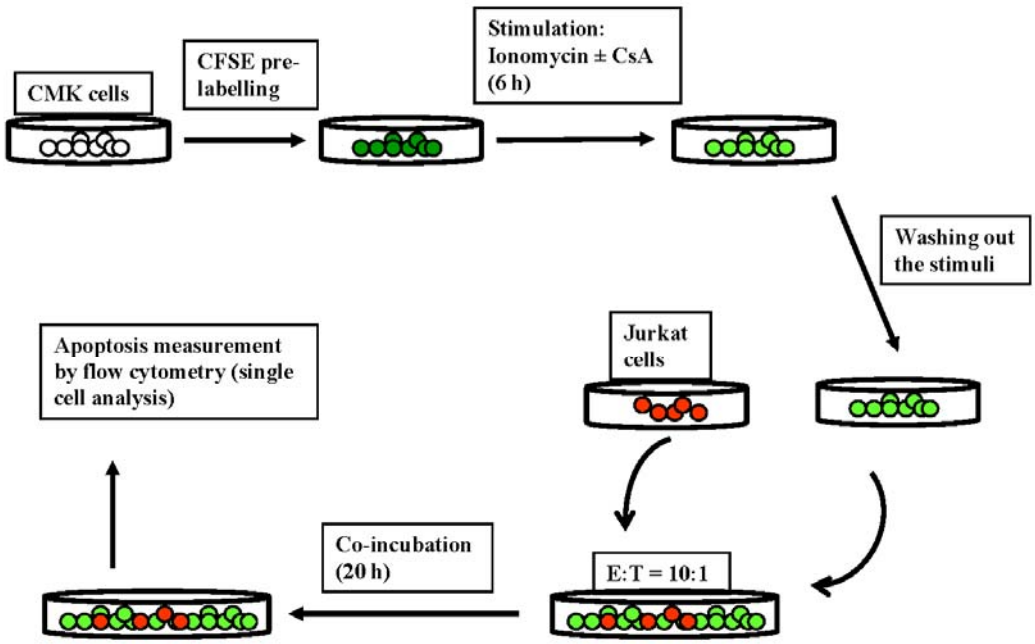
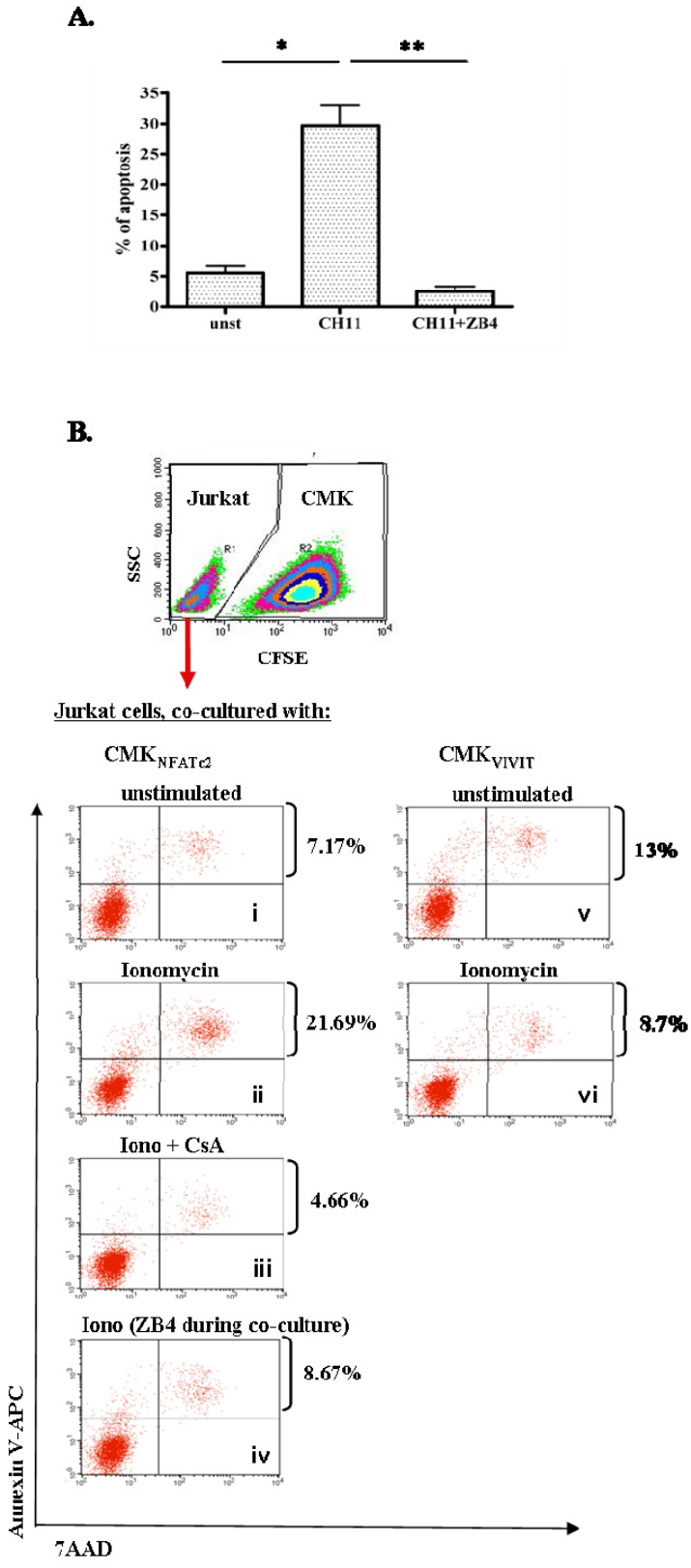
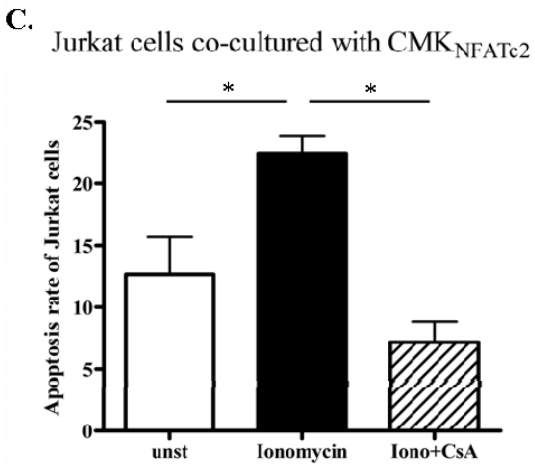
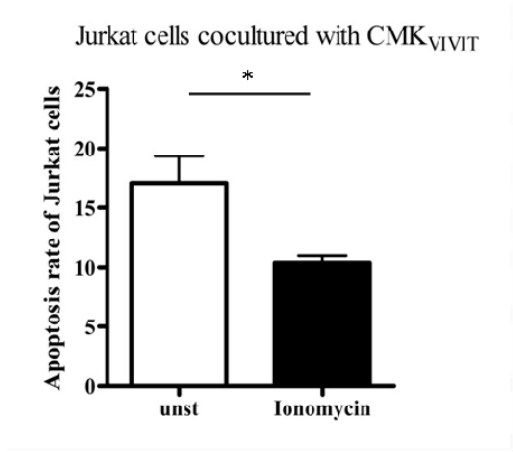


Figure 5.13. Schematic representation of CMK/Jurkat co-cultures. CMK cells (effectors) were first prelabeled with CFSE and then remained either untreated or were stimulated with 1 µM ionomycin in the presence or absence of CsA for 6 h. After washing, the cells were then mixed with Jurkat cells (targets) in an effector-to-target ratio (E: T) of 10:1. After 20 h of co-incubation, the cells were harvested, stained with annexin V/7AAD, and the apoptosis rate of the Jurkat cells was analysed by flow cytometry.


A.
*
**
% of apoptosis
unst
CH11
CH11+ZB4
B.
Jurkat
CMK
SSC
CFSE
Jurkat cells, co-cultured with:
CMK NFATc2
CMK VIVIT
unstimulated
7.17%
13%
Ionomycin
21.69%
8.7%
Iono + CsA
4.66%
Iono (ZB4 during co-culture)
8.67%
Annexin V-APC
7AAD


C.
Jurkat cells co-cultured with CMK NFATc2
Apoptosis rate of Jurkat cells
unst
Ionomycin
Iono+CsA


D.
Jurkat cells cocultured with CMK VIVIT
Apoptosis rate of Jurkat cells
unst
Ionomycin

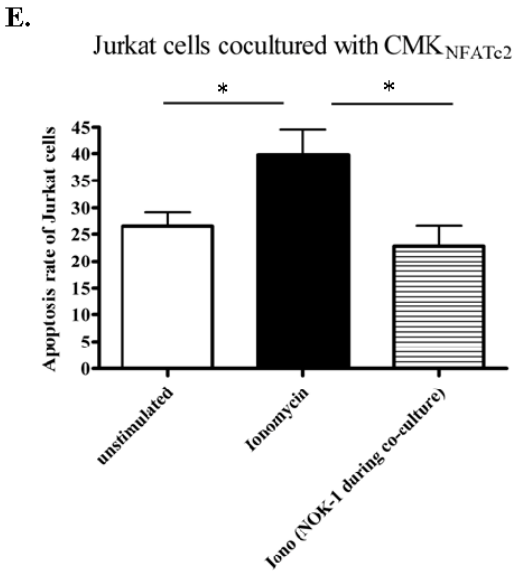

E.
Jurkat cells cocultured with CMK NFATc2
Apoptosis rate of Jurkat cells
unstimulated
Ionomycin
Iono (NOK-1 during co-culture)

