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1. Introduction

1.1. Myeloid lineage commitment: granulopoiesis versus monopoiesis

All living organisms are continuously exposed to substances that are capable of causing them harm. To protect themself higher organisms have developed a general defence mechanism, the immune system, which is made up of a complex network of cells, tissues, and organs that work together. The key players mediating the organism's protection are specialized immune cells that arise from multipotent hematopoietic steam cells (HSCs). Through the life, these highly proliferative and self-renewal HSCs are responsible for the development, maintenance, and regeneration of a multifaceted repertoire of mature blood cells that differentiate during a highly regulated process known as haematopoiesis. As the first step of such a differentiation process multipotent HSCs commit either to the myeloid or lymphoid lineage by differentiating to common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) [1;2]. The CLP in turn gives rise to T, B and natural killer (NK) cells, whereas the CMP further differentiates into the granulocyte monocyte progenitor (GMP) or the megakaryocyte erythrocyte progenitor (MEP). Finally the GMP dives birth to the myelopoietic cell types we are interested in - granulocytes and monocytes [3;4] (Fig. 1).

The lineage choice of the bi-potent GMP precursor to either granulocytic or monocytic direction was shown to be driven by the differential expression of complex network of lineage specific transcription factors [5]. Thus, C/EBPα was found to be preferentially expressed in and required for granulocytic commitment, whereas, for example, cJun was shown to promote monocyte differentiation [6;7]. An overview on the most critical transcription factors driving the granulocytic versus monocytic differentiation is illustrated in Figure 2.