Figure 5.14. Calcineurin/NFAT-induced FASLG expression in megakaryocytes induces apoptosis in T lymphocytes. (A) Jurkat cells (mono-culture) were left untreated or treated with an activating anti-Fas antibody (CH11) in the presence of absence of a neutralizing anti Fas antibody (ZB4). The apoptosis rate was determined by flow cytometric analysis of annexin V-positive cells. **(B–E)** CMK cells stably transfected with either NFATc2 (CMK_{NFATc2}) or VIVIT (CMK_{VIVIT}) were first labeled with CFSE and then either left untreated or stimulated for 6 h with 1 μM ionomycin in the presence or absence of CsA, as indicated. The prelabeled and prestimulated cells were then washed and co-incubated with Fas-expressing Jurkat T cells for 20 h, after which the apoptosis rate of Jurkat cells was analyzed by flow cytometry (see figure 5.13 for a schematic representation of the experimental design). Note that CFSE-positive CMK effector cells were gated out of the analysis (figure 5.14B, top dot blot). Where indicated (figure 5.14B, panel iv; figure 5.14E), co-cultures were performed in the presence of neutralizing antibodies against Fas (ZB4) or FASLG (NOK-1). **(B)** Dot blots represent Jurkat cells after 20 h of co-culture with CMK_{NFATc2} or CMK_{VIVIT} cells, which had been pretreated as indicated in the respective panel. **(C–E)** Statistical analysis of at least three independent experiments as in figure 5.14B. Results are shown as means ± SEM. *P<0.05, **P<0.001.

5.3 NFATc2 target genes in megakaryocytes

We demonstrated for the first time that the expression of FASLG protein in MKs is induced by NFAT signaling, and we extended this finding to a functional level, i.e., the induction of apoptosis in bystander cells mediated by NFATc2-induced FASLG. This novel finding prompted us to look for further NFATc2 target genes in MKs globally, in order to further define the role of NFAT in these cells. For this purpose, we performed an RNA sequencing study, in which the global gene expression profile in NFATc2-expressing (WT) and –deficient (KO) MKs was compared.

5.3.1 Generation of *ex vivo* pure and intact murine megakaryocytes

MKs can be isolated from the BM or spleen of adult mice, but they are rare. Moreover, the isolation of murine MKs by elutriation or cell sorting has resulted in poor yield of intact cells, and such cell populations are not sufficiently pure which limits their application in, for example, RT-PCR analysis of gene expression at the mRNA level.[66] However, expansion and differentiation of MK progenitors is possible due to the discovery of thrombopoietin (TPO) as a growth factor, which promotes lineage-specific differentiation and allows to obtain sufficient cell numbers.[185]

First, it was necessary to modify our *ex vivo* differentiation system that we carried out in human MKs (section 5.2), because of the very low number of cells that can be obtained from mice. To obtain a pure population of intact and mature murine MKs and to remove contaminating non-MK cells, the BM cell suspension was enriched for lineage-negative cells using immunomagnetic beads conjugated to an anti-lineage$^+$ mAb cocktail before initiating the *ex vivo* differentiation culture. Lineage-negative cells were then cultured for 3-4 days with 100 ng/ml TPO to obtain an enriched population of MKs. Efficiency of the differentiation process was examined by the morphology of the cells (May-Grünwald-Giemsa staining, figure 5.15) as well as by analysis of surface expression of megakaryocytic differentiation markers by flow cytometry (data not shown). Typical mature MK morphology was characterized by a significant increase in size; basophilic

cytoplasm in immature cells which turns to granulated and acidophilic in mature and differentiated MKs; as well as a lobular and polyploid nucleus and a demarcated membrane. The MK-enriched culture was resuspended and gently overlaid on a density gradient (1.5% and 3% BSA) with minimal agitation. After 30-45 minutes of incubation, white cells sedimenting through the lower BSA phase can be observed by eye. The upper phase was removed, the pellet resuspended and analyzed for purity (>95%) and integrity (figure 5.15). The MKs then were appropriate to be used for stimulation and RNA isolation.

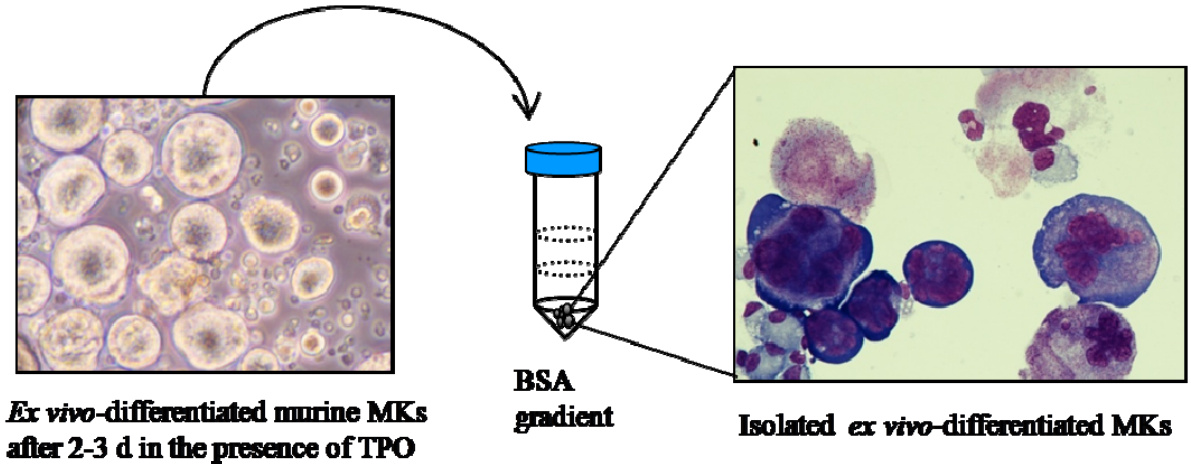


Figure 5.15. Differentiation and isolation of murine megakaryocytes. The left image illustrates the cell culture appearance of the lineage-negative fraction of murine BM after 2-3 d in the presence of TPO. Large cells represent immature and mature MKs. Cells were then loaded over a BSA gradient. After 30 minutes of incubation, the cells reaching the bottom of the tube were collected and stained with May-Grünwald Giemsa (right image). Purity of MKs was determined by microscopic analysis of stained slides.

5.3.2 RNA-sequencing (next generation sequencing) experimental design

Since NFAT needs to be activated in order to translocate to the nucleus and bind to the promoter of its target genes, for differential expression analysis of this study we were

interested in comparison between untreated and ionomycin-treated murine MKs isolated from WT and NFATc2-KO mice. RNA-Seq was performed on six biological replicates in each group (untreated WT [WT], untreated KO [KO], ionomycin-treated WT [WTst], and ionomycin-treated KO [KOst]).

5.3.3 Primary analysis: Bioinformatics and statistical analysis (performed by bioinformaticians)

Before performing differential expression tests, sequences generated from RNA-Seq experiment should be mapped to libraries of known exons in known transcripts. This step is absent in microarray expression analysis. Moreover, some basic statistical tests such as correlation of samples and variance estimation are necessary to evaluate the accuracy of experimental design and data analysis process. An overview of primary analysis is presented below:

5.3.3.1 Mapping

Millions of reads were generated by single end sequencing from 24 cDNA libraries – comprising four different sample classes ("WT", "WTst", "KO", "KOst") – with 6 replicates each. A range of 73%-79% of the sequenced reads were successfully mapped to the murine reference genome (mm9 transcriptome).

5.3.3.2 Correlation of the samples

Correlation (sample-to-sample linear dependence) between each two samples was calculated (figure 5.16). "The correlation coefficient ranges from −1 to 1. A value of 1 implies that a linear equation describes the relationship between X and Y perfectly, with

all data points lying on a line for which *Y* increases as *X* increases. A value of −1 implies that all data points lie on a line for which *Y* decreases as *X* increases. A value of 0 implies that there is no linear correlation between the variables" (adapted from Wikipedia; http://en.wikipedia.org/wiki/Pearson_product-moment_correlation_coefficient). Figure 5.16 shows the correlation coefficient of ~1 (0.97-0.99) calculated for all samples. The results strongly show that these experiments are accurately designed and are highly reproducible.