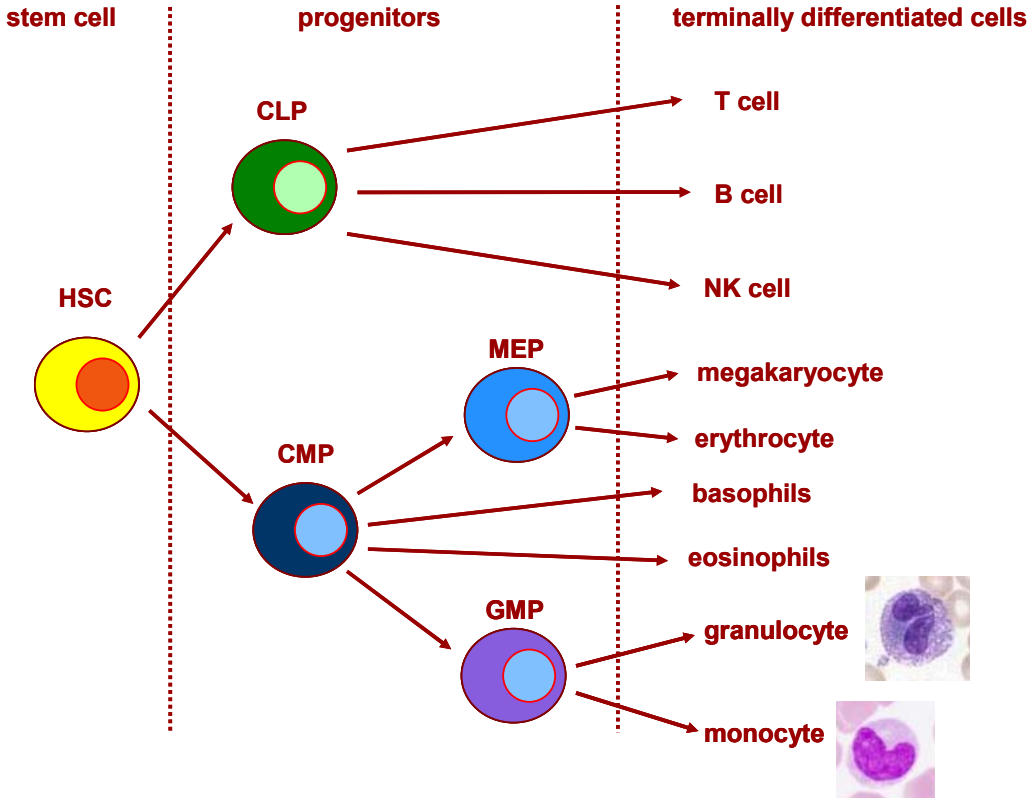

Figure 1. Haematopoiesis - schematic representation of the lineage differentiation. The hematopoietic stem cell (HSC) gives rise to the common myeloid progenitor (CMP) or common lymphoid progenitor (CLP). CLP further differentiates to T, B or natural killer (NK) cells, whereas the CMP gives rise to the granulocyte monocyte progenitor (GMP) or the megakaryocyte erythrocyte progenitor (MEP). Next, the MEP commits either to the megacaryocyte or the erythrocyte lineage, whereas the GMP further differentiates into granulocytes or monocytes. Furthermore basophils and eosinophils arise from the CMP. Figure adapted from Myatt S. et al [8].

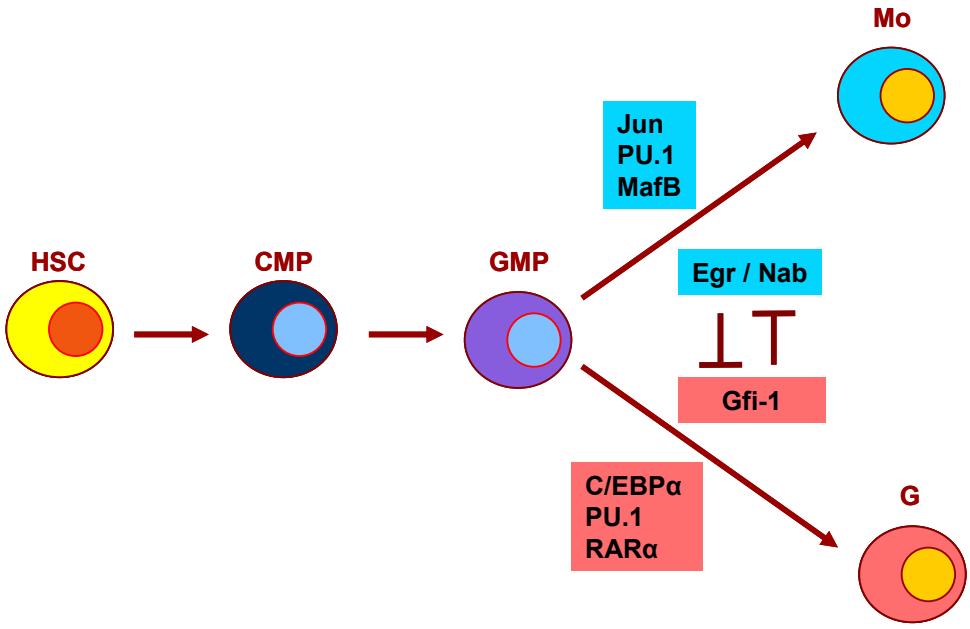

Figure 2. Transcriptional network of monopoiesis versus granulopoiesis. Transcription factors, critical for the monocytic versus granulocytic lineage differentiation, are listed in the corresponding boxes.

The process of myeloid differentiation was shown to be regulated by signals of the local microenvironment including the hematopoietic cytokines, which are found in the bone marrow where they act on the different progenitors such as the CMPs and drive their differentiation into mature myeloid cells. Cytokine binding to the cognate cell-surface receptors in a stage- and lineage-specific manner triggers the activation of the intracellular signal transduction pathways that facilitate the differentiation process. The diverse signalling pathways may thereby promote myelopoiesis by stimulating expansion of a progenitor pool, supporting cellular survival, or by directly driving the phenotypic changes associated with differentiation. Ultimately, the specific signalling mediators that trigger the differentiation process converge on myeloid transcription factors that are critical for terminal commitment [4;9;10].

1.2. Activation and signalling of the MAP kinase pathway

Cellular behaviour in response to extracellular stimuli is mediated through intracellular signalling pathways such as the mitogen-activated protein kinase (MAPK) pathways. The MAPK pathways transduce a variety of external signals, leading to a wide range of cellular responses, including growth, differentiation, inflammation and apoptosis [11;12]. Like other cascades the MAPK signalling cascade serves as an amplifying mechanism to enhance the upstream signal intensity on the way to the downstream targets found in the nucleus. The signal transduction thereby begins from the MAP kinase kinase kinases (MAPKKKs), which are activated by an external stimulus [13]. The MAPKKKs next activate the MAP kinase kinases (MAPKK), which in turn activates the downstream MAP kinases (MAPK) [14] (Fig. 3). MAPK are among the most ancient and evolutionarily conserved signal transduction pathways transferring stimuli from the cell surface to the nucleus [15]. Three major groups of MAPKs have been identified in mammalian cells: the extracellular signal-regulated protein kinases (ERK), the cJun N-terminal or stress-activated protein kinases (JNK/SAPK), and the p38 MAPK. The JNK family is thereby composed of JNK1, JNK2, and JNK3, while the ERK kinase group includes two members (ERK1 and ERK2). The p38MAPK family consists of 4 different isoforms (p38α, p38β, p38γ, and p38δ) that share significant structural homology [16]. Activation of the various MAPKs is mediated via dual phosphorylation on threonine (Thr) and tyrosine (Tyr) residues present in specific motifs (ThrXaaTyr). Such phosphorylation is regulated by upstream dual-specificity kinases, the MAPKKs, which are capable of phosphorylating

MAP kinases on both serine/threonine, as well as on tyrosine residues. The MAPKKs thereby exhibit relative specificity for the substrate MAPK proteins that they phosphorylate. MKK1 and MKK2 phosphorylate ERK kinases; MKK3, MKK4, and MKK6 phosphorylate p38 kinases, while MKK4 and MKK7 regulate dual phosphorylation of JNK kinases [17;18] (Fig. 3). Interestingly, MAPKKs also exhibit relative specificity for the different isoforms that they target within each group of MAPKs. For instance, among the different p38MAPK subtypes, MKK6 functions as a common activator of all four isoforms (p38α, p38β, p38γ, and p38δ), while MKK3 activates p38α, p38γ, and p38δ, but not p38β [19].