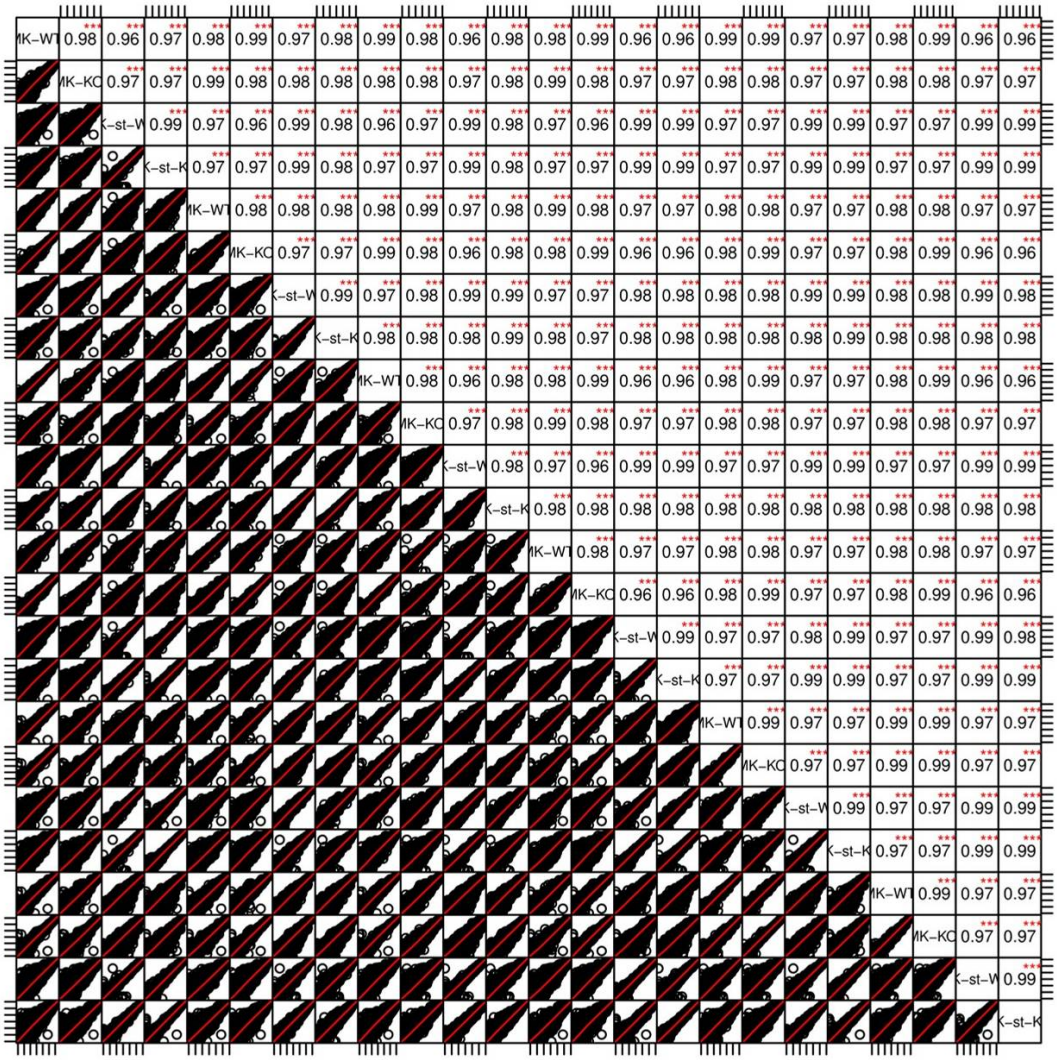


Figure 5.16. Correlation of the samples. Correlation plot together with coefficients (numbers) for all samples (on log scale). *** indicates significant linear correlation between samples. Red line presents linear regression.

5.3.3.3 Variance estimation

To check the dispersion among the replicates, variance estimation was performed. Typically, many of the values below the red curve are underestimates of the true dispersions, so DESeq biostatistics package uses the fitted values for them. However, not all values above the red curve are overestimates, so the conservative choice is to keep them (instead of using the fitted values). Figure 5.17 contains the variance estimation plots for all conditions ("WT", "WTst", "KO", "KOst") and indicates how the expression value of biological replicates are distributed (how far the expression values lie from the mean value). The less biological samples are used, the higher is the scattering of the estimates around the true values.

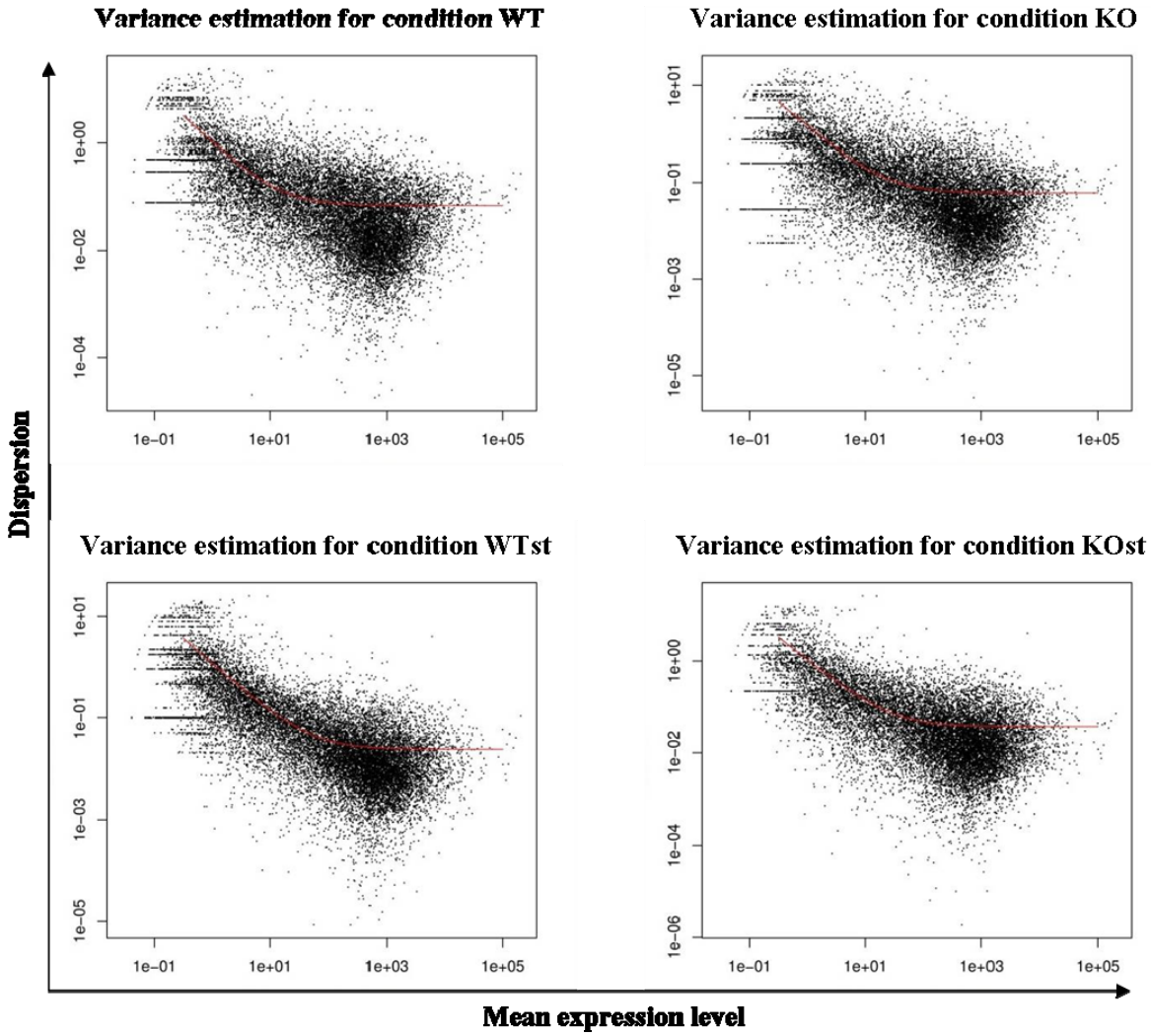


Figure 5.17. Variance estimation plots for all conditions. Empirical (black dots) and fitted (red line) dispersion values plotted against mean expression level (log scale) for all conditions: WT, KO, WTst, KOst. Dispersion is calculated by the square of the coefficient of biological variation (e.g., if a gene's expression differs from replicate to replicate by 20%, its dispersion is 0.20^2 = 0.04).

5.3.4 Secondary analysis: Genes of interest (significantly differentially expressed NFATc2 target genes).

To test for differential expression, we did the following comparisons among four experimental conditions using R statistical package DESeq (plots in figure 5.18, figure 5.19 and table 5.2):

_ resigDEMK1: WT vs. WTst

_ resigDEMK2: WT vs. KO

_ resigDEMK3: KO vs. KOst

_ resigDEMK4: WTst vs. KOst

First, we calculated the number of significantly differentially expressed genes in resigDEMK1 and resigDEMK2, which resulted in 6161 and 22 genes, respectively. Then, significantly differentially expressed genes were analyzed in comparison of WTst vs. KOst (resigDEMK4), which resulted in 592 genes.

NFAT is a transcription factor, which is inactive without stimulation (see introduction, section 2.1). Genes regulated by NFAT therefore, are not expected to be expressed at considerable levels in unstimulated samples (WT and KO). As "genes of interest" therefore were those genes considered which appear in resigDEMK4 but not in resigDEMK2, because genes differentially expressed between unstimulated-WT and -KO samples most likely represent artefacts (for example, unspecific consequence of a gene knockout). Finally, to confirm the accuracy of the presumed “genes of interest”, we analyzed if they also appeared in resigDEMK1 (i.e., were upregulated by stimulation).

492 of 580 genes showed the same behavior, and the rest was excluded from further analysis (table 5.2 displays a summary of differentially expressed results).