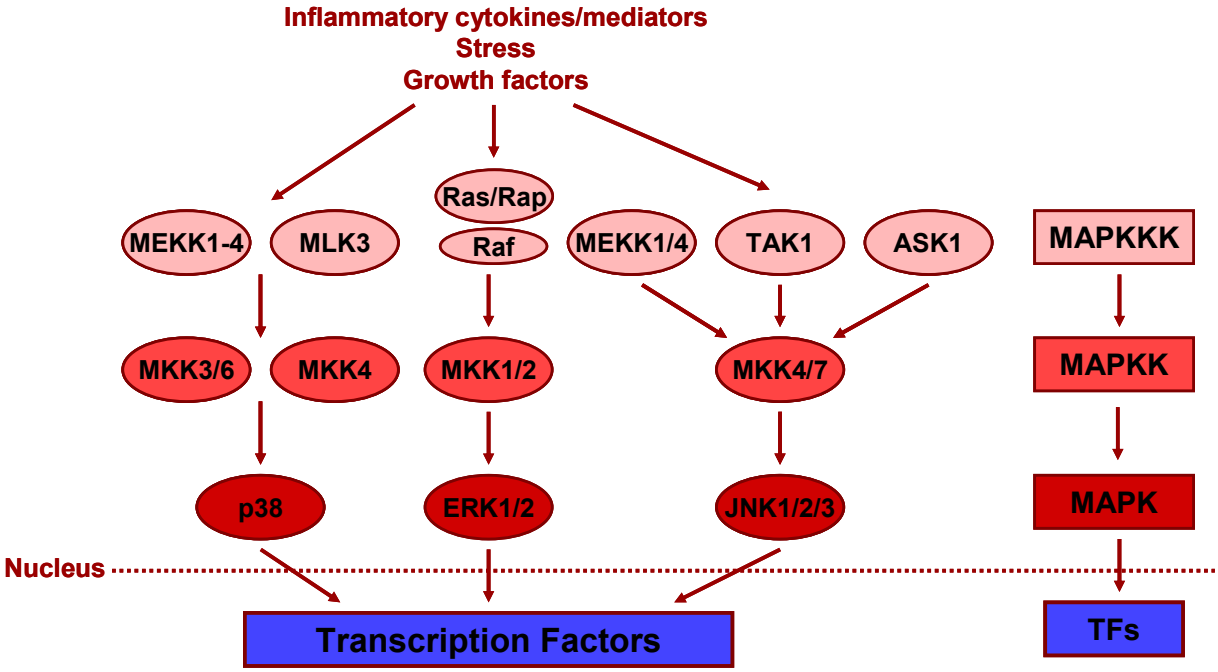

Figure 3. The MAPK signalling cascades.
External stimuli trigger the activation of MAPKKK, which in turn activates a MAPKK. Next, MAPK is activated and transduces the signal into the nucleus, modulating transcriptional networks. Figure adapted from Thalhamer T. et al [20].

The proliferation and differentiation of hematopoietic cells is known to be regulated by various cytokines and growth factors that activate the MAPK signalling pathways to generate their effects. Among them, pro-inflammatory cytokines were shown to modulate myeloid differentiation via the MAPK signalling cascade [21;22].

1.3. Transcriptional mechanism of granulocytic versus monocytic differentiation – the role of C/EBPα and cJun

The process of myeloid differentiation is tightly regulated by differential expression of lineage specific transcription factors. Generally, lineage commitment has been considered to be an irreversible event: once a progenitor loses its multipotency, it never regains the lost developmental potentials. However, resent studies provide increasing evidence for lineage plasticity [10].

The CCAAT enhancer binding protein α (C/EBPα) is one of the key transcription factors that mediate lineage specification and differentiation of multipotent myeloid progenitors into mature granulocytes. It is a member of the basic-region leucine zipper (bZIP) DNA binding transcriptions factors. This family is characterized by the presence of a basic region that mediates DNA binding and a leucine zipper that allows dimer formation. In addition to forming homodimers, C/EBPα also dimerizes with other members of the C/EBP family and interacts with other proteins [23]. A recent study demonstrated a direct interaction of C/EBPα with the monocytic transcription factor cJun during granulocyte differentiation. The C/EBPα/cJun dimerization blocks the positive feedback regulation of the cJun expression, therefore allowing granulocytic lineage commitment [24]. In addition, several other investigations highlighted the indispensability of C/EBPα for granulocytic differentiation. For example, C/EBPα null mice were shown to lack mature granulocytes, while all other blood cell types were present [25]. In contrast, increased levels of C/EBPα, expressed from an inducible promoter construct, directed the differentiation of bi-potential myeloid cells along the granulocytic pathway. Furthermore, it was demonstrated that the ectopic expression of C/EBPα can prevent TPA-induced monocytic differentiation of bi-potential myeloid progenitor cells [26].

AP-1 (activating proteins 1) are sequence-specific bZIP transcription factors composed of homodimers or heterodimers of the Jun family (cJun, JunD, and JunB) or heterodimers of the Jun family member with any of the Fos family members (c-Fos, FosB, Fra1, and Fra2) or other transcription factors [7;27]. Members of the AP-1 family are regulated both at the transcriptional and the posttranscriptional level by MAPKs [28]. The activity of the Jun family member we are interested in, cJun, is regulated by posttranslational modifications such as phosphorylation [29]. The phosphorylation of Ser-63 and Ser-73, located within

the N-terminal transactivation domain, was shown to potentiate the ability of cJun to activate transcription [30]. Furthermore, it could be demonstrated that such activated cJun is able to autoregulate its expression through a cJun/AP-1 enhancer element in its own promoter [31]. Numerous studies highlighted cJun as a critical regulator of monocytic differentiation. As an example exogenous cJun expression was shown to induce monocyte differentiation of bi-potential myeloid cells. Furthermore, commitment along the monocytic lineage is known to be accompanied by cJun upregulation [32;33].

1.4. The role of MKK6-p38MAPK signalling in chronic inflammatory diseases such as rheumatoid arthritis

Rheumatoid arthritis (RA) is the prototype of a chronic inflammatory disease in humans. Chronic inflammatory processes are based on a sustained and tightly regulated communication network among different cell types. This network comprises extracellular mediators such as cytokines, chemokines, and matrix degrading proteases, participating in inflammatory processes as well as in the bone and cartilage destruction, which is characteristic for RA [34;35].

The development and severity of RA was found to be dependent on pro-inflammatory cytokines such as IL-1 and TNFα that can activate a broad array of intracellular signal transduction mechanisms [36]. Among the cytokine- and stress-activated pathways, MAP kinases are especially important in such disorders as they were shown to increase the production of several mediators of inflammation and cartilage damage [37]. The p38MAPK is of particular interest in RA as it is know to play a major role in the production of pro-inflammatory cytokines including IL-1 and TNFα by activating transcription factors binding to the corresponding promoter regions [38]. Furthermore, p38MAPK participates in other inflammation-related events, such as neutrophil activation, apoptosis, and nitric oxide synthase induction [39-41]. Interestingly, p38MAPK was shown to be expressed and activated in the inflamed synovial tissue, suggesting a critical role in RA [42].

A differential tissue expression and activation of the described p38MAPK isoforms (p38α, p38β, p38γ, and p38δ) was found in the inflamed tissues of patients with RA. The isoforms p38α and p38γ were the most abundantly expressed and predominantly activated. The expression of p38MAPKs was localized to the synovial lining layer and to the blood vessels. Furthermore, monocytes/macrophages, synovial fibroblasts and granulocytes

found in the inflamed tissue showed p38MAPK expression, whereas lymphocytes were rarely positive for any p38 isoform [42]. Interestingly, the distinct p38 isoforms were found to have opposite effects on the regulation of cJun transcription as well as activation in terms of phosphorylation. Thus, p38β was shown to induce cJun phosphorylation whereas, in contrast, the γ and δ isoforms seem to inhibit cJun *trans*-activation by upstream MAPKK [27].

Although the impact of p38MAPK in the development of chronic inflammatory diseases was extensively examined little is known about upstream kinases, that activate these pathways in joint tissues [43]. Among the MAPKKs, MKK3 and MKK6 are thought to be especially important regulators of p38MAPK and represent potential therapeutic targets to modulate pro-inflammatory cytokine production [44]. Both MKK3 and MKK6 are activated upon phosphorylation of serine and threonine residues by upstream MAPKK kinases. Upon activation, MKK3 selectively phosphorylates p38α, γ, and δ whereas MKK6 activates all four p38 isoforms (α, β, γ, and δ) [45;46], which were shown to have opposing effects on cJun activation [27]. Due to the fact, that the MAP kinase kinase MKK6 is able to activate all known p38 isoforms, the response downstream of p38MAPK is thought to be determined by the specific expression patterns of the p38MAPK family members.

1.5. *In vitro* differentiation of distinct myeloid cell subsets

A variety of myeloid cell subsets can be differentiated *in vitro* from hematopoietic stem cells. An established system used in our lab allows the generation of distinct myeloid lineages out of $CD34^+$ cord blood stem cells. As the first step of the differentiation process the multipotent progenitor cells are expanded in the presence of a defined cytokine cocktail mimicking the natural environment found in the bone marrow [5]. After expansion, differentiation into desired myeloid cell subsets is achieved by the corresponding critical cytokines depicted in Figure 4. As an example, the differentiation of granulocytes and monocytes is driven by G-SCF and M-CSF/IL-6, respectively. Validation of differentiation can be achieved by morphological examination monitored by microscopy, as well as by the determination of the expression of the subset specific surface markers (Fig. 4) with FACS analysis. The described differentiation procedures illustrate a facile system for the *in vitro* generation of various myeloid cell subsets [47].