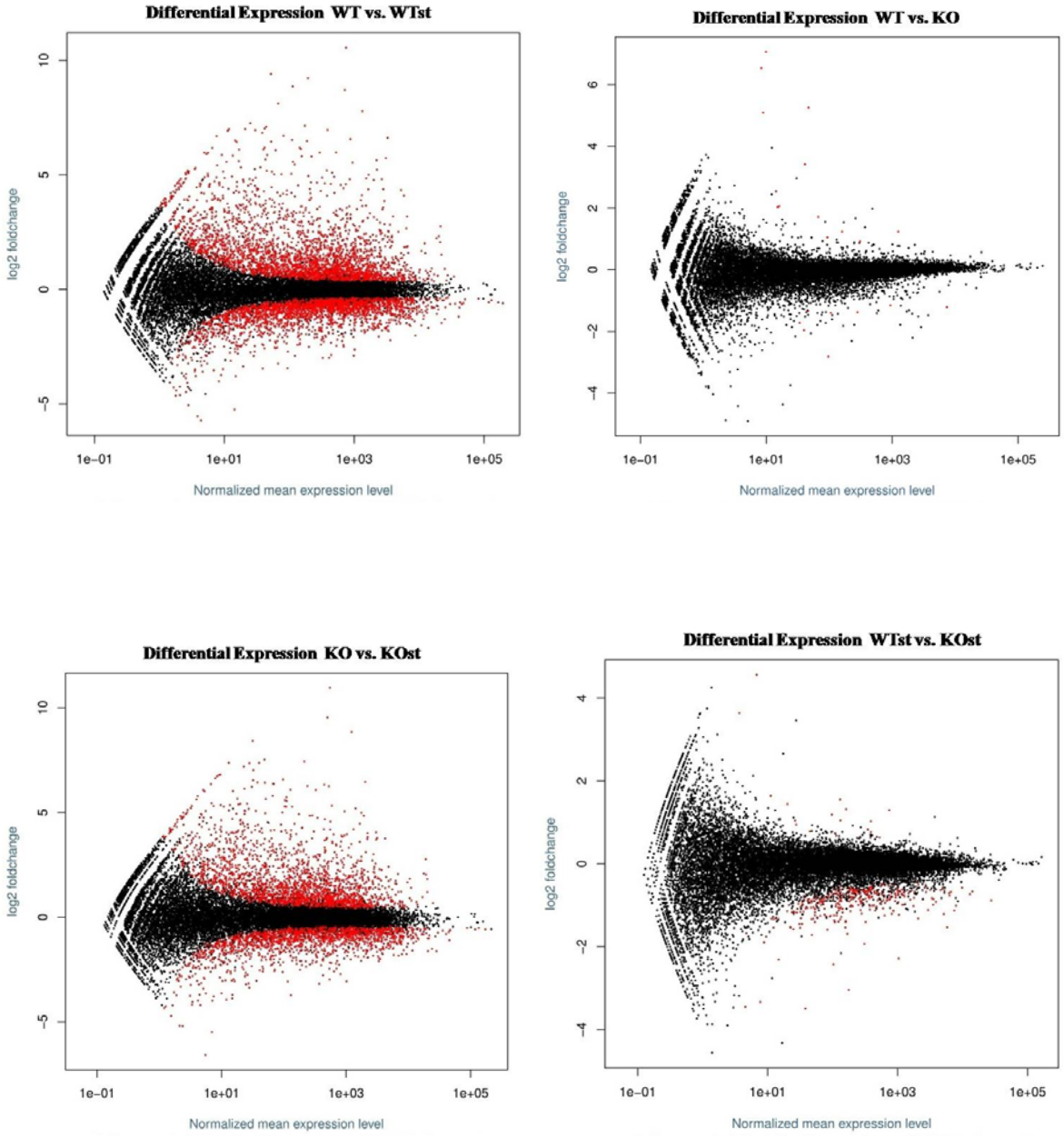


Figure 5.18. Plots of the significantly differentially expressed (DE) genes. Differentially expressed genes were obtained by plotting "log2 fold change" against "normalized mean expression level". Red dots represent significantly DE genes. WT vs. WTst, 6161 significantly DE genes. WT vs. KO, 22 significantly DE genes. KO vs. KOst, 4327 significantly DE genes and WTst vs. KOst, 592 significantly DE genes.

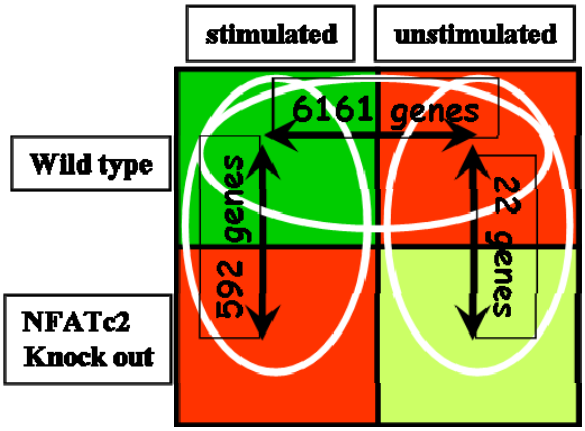


Figure 5.19. Schematic representation of the analysis procedure for the selection of differentially expressed genes (DEGs).

Comparison	DEGs
resigDEMK1: WT vs. WTst	6161
resigDEMK2: WT vs. KO	22
resigDEMK3: KO vs. KOst	4327
resigDEMK4: WTst vs. KOst	592
Set difference	**Different DEGs**
resigDEMK4- resigDEMK2	580
OK genes*	492
excluded genes	88

* genes of interest

Table 5.2. Summary of differentially expressed genes. The DEGs column contains the number of significantly differentially expressed genes at the given comparison. The second part of the table (set difference) lists the number of "interesting" genes that appear in resigDEMK4 but not in resigDEMK2. Out of these 580 "interesting" genes, 492 genes ("OK genes") showed the same behavior in resigDEMK1 (i.e., were upregulated during the stimulation in WT), whereas 88 genes ("excluded genes") did not and therefore were excluded.

5.3.4.1 Confirmation of results by known NFATc2 target genes in Megakaryocytes

In order to confirm the reliability of the final list of genes, we analyzed them for the presence of known NFATc2 target genes in MKs. The DSCR1 gene, which has been recently demonstrated as an NFATc2 target gene in MKs,[143] was found in the final list of "genes of interest" (an excerpt of the list is shown in figure 5.20A). In addition, a PCR-array experiment using a different set of samples of stimulated/unstimulated WT and KO cells identified a number of NFATc2-target genes in MKs (IGF1, CD36, NF-κB, ITG-αm and ITG-αv), which also appeared in the list of RNA-Seq experiment (figure 5.20C). These results confirm the accuracy and reliability of the method and analysis procedure used in this experiment.