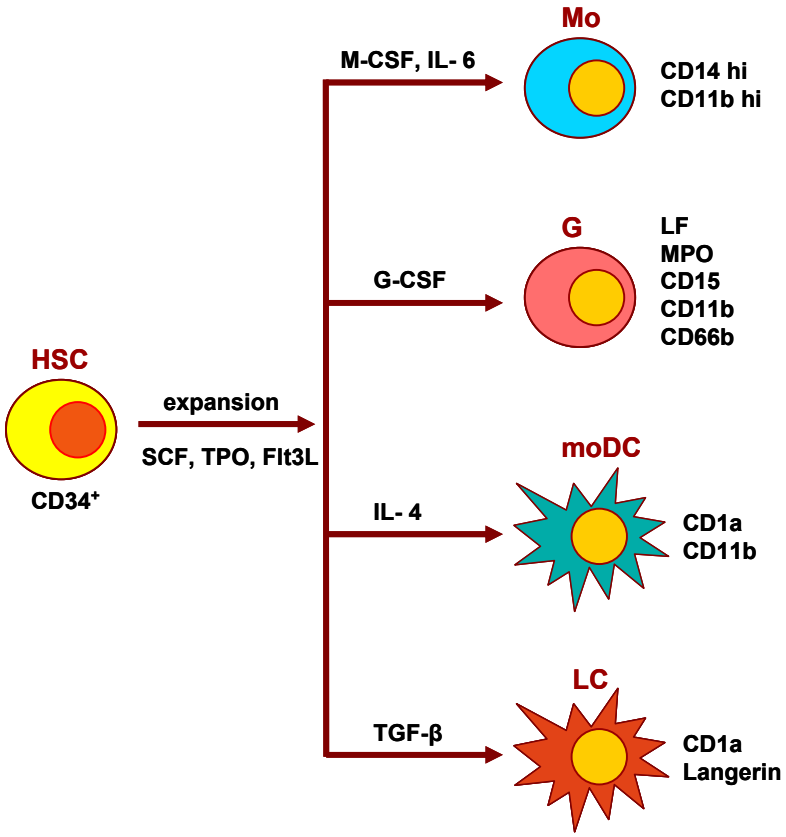

Figure 4. *In vitro* differentiation system for the generation of distinct myeloid cell subsets. Cytokines critical for the commitment of the corresponding myeloid lineage (monocytes (MO); granulocytes (G); monocyte-derived dendritic cells (moDC); Langerhans cells (LC)) as well as the subset specific surface markers are depicted in black.

1.6. Lineage characteristic cell surface markers

To characterize the lineage specificity of the *in vitro* differentiated myelopoietic cells the expression levels of subset specific markers have to be determined by FACS analysis. In the current study we are interested in two particular lineages, granulocytes as well as monocytes. As depicted in Figure 3, granulocytes show expression of CD15, CD11b, CD66b, lactoferrin (LF), and myeloperoxidase (MPO), whereas high expression levels of CD14 and CD11b are characteristic for monocytes. An overview highlights the prominent, cell type characteristic functions of the G- versus Mo-specific markers (Table 1).

Table 1. Expression markers characteristic for the granulocytic and monocytic lineages

CD15	carbohydrate adhesion molecule; expressed on glycolipids and glycopoteins on the neutrophils surface and to some extend on monocytes; mediates chemotaxis, cell-cell interactions and phagocytosis [48-50].
CD66b	GPI-anchored glycoprotein; exclusively expressed on human granulocytes; an granulocytic activation marker; localizes in lipid rafts and interacts with other cell surface molecules, such as CD11b; mediates the adhesion to E-selectin [51;52].
Lactoferrin (LF)	Glycoprotein; member of the transferrin family; found in the secondary granules of neutrophils and broadly distributed within body fluids; important component of the innate immune system with an antimicrobial activity; broad spectrum of functions: regulation of iron homeostasis, host defence against a broad range of microbial infections, anti-inflammatory activity, regulation of cellular growth and differentiation [53-56].
Myeloperoxidase (MPO)	Heme protein; major component of neutrophil azurophilic granules; produces hypohalous acids, a potent oxidant implicated in the microbicidal activity of neutrophils and tissue destruction [57;58].
CD11b	Integrin alpha M chain; important for the adherence of neutrophils and monocytes to stimulated endothelium, and phagocytosis of complement coated particles; forms transmembrane signalling complexes with GPI-anchored glycoproteins [59;60].
CD14	Myeloid-specific leucine rich repeat (LRR) protein; abundant on mature monocytes and macrophages; pattern recognition receptor, acts as a co-receptor for LPS [61;62].
CD86	Type I membrane protein; expressed by antigen-presenting cells; ligand for CD28 and CTLA-4 at the surface of T cells [63-65].

1.7. Inducible gene expression - the Tet-on retroviral gene transductions system

The *in vitro* differentiation approach enables the generation of a variety of myeloid cell subsets from $CD34^+$ cord blood stem cells. To analyse the impact of distinct signalling cascades on commitment as well as on function of myelopoietic cells the Tet-on gene transduction system can be used. This method allows the retroviral tranduction of $CD34^+$ progenitors with a gene of interest, followed by the induction of gene expression at any time point of the differentiation process. In the described system $CD34^+$ cord blood stem cells are sequentially transduced with two retroviral vectors. The first vector encodes a tetracycline activator (TA) and the second vector bears the gene of interest under the control of a tetracyclin response element (TRE) (Fig. 5). To induce gene of interest expression doxycycline has to be added. For the examination of the impact of a factor of interest on cell commitment the transduced gene can be turned on at the desired time point of the differentiation process, whereas for functional analysis within terminally differentiated cells gene induction can be performed at the end of the differentiation period. In addition, this established procedure for primary cells can be furthermore adapted for any cell line.

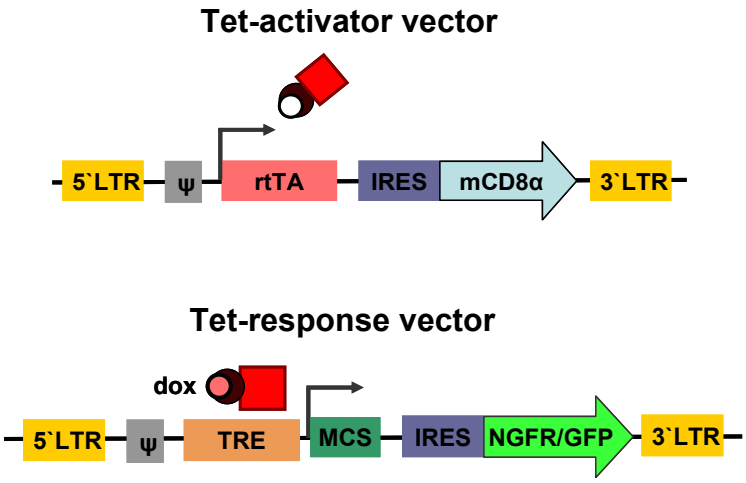

Figure 5. Tet-inducible gene transduction system.
Sequential transduction with two retroviral vectors: the Tet-activator and the Tet-response vector. Tet-activator vector encodes the tetracycline activator (TA) and the expression marker mouse CD8α. The Tet-response vector contains a multiple cloning site (MCS) for the introduction of a gene of interest; a tetracycline response element (TRE) controlling the expression of the introduced gene; the expression marker NGFR or GFP.

2. Aim of the study

In the current study we aim to assess the role of the MAP kinase signalling pathway within myelopoiesis. We focus on one prominent family member, MKK6, as well as its downstream effector p38MAPK and their impact on the plasticity of the myelopoietic sublineage differentiation. Preliminary results from our laboratory suggest the MKK6 signalling cascade to be able to redirect granulocytic cells to the monocytic lineage. Central to this study is the investigation of the molecular mechanisms that underlie the conversion process. Of particular interest are MKK6-p38MAPK induced alterations of transcriptional regulators. Furthermore, we aim to elucidate external stimuli capable to trigger the conversion process and test various combinations of pro-inflammatory cytokines, known to induce MAPK signalling, for their ability to reprogram granulocytic cells to monocytes. Based on the obtained findings we provide a model whereby committed granulopoietic cells undergo monocyte differentiation under inflammatory conditions.