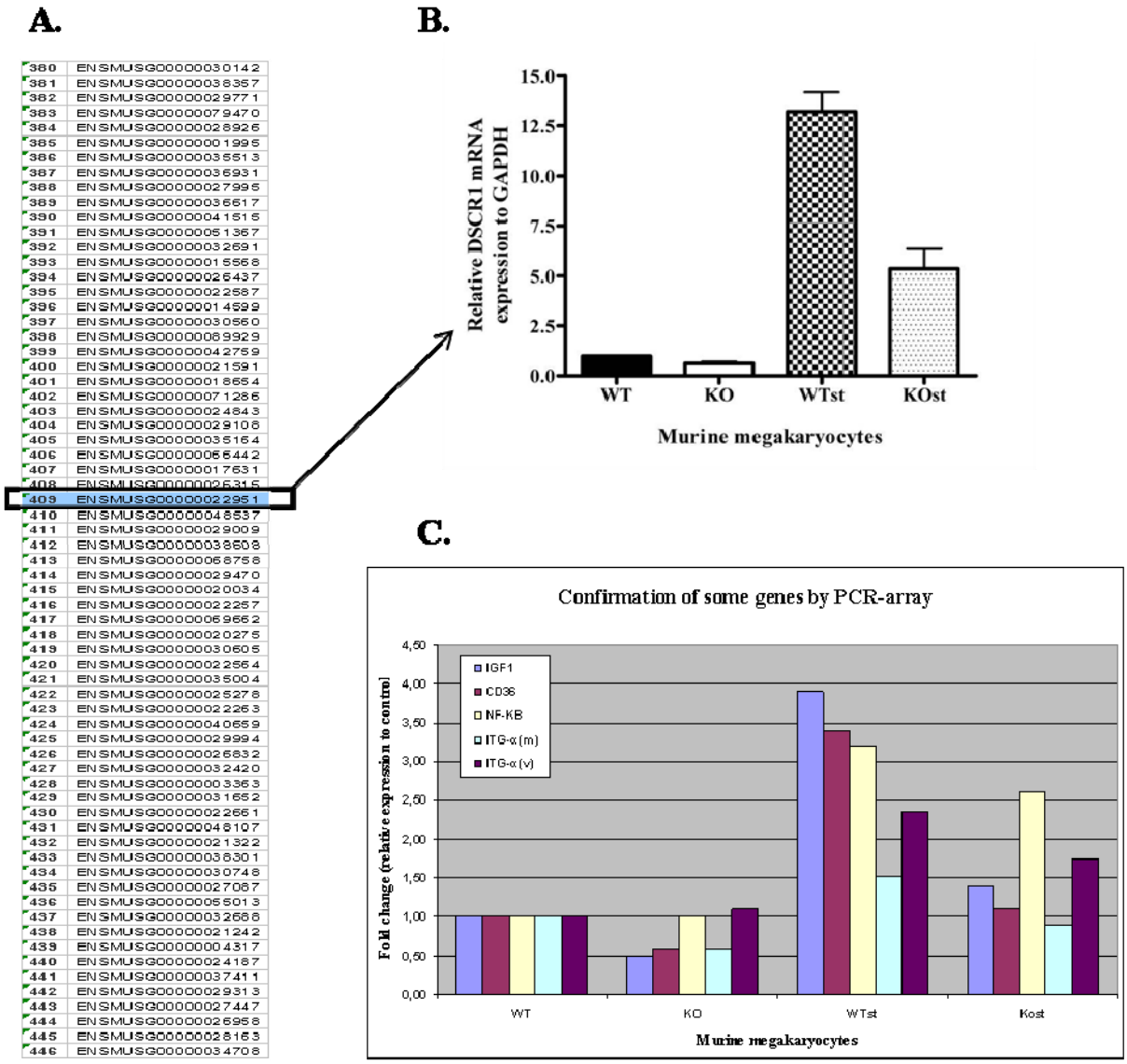


Figure 5.20. Confirmation of RNA-Seq data. (A) DSCR1 gene (gene Ensembl ID: ENSMUSG00000022951) appeared in the list of "interesting gene". **(B)** Total RNA isolated from either WT or NFATc2-KO murine MKs were analyzed for DSCR1 gene expression by RT-PCR. Values were normalized for expression of GAPDH as a reference gene and expressed relative to the value of the unstimulated sample. **(C)** The expression of IGF1, CD36, NF-κB, ITG-αm and ITG-αv were evaluated relative to control sample by PCR-array.

5.3.5 Functional annotation (performed by Miltenyi Biotec Company)

The functional grouping and annotation analysis provides an overview of the different biological processes and pathways that are regulated by the provided gene set. The set of 492 genes was subdivided into an upregulated group (significantly differentially expressed genes, which are upregulated by the activation of NFATc2, i.e., in WTst vs. KOst) and a downregulated group. For the functional annotation and grouping analysis, the genes were annotated with information from various databases in order to find common features among the genes sharing similar expression characteristics. The annotations used were derived from Gene Ontology (GO), which provides information on molecular function, as well as from various pathway resources for information on involvement in biological signaling pathways. Subsequently, the gene list(s) are used for database queries searching for annotations related to functional categories or pathway membership. In addition, the gene lists obtained are compared to known targets of certain signaling pathways. In the present project, functional grouping analysis was carried out for two gene sets (genes upregulated and downregulated by NFATc2). Table 5.3 containing summarized functional annotation results are demonstrated.

This assessment cannot replace a full pathway enrichment analysis, which uses a much more extensive set of pathways and biological categories. In the present analysis, the upregulated gene list exhibits a large number of highly significant associations with broad categories such as innate immunity, inflammation, T-cell immunity, B-cell immunity and with more specific pathways like cytokine signaling, NF-κB signaling, death-receptor signaling, the IL-4, IL-6, wnt and TGF-ß pathways (table 5.3). By contrast, the (much smaller) list of downregulated genes does hardly show any meaningful association in this type of analysis. Obviously, a more fine-grained analysis will be required to detect more subtle biological connections between the regulated genes.

Table 5.3. Pathway components from gene ontology (GO) within the gene set upregulated by NFATc2.

Pathway components(Gene Ontology)	No. of genes
cytokine/chemokine signaling	20
NF-κB signaling	19
death receptor signaling	13
EGF receptor	10
Toll signaling	9
TNF-α pathway	8
Antigen presentation	7
Wnt signaling	7
Integrin mediated signaling	6
IL-4 pathway	6
IL-6 pathway	6
B cell receptor pathway	6
T cell receptor pathway	6
TGF-ß signaling	5
TGF-ß receptor	5
IL-3 pathway	5
BMP signaling	4
IL-1 pathway	4
IL-2 pathway	4
PDGF signaling	3
p53 signaling	3
hormone/steroid signaling	3
EGF signaling	2
ILGF/insulin receptor signaling	2

Further analysis on biological pathways indicated that while the majority of the enriched categories are indicative of an actively ongoing immune response (or at least an activated state of various immune cells), there are a few enriched categories without a direct link to the immune system. Among them is a 10-fold enrichment of the nitric oxide (NO) biosynthesis gene, Nos2 (encoding the inducible nitric oxide synthesis iNOS). Nos2 is seen on the top of the gene list of enriched categories (table 5.4) that was sorted by the transcriptional effect of stimulation in the WT cells. Ch25h (encoding a cholesterol hydroxylase) and Arg1 (encoding arginase) were also among the top-induced genes. Interestingly both Nos2 and Arg1 are implicated in NO signaling and so are Slc7a2 (encoding an arginine transporter) and Cav1 (encoding caveolin-1), which are both highly induced.

Table 5.4. Genes sorted based on the transcriptional effect of stimulation in WT relative to KO (only the top twenty genes are shown).

Ensembl ID	Gene symbol	effect
ENSMUSG00000020826	**Nos2**	10.28
ENSMUSG00000050370	**Ch25h**	9.36
ENSMUSG00000019987	**Arg1**	9.14
ENSMUSG00000031596	**Slc7a2**	7.20
ENSMUSG00000025321	**Itgb8**	5.61
ENSMUSG00000007655	**Cav1**	5.51
ENSMUSG00000050578	**Mmp13**	5.51
ENSMUSG00000041324	**Inhba**	5.43
ENSMUSG00000049511	**Htr1b**	5.20
ENSMUSG00000026981	**Il1rn**	4.85
ENSMUSG00000074170	**Plekhf1**	4.80
ENSMUSG00000028583	**Pdpn**	4.74
ENSMUSG00000030162	**Olr1**	4.60
ENSMUSG00000053338	**Tarm1**	4.54
ENSMUSG00000060044	**Tmem26**	4.36
ENSMUSG00000051367	**Six1**	4.36
ENSMUSG00000033213	**AA467197**	4.09
ENSMUSG00000051439	**Cd14**	4.04
ENSMUSG00000035208	**Slfn8**	3.97
ENSMUSG00000073489	**Ifi204**	3.93

6 Discussion

6.1 Lack of the transcription factor NFATc2 in hematopoietic stem cells results in profound hematological abnormalities in mice

NFATc2 has been shown to be expressed in hematopoietic stem cells. Therefore, understanding the molecular mechanisms regulated by this transcription factor is of importance and may assist to exploit the NFAT pathway for therapeutic approaches. In this study, we evaluated the role of NFATc2 in hematopoiesis, using NFATc2$^{-/-}$ mice as a model system.

We have previously shown that aged mice lacking the transcription factor NFATc2 develop BM hypoplasia, anemia, thrombocytopenia and extramedullary hematopoiesis in spleen and liver.[84, 106] The proliferation and differentiation of NFATc2-deficient HSCs *ex vivo*, however, was found to be intact. In this study we aimed to address the question whether the disturbed hematopoiesis in NFATc2-deficient mice was caused by the hematopoietic or the stroma component of the BM hematopoietic niche. Therefore, we performed reciprocal transplantation experiments in which NFATc2$^{-/-}$ HSCs were transplanted into WT mice and vice versa. Subsequently, post-transplantation alterations were investigated and compared to WT and KO mice transplanted with control cells.

6.1.1 HSC transplantation

At present, BM and peripheral blood hematopoietic stem cell transplantation are considered to be potentially therapeutic ways for a variety of BM disorders. Following transplantation, HSCs are able to reconstitute the recipient.[186] However, the use of whole BM as a source of stem cells includes the risk of transplantating contaminating stroma cells, which for our experimental purposes was not suitable.[89]

To circumvent this problem, Lin^{-}/Sca-1^{+}/cKit^{+} cells, commonly referred to as the LSK compartment of HSCs, are often used for transplantation. This population has the ability

to give rise to hematopoietic subpopulations (common lymphoid progenitors, CLP, and common myeloid progenitors, CMP) and repopulate long-term blood-lineage in irradiated recipients.[166, 168] Thus, we employed LSK cells for the transplantation experiments.

6.1.2 NFATc2 deficiency in HSCs leads to hematological and stroma abnormalities

In the current study, we demonstrate that transplantation of NFATc2$^{-/-}$ HSCs into WT mice leads to the induction of hematologic abnormalities, similar to those which had been observed in (non-transplanted) aged KO mice. The hematologic abnormalities were significant with respect to the induction of anemia and thrombocytopenia as well as extramedullary hematopoiesis in spleen and liver. In addition, KO mice transplanted by WT HSCs demonstrated a remarkable reduction in the hematologic abnormalities, which had been observed in non-transplanted KO mice.

Hitherto, it had been established that NFATc2 is expressed in HSCs and that its expression is maintained during megakaryopoiesis.[78, 79] In contrast, it had not been described as a crucial transcription factor required for hematopoiesis. In mice with NFATc2 deficiency, the hematopoietic organs (blood, BM and spleen) are abnormal, but the differentiation potential of HSCs is preserved.

Even though a lot of evidence indicates its importance in the lifespan of HSCs, the BM niche is not the only mechanism for HSC regulation. The stem cell itself has crucial impact. Fetal liver-derived HSCs, for instance, have been shown to exhibit a more efficient regeneration potential than similar cells from adult BM when transplanted into irradiated recipients.[187] Bowie et al recently revealed the existence of "intrinsically regulated master switch", in which HSCs rapidly change from a fully cycling population to a quiescent one.[188] These changes seem to be independent of the niche as they are not affected by transplantation into adult recipients.[189]

The BM of aged NFATc2-deficient mice exhibits hematopoietic insufficiencies, which is partly compensated by extramedullary hematopoiesis in spleen and liver. The observation from the current study, in which transplantation of HSCs from WT mice rescues the hematological abnormalities of NFATc2-deficient mice and vice versa

indicates that the abnormalities observed in the KO mice are caused by an endogenous HSC defect.

Supporting these data, there is evidence from studies which indicate a role of NFAT in cell cycle and proliferation. A large number of genes associated with cell proliferation and apoptosis are induced by NFAT.[190] In some systems, NFATc2 appears to negatively control cell proliferation,[18, 31, 191] since its deficiency (despite the presence of other NFAT family members) leads to uncontrolled cell growth. Contrary to these observations, NFATc2 is known as a regulator of apoptotic mechanisms by induction of the expression of a number of pro-apoptotic molecules (e.g. TNF-α, FASLG and others).[5, 191] Therefore, the role of NFATc2 in proliferation and apoptosis is complex and depends on the cell and the cellular context.

Of note, the hematopoietic abnormalities induced by NFATc2-deficient HSCs are markedly apparent in erythroid and megakaryocytic lineages, consistent with the anemia and thrombocytopenia observed in the peripheral blood of NFATc2$^{-/-}$ mice. These abnormalities are rescued by transplantation of WT HSCs. Therefore, one can speculate that NFATc2 has a stronger impact on megakaryocyte-erythroid lineage commitment than on other myeloid lineages such as granulocyte-macrophage lineages. However, NFATc2 activation is not required for erythrocyte and MK differentiation.[84] Therefore, an attractive hypothesis may be that NFATc2 mediates cell cycle and proliferation of erythro-megakaryocytic committed lineages rather than of other myeloid lineages or of lymphocytes.

Nevertheless, the phenotype observed in NFATc2-deficient mice does not exclude an aberrant HSC-stroma crosstalk. One of the possible mechanisms might be perturbed homing caused by the absence of NFATc2. In order to efficiently establish hematopoiesis after transplantation, HSCs must home into hematopoietic organs, especially the BM.[192, 193] We tested our hypothesis by performing homing assays, in which hematopoietic stem/progenitor (lineage-negative) cells of the BM were isolated and labeled with a viable dye (CFSE). This dye allows the detection of fluorescent cells for up to seven cell divisions, which is adequate for the time of homing (~20 h) in our system. The results revealed no differences between WT and KO HSC in number of donor-derived hematopoietic stem/progenitor cells homing to the marrow of recipient mice (figure 5.6). These results could be confounded by the fact that cells passing through the BM intravenous system may be miscounted as “homed cells”. However, some studies

suggest that the extreme adhesion capacities of injected stem cells force them to immediately leave the central vein and reach the BM niche within a few hours.[194] Therefore, in the current experiment, we chose 20 h after injection as the time point for the evaluation of homing cells in BM. Secondly, we found clearance of labeled cells from the peripheral blood of the transplanted mice at this time point, which declines the possibility of miscalculating intravenous cells as cells homed to the BM niche (figure 5.6C). Furthermore, the fluorescent intensity of engrafted cells detected after 20 h shows the presence of daughter cells as well as of injected cells (figure 5.6, compare $CFSE^+$ cells in A and B) which correlates with the fact that stem cells divide every 12 h, and allows to verify the appropriate time of detection in this experiment. Our results suggest that homing of NFATc2-deficient HSCs is normal. Nevertheless, due to the limitations of the experimental systems used, homing deficiency cannot be excluded as mechanism of the phenotype of KO or KO→WT mice. It has to be emphasized that hematopoiesis in NFATc2-KO mice is characterized by a "relocation" of the hematopoietic progenitor cells from the BM to the spleen and liver (figure 5.5). Most evidence, therefore, points to the hypothesis that NFATc2-deficient HSCs suffer from a cell intrinsic defect which disturb their interaction with the BM (but not with spleen/liver) stroma.

Homing is a highly regulated process,[93] in which the expression of cell adhesion molecules and the impact of intrinsic and extrinsic pathways which mediate the interaction of HSCs with the niche has to be considered. HSC engraftment has been shown to take place in perivascular spaces,[195, 196] which draws the focus on a role for molecules such as angiopoietin-1 receptor Tek and adhesion molecule Integrin-ß1 in maintenance and homing of transplanted HSCs.[197] The expression of adhesion molecules or chemokine receptors in NFATc2-deficient HSC has not been studied so far in detail, but may be a focus for further experiments in the future. Finally, experiments monitoring the proliferation of transplanted HSCs *in vivo* are essential to be also considered in future plans.

Previously, we have shown that the marrow of aged KO mice exhibit intramedullary sclerosis, although to a variable extent. The next interesting question was whether, in addition to their impact on hematopoiesis, donor-derived HSCs are able to transfer their phenotype to the BM stroma as well. Transplantation of KO HSCs to WT recipients was found to modify the marrow structure. We showed that WT mice transplanted by KO HSCs (KO→WT) (figure 5.3) demonstrate fibrosis as well as ossified BM which is also

the case in the marrow of KO mice receiving KO HSCs (KO→KO) but not in WT mice transplanted by WT HSCs (WT→WT) (figure 5.3 D, E). This phenomenon was however incomplete. Though in some cases of KO→WT mice a significant myelofibrosis and formation of trabeculae is observed, in others either the modifications were moderate or were not observed. We reasoned that these symptoms are apparently cumulative and can be detected in higher ages.

This phenotype of NFATc2$^{-/-}$ mice exhibits a noticeable resemblance to human myelofibrosis (WHO2008: primary myelofibrosis). Myelofibrosis is a myeloproliferative disease in which the proliferation of an abnormal type of BM stem cells leads to thrombocytopenia, secondary intramedullary collagen fibrosis, splenomegaly and occurrence of extramedullary hematopoiesis. To date, the mechanisms underlying the secondary marrow changes are not completely understood.[106, 198] Mouse models with overexpression of TPO (TPO^{high} mice) or low expression of GATA-1 (GATA-1^{low} mice) have also been shown to exhibit myelofibrosis. In these animals, a significant contribution of transforming growth factor-beta 1 (TGF-ß1) originating from hematopoietic cells was found to induce marrow fibrosis. Other factors, including osteoprotegerin (OPG), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and vascular-endothelial growth factor (VEGF) have also been implicated in mediating osteosclerosis, fibrosis and angiogenesis in this disease. It is currently assumed that various cytokine families, notably TGF-ß, are associated with alterations in MKs.[199-201] Since TPO (extrinsically) and GATA-1 (intrinsically) both control MK differentiation, the development of myelofibrosis in above-mentioned mouse models (TPO^{high} and GATA-1^{low}) is the result of high TGF-ß levels originating from MK dysfunction. The similar phenotypes observed in NFATc2$^{-/-}$ and GATA-1^{low} mice might provide a clue to the underlying mechanism. GATA-1, known as a transcriptional partner of NFAT, is established as a regulator of megakaryopoiesis (section 2.4.1 and figure 2.2). NFAT and GATA families of transcription factors are shown to cooperatively control the expression of certain genes (e.g. IL-4, IL-5 and IL-13) in Th2 (T helper 2) and mast cells.[202] A crosstalk of NFAT and GATA-1 could also be illustrated in patients with Down's syndrome, in which MK dysfunction might be the consequence of the combinatorial effects of GATA-1 mutation and NFAT inactivation caused by overexpression of DSCR1 (down syndrome critical region 1).[173] As a conclusion, NFAT

and GATA-1 might have roles in the transcriptional regulation of hematopoietic cells which themselves can influence the stroma compartment of the BM as well.

At present, allogenic stem cell transplantation is considered as one of the most efficient treatments for the patients with myelofibrosis.[203, 204] An interesting outcome of this study also reveals that KO mice harboring myelofibrosis are at least in part rescued by WT-derived HSCs (WT→KO). Conversely, myelofibrosis is induced in WT mice by transplanting KO HSCs (KO→WT).

These findings should promote more investigation in the transcriptional regulation of NFATc2, particularly in the erythro-megakaryocytic lineage. Of particular interest apparently are MKs since these cells are involved in a number of diseases, for instance myelofibrosis (osteomyelofibrosis) which is observed in NFATc2$^{-/-}$ mice as well. Therefore, these data prompted us to investigate the role of NFATc2 in MKs.

6.2 NFATc2 regulates apoptosis in megakaryocytes

Signal transduction pathways regulating gene expression in MKs are poorly understood. Studies in MKs are complicated by the problem of their low abundance in the BM (~0.5%) and the difficulties in obtaining sufficient numbers for experiments.[185, 205] Moreover, MKs isolated by these methods are not sufficiently pure and intact. Researchers have overcome these problems either by employing megakaryocytic cell lines or by differentiating MKs *ex vivo* out of their progenitor cells. Megakaryocytic cell lines (for example, CMK, Meg-01, M-07, K562) provide a unique opportunity to study MKs.[205, 206] However, they are leukemic cells with a modified karyotype and represent an immature state of MKs. Therefore, the physiological significance of any finding in these systems needs to be confirmed in primary cells. Thus, in addition to megakaryocytic cell lines, we considered it important to confirm our principal findings in culture-derived primary MKs (*ex vivo*-differentiated MKs). The availability of thrombopoietin (TPO), a cytokine that stimulates megakaryopoiesis enabled us to expand MKs efficiently *ex vivo* for research purposes.[66, 117] Therefore, MKs were differentiated over several days out of either primary $CD34^+$ hematopoietic progenitor cells (for human MKs) or Lin^- cells (for mouse MKs, section 6.3) in the presence of TPO for our experiments.

6.2.1 NFAT regulates inducible FASLG protein expression in megakaryocytes

In the present study, we identify the phosphatase calcineurin and the transcription factor NFAT as part of a calcium-dependent signal transduction pathway that is central for inducible gene expression in MKs. From earlier studies, it was known that MKs express both calcineurin and NFAT proteins, and that the expression of NFAT during megakaryopoiesis was regulated in a lineage-specific manner.[78, 79] Neither activation of calcineurin nor of NFAT, however, proved to be necessary for the differentiation of MKs from hematopoietic progenitor cells.[142] We then focused on the question if the

calcineurin/NFAT pathway was involved in the regulation of gene expression in mature MKs. Indeed, we and others could subsequently identify the CD154, DSCR1 and FASLG genes as NFAT transcriptional targets in these cells.[79, 126, 143]

The present study for the first time extended these findings and advanced them from a descriptive to a functional level. We demonstrate that activation of the calcineurin/NFAT pathway in MKs induces expression of the FASLG protein. Immunoblot analysis revealed that FASLG protein, in stimulated *ex vivo* differentiated MKs as well as in transfected megakaryocytic CMK cells, was detected within hours and exactly followed the pattern of its expression in stimulated Jurkat T cells. Conversely, FASLG protein expression was completely undetectable, when cells were stimulated in the presence of calcineurin inhibitor CsA, which indicates FASLG expression is mediated by calcineurin. Since calcineurin can affect other pathways via different substrates, CMK megakaryocytic cells transfected with NFAT-specific peptide-inhibitor of calcineurin VIVIT were used and also showed an inhibition of FASLG protein expression. Therefore, the expression of FASLG protein in MKs is mediated by NFAT.

Similar to other proteins of the TNF-family, FASLG is also found in two variants, namely membrane-bound FASLG (molecular weight of 37 KDa) and soluble FASLG (sFASLG, 26-29 KDa). It has been shown that sFASLG is generated by proteolytic cleavage of membranous FASLG by matrix metalloproteinases.[207] Tanaka et al. have demonstrated that sFASLG acts to prevent the cytotoxic activity and reduce the effectiveness of membrane-bound FASLG.[181, 208] Therefore, membranous FASLG is the functional form of the protein. As we show in our study, NFAT-mediated FASLG protein in stimulated MKs is located at the membrane of the cells. Fas/FASLG interaction is essential for triggering programmed cell death. It has been shown that functional FASLG is partially localized in membrane rafts and is remarkably increased, once it interacts with its receptor for the induction of apoptosis. Immunofluorescence staining of FASLG demonstrates the accumulation of FASLG protein in the proximity or on the membrane of mature MKs when the calcineurin/NFAT pathway is activated.[182, 183] These findings raised the hypothesis that FASLG protein expressed via calcineurin/NFAT-mediated pathway might be functional.

6.2.2 Calcineurin/NFAT-dependent expression of FASLG in megakaryocytes is functional

Of note, FASLG expression is also found in platelets.[209, 210] As platelets are anuclear cells and receive their genetic repertoire from their MK precursors, it had to be considered that FASLG expression in MKs may serve the sole purpose to transfer it to their progeny.[126, 127] In addition, platelets express biologically active FASLG on their surface which is able to induce apoptosis in Fas-positive cells, thus it was crucial to investigate whether NFAT-induced FASLG protein in MKs is able to exert biological activity as well.

One of the main findings of our study suggests that FASLG expression in MKs is of intrinsic biological significance for these cells. First, transcription of the FASLG gene in MKs is not only followed by expression of the protein, but the protein is also appropriately transported to the MK surface (figure 5.11). Second, we demonstrate that expression of the membrane protein is functional, as it conferred to the MK the ability to induce programmed cell death in bystander cells (figures 5.12 and 5.14).

Of note, as we demonstrate in this study, the propensity of MKs to induce apoptosis is not a constitutive characteristic of these cells, but requires prior stimulation with agents that increase the intracellular calcium to levels sufficient to activate the calcineurin/NFAT pathway. We also provide the explanation for this observation on a molecular level, as we show that expression of the FASLG gene in MKs follows the same inducible, calcineurin/NFAT-dependent pattern as in T lymphocytes. The physiological ligand which is capable to activate MKs *in vivo* is presently unknown and may depend on the respective microenvironment. Collagen (an extracellular matrix protein present in the BM) as well as thrombin are possible candidates, as treatment of MKs with either agent is able to induce activation of the calcineurin/NFAT pathway *in vitro* and regulates proplatelet formation.[143] Furthermore, the majority of MKs spend their lifetime in BM until they undergo apoptosis and release platelets into the blood stream. Collagen, the main component of connective tissue, is abundantly found in the extracellular matrix of the BM microenvironment and blood vessels, thus it is possible that this protein functions as physiological ligand for MK stimulation.

It has been observed that MK apoptosis and proplatelet formation is augmented by treatment of MKs with an agonist anti-Fas antibody which in fact mimics the action of

FASLG.[184] Interestingly, our finding shows that MKs are also able to induce apoptosis in other MKs. The biological relevance of this phenomenon is unknown and merits further investigation. Practically, activation of the Fas/FASLG pathway between MKs would require that they are located in close proximity to each other. Considering their rarity, this is unlikely to occur during megakaryopoiesis. However, it has been shown that some cell types express both Fas and FASLG concurrently that could potentially lead to autocrine cell death.[211, 212] After conclusion of their maturation, MKs are known to migrate within the BM to blood vessels, where they release their platelets in a process called proplatelet formation. As an intriguing hypothesis, MK apoptosis under these circumstances might be induced by other, co-migrating MKs, which had been activated within the BM vascular niche to upregulate expression of FASLG on their membrane.

It has to be noted, however, that blocking antibodies against Fas and FASLG are only partially able to suppress apoptosis induced by ionomycin treatment in MKs (figure 5.12). Therefore, other pathways than Fas/FASLG probably contribute to the regulation of apoptosis in MKs as well. Possible candidates are members of the Bcl-2 family, which have been identified to regulate programmed cell death in platelets *in vivo*, possibly by inhibiting calcineurin activation.[213] Overexpression of Bcl-2 for instance, results in decreased proplatelet formation and lower platelet counts in mice, whereas MK numbers remained unchanged.[214-216]

Finally, our results suggest that MKs may be capable of inducing apoptosis in other Fas-bearing cells, notably lymphocytes. FASLG, via interaction with its receptor Fas, plays a key role in the regulation of programmed cell death, for example in cells of the immune system. It is now obvious that the cells in the BM are not only connected in terms of proximity but by functionality, and it is increasingly recognized that MKs, despite their rarity, exert biological functions independent from their role as platelet producers. Several studies, for example, show that they are involved in the maintenance of bone homeostasis.[217-219] They produce cytokines that induce fibrosis in the BM and other organs including spleen and lung.[220-222] Growth factors and cytokines such as PDGF, bFGF and TGF-ß which are produced by MKs, are found to be the major mediators of increased reticulin fibers in myelofibrosis disorders.[221, 223] Under certain circumstances, they appear in blood, spleen, liver and lung, where they migrate to sites of active inflammation.[122, 124, 220] For example, in patients suffering from burns or in experimental models of shock, an accumulation of MKs has been described in the lung.[124]

Hence, MKs interact with their microenvironment, but their cellular counterparts and the mechanisms of cellular interaction are incompletely understood. In this respect, our study provides interesting findings. The physiological relevance of Fas/FASLG-mediated apoptosis induction by MKs in bystander cells, however, needs to be elucidated in further studies.

6.3 NFATc2 regulates multiple genes in megakaryocytes

In order to obtain a comprehensive picture of the NFAT function in MKs, it is necessary to discover other NFAT target genes in this cell type. For the identification of additional NFATc2 regulated genes, an RNA-Seq analysis using *ex vivo* differentiated murine MKs was performed.

Though the list of genes which were dependent on the presence of NFATc2, was clear and significant, at the same time it was quite unexpected. An interesting finding was a strong and broad immune-activation phenotype regulated by NFATc2, going along with the strong induction of many genes involved in various aspects of the immune response. While such an immune-stimulatory effect would have been expected as a response to NFAT stimulation, the genes involved in the observed response do hardly overlap with the canonical NFAT target genes. Interestingly, the pathways induced by the treatment are mostly identical to the known NFAT-induced pathways (IL-2, IL-4, IL-5, TNF, IFN), but the observed transcriptional regulation mainly affects the receptors rather than the cytokines.

Another interesting finding of this study was the strong induction of genes involved in nitric oxide (NO) biosynthesis. Nos2, Ch25h, Arg1, Slc7a2 and Cav1 genes are seen on the top of the gene list that were sorted by the transcriptional effect of ionomycin-stimulation in the WT cells. Interestingly, four out of six most strongly induced genes have a role in NO biosynthesis from arginine. This pathway is known to play an important role in macrophages, however, the upregulation factor of the NO biosynthesis genes is much higher than that of most other macrophage-specific markers, which suggests that this pathway might be important in MKs.

The current findings contribute a novel understanding of NFAT transcriptional regulation in mature MKs. However, data validation by quantitative PCR or PCR-array as well as by functional assays of differentially expressed genes is still necessary and should be scheduled for future studies. Furthermore, these data represent a useful source for identification of new regulators which cooperatively work with NFAT in different cellular processes of MKs.

7 Conclusion

The NFAT transcription factors are primarily known for their central role in transcriptional regulation of lymphocytes. Recently, the family member NFATc2 has been shown to be expressed in hematopoietic stem cells as well as in specific myeloid cell types, especially in MKs. The function of this transcription factor in this context, however, was unknown. The aim of the present work was to contribute to the understanding of the role of NFAT in hematopoietic cells, with a particular focus on MKs.

We show that lack of NFATc2 in hematopoietic stem cells results in perturbed hematopoiesis in mice, namely BM hypoplasia, peripheral blood cytopenia and extramedullary hematopoiesis. Our results point to disrupted interaction of the NFATc2-deficient stem cell with its BM hematopoietic niche. One possible mechanism is an inefficient homing capability of HSCs, but this could not be experimentally proven. Alternative possibilities include cell-intrinsic mechanisms such as cell cycle or proliferation, but the *ex vivo* characteristics of NFATc2$^{-/-}$ seem to be normal.

While the activation of NFATc2 has shown to be dispensable for megakaryocytic differentiation, previous studies had suggested that NFATc2 plays a role in the regulation of inducible gene expression in mature MKs. Here, we demonstrate for the first time that expression of the Fas ligand protein in MKs occurs in an inducible, calcineurin/NFAT-dependent manner, and is transported to the MK membrane. Most importantly, we prove that Fas ligand protein induced by NFAT signaling is biologically active and able to induce apoptosis in Fas-bearing bystander cells.
Finally, we have identified by RNA sequencing analysis of NFATc2-expressing and -deficient cells the entire set of genes which is induced by NFATc2 in stimulated MKs. Functional pathway analysis suggests an involvement of NFATc2 in pro-inflammatory pathways in these cells. The significance of these findings has to be addressed in further studies.

8 Thesis

- NFATc2, a member of the NFAT (Nuclear Factor of Activated T cells) family, has a biological role in hematopoiesis. Lack of NFATc2 in hematopoietic stem cells leads to hematological and osteogenic abnormalities in mice.

- Hematopoietic stem cells with NFATc2 deficiency are able to transfer the phenotype of aged NFATc2-deficient mice to wild type mice.

- NFATc2 may influence the cross-talk between hematopoietic stem cells and the BM stroma cells.

- NFATc2 deficiency is associated with hematological abnormalities, and therefore might be exploited as therapeutic target for the development of new drugs for treatment of hematological disorders in the future.

- NFATc2 is a transcriptional regulator of gene expression in mature megakaryocytes.

- Activation of the calcineurin/NFAT-pathway leads to the expression of the Fas Ligand gene and subsequently, its protein.

- Fas Ligand protein induced by the activation of calcineurin/NFAT pathway is biologically functional as it is transferred to the membrane of megakaryocytes and is able to induce programmed cell death in Fas-positive bystander cells.

- Megakaryocytes fulfill a cell-intrinsic function which is independent of platelet production. They may contribute to a number of physiological mechanisms such as bone hemostasis or inflammation.

- Further studies should aim to investigate the regulatory role of NFAT in hematopoiesis, in hematopoietic cells of various lineages (specifically megakaryocytes) and the stroma components of the hematopoietic niche.

- The expression profile of genes regulated by NFATc2 in stimulated megakaryocytes suggests a role of this factor in pro-inflammatory conditions. The biological significance of this finding has to be verified in further studies.

9 List of publications

Arabanian, L.S., Haase, M., Habermann, I., von Bonin, M., Rauner, M., Ehninger, G., Kiani, A. Lack of the transcription factor NFAT (Nuclear Factor of Activated T cells) c2 in hematopoietic progenitors cells results in profound hematological abnormalities in mice. *In preparation.*

Arabanian, L.S., Kujawski, S., Habermann, I., Ehninger, G., Kiani, A. Regulation of fas/fas ligand-mediated apoptosis by nuclear factor of activated T cells in megakaryocytes. *British Journal of Haematology* (2012) Feb; **156**(4):523-34.

Bauer,W., Rauner, M., Haase, M., Kujawski, S., Arabanian, L.S., Habermann, I., Hofbauer, L.C., Ehninger, G., and Kiani, A. Osteomyelosclerosis, anemia and extramedullary hematopoiesis in mice lacking the transcription factor NFATc2. *Haematologica* (2011) Nov; **96**(11):1580-8.

Contribution to scientific seminars and conferences

Oral and poster presentation: Regulation of Fas/Fas ligand-mediated apoptosis by nuclear factor of activated T cells in megakaryocytes. Laleh S. Arabanian, Satu Kujawski, Ivonne Habermann, Gerhard Ehninger, Alexander Kiani.
American Society of Hematology, Annual Meeting 2009
New Orleans, Luisiana, USA

Participitation in Stem Cell Congress 2010, Dresden, Germany and Berlin Max Delbrück Center (MDC) conference, 'Stem cells in Developement and Diseases' 2011, Berlin, Germany.

Poster presentation: Lack of transcription factor NFATc2 leads to profound hematological abnormalities in mice. Laleh S. Arabanian, Michael Haase, Ivonne Habermann, Malte von Bonin, Claudia Waskow, Rainer Ordemann, Gerhard Ehninger and Alexander Kiani.
American Society of Hematology, Annual Meeting 2011
San Diego, California, USA

Oral and poster presentations at SFB 655 'from cells into tissues ' retreats of 2009 and 2011.

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11 Declaration

Declaration According to the §5.5 of the Doctorate Regulations

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of such aids other than specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from March 2008 to June 2012 under the supervision of PD. Dr. Alexander Kiani at the University Hospital Carl Gustav Carus at the Technical University of Dresden, Department of Medicine I (director: Prof. Dr. Gerhard Ehninger).

I declare that I have not undertaken any previous unsuccessful doctorate proceedings.

I declare that I recognize the doctorate regulations of the *Fakultät für Mathematik und Naturwissenschaften* of the *Technische Universität Dresden*.

Dresden, June 2012 _____

Laleh Sadat Arabanian

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I. List of figures and tables

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II. List of abbreviations

APC, allophycocyanin

bFGF, basic fibroblast growth factor
BFU-E, burst-forming unit of erythrocyte
BM, bone marrow
BSA, bovine serum albumin

CaN, calcineurin
CB, cord blood
cDNA, complementary DNA
CFU-G, colony forming unit of granulocyte
CFU-GM, colony forming unit of granulocyte-macrophage
CFU-M, colony forming unit of macrophage
CFU-Mk, colony forming unit of megakaryocyte
CLP, common lymphoid progenitor
CMP, common myeloid progenitor
CsA, cyclosporin A

DAPI, 4', 6-diamidino-2-phenylindole
DE, differentially expressed
DEG, differentially expressed gene
DNA, deoxyribonucleic acid

ECM, extra cellular matrix
EDTA, ethylene diamine tetraacetic acid

FACS, fluorescence activated cell sorting
FASLG, Fas ligand

FCS, fetal calf serum
FDR, false discovery rate
FITC, fluorescein isothiocyanate
foldChange, fold change from condition A to condition B

GO, gene ontology
GM-CSF, granulocyte-macrophage colony stimulating factor

HGB, hemoglobin
HPC, hematopoietic progenitor cell
HRP, horseradish peroxidase
HSC, hematopoietic stem cell
HSCT, hematopoietic stem cell transplantation

id, gene id (e.g. ENSMUSG00000047642)
IL-1, interleukin 1
Iono, ionomycin

KO, knockout
KOst, knockout stimulated

log2FoldChange, logarithm (to basis 2) of the fold change
LSK, Lin-/Sca-1+/cKit+

mAb, mouse anti-body
MDS, myelodisplastic syndrome
MK, megakaryocyte
MPB, mobilized peripheral blood
MPi, metalloproteinase inhibitor
mRNA, messenger ribonucleic acid

NFAT, nuclear factor of activated T cells
NF-E2, nuclear factor–erythroid 2

NGS, next-generation sequencing

OPG, osteoprotegerin

PBS, phosphate buffered saline
PCR, polymerase chain reaction
PDFG, platelet-derived growth factor
PE, phycoerythrin
PFA, paraformaldehyde
PI, propidium iodide
PMA, phorbol 12-myristate 13-acetate
p, p value
pval, p value for the statistical significance of the fold change

RNA, ribonucleic acid
rpm, revolutions per minute
RT, room temperature
RT-PCR, reverse-transcription PCR
RUNX, runt-related transcription factor

SCF, stem cell factor
sFASLG, soluble Fas ligand

TPO, thrombopoietin

VEGF, vascular-endothelial growth factor

WT, wild type
WTst, wild type stimulated