3. Reagents, buffers, and solutions

Table 2. Self-made buffers and solutions

Buffer/Solution	Components
X-vivo + Glutamax + Pen/Strep	X-vivo15 medium (BioWhittaker) + GlutaMAX (2.5 mM; Gibco/Invitrogen) + penicillin/streptomycin (125 U/mL each; Sigma)
DMEM + 10% FCS + Glutamine + Pen/Strep	DMEM (Sigma) + 10 % Foetal Bovine Serum (Gibco) + L-Glutamin (2 mM; Sigma) + penicillin/streptomycin (125 U/mL each; Sigma)
RPMI + 10% FCS + Glutamine + Pen/Strep	RPMI (Sigma) 10 % Foetal Bovine Serum (Gibco) + L-Glutamin (2 mM; Sigma) + penicillin/streptomycin (125 U/mL each; Sigma)
dH_2O	Aqua bidest. "Fresenius" (Fresenius Kabi)
2xHBS	8 g NaCl; 6.5 g HEPES; 105 mg Na_2HPO_4 in 500 ml H_2O (pH 7.0)
RetroNectin	90µl RetroNectin (1mg/ml; Takara Bio) in 3 ml 1xPBS
Beriglobin	Beriglobin (CSL Behring) diluted 1:8 in PBS/BSA/Azid
4xSampleBuffer	0.5M Tris/HCl (pH 6.8); 40% glycerol (Roth); 4% SDS (Roth); 5 µl/ml Bromphenolblue (Roth); 5% Mercartoethanol (Sigma)
2xSamleBuffer/Tris-Base	0.5 ml 4xSample Buffer; 0.5 ml Tris-base (pH 11)
Separating gel (10%)	2667 µl 30% Acrylamid/Bis (29:1) (BioRad); 2000 µl Tris/HCl pH 8.8; 3203 µl H_2O; 40 µl 20% SDS (Roth); 80 µl 10% APS (Roth); 10 µl TEMED (BioRad)
Stacking gel (4%)	396 µl 30% Acrylamid/Bis (29:1) (BioRad); 378 µl Tris/HCl pH 6.8; 2196 µl H_2O; 15 µl 20% SDS (Roth); 20 µl 10% APS (Roth); 5 µl TEMED (BioRad)
10x SDS-PAGE runnig buffer	30.2 g Tris (Roth); 144 g Glycin (Roth); 10 g SDS (Roth) in 1000 ml H_2O
10x Blotting buffer	30,3 g Tris (Roth); 144 g Glycin in 1 L H_2O
Stripping buffer	100 ml SDS (Roth); 62.5 ml 1M Tris HCl pH 7.5; 7.3 ml β-Mercaptoethanol (Sigma); 830.2 ml dH_2O

10x PBS	400 g NaCl (Roth); 10 g KCl (Roth); 72 g Na_2HPO_4 (Roth); 10 g KH_2PO_4 (Roth); adjust to pH 7,4; in 5 L H_2O
1x PBST	1xPBS/ 0.05% Tween (Tween20; Roth)
10x TBS	24.2 g Tris Base; 80 g NaCl in 1 L dH_2O (pH adjusted to 7.6)
1x TBST	1xTBS/0.05% Tween (Tween20; Roth)
1x PBS/BSA/Azid	1xPBS; 20% BSA (Roth); 0,4% Na_3N (Roth)
Hunt Buffer	20 µM Tris-HCl (pH 8); 100 µM NaCl (Roth); 1 mM EDTA (Roth); 0,5% NP-40 (Calbiochem Novabiochem Corporation)
Dephosphorylation Buffer	50 mM Tris-HCl (pH 8); 1 mM $MgCl_2$; 0.5% TX-100 in dH_2O
50xTAE Buffer	242 g Tris/HCl; 57.1 ml acetic acid; 37.2 g $Na_2EDTA.2H_2O$ in 1 L H_2O
5X KCM Buffer	0.5 M KCl ; 0.15 M $CaCl_2$; 0.25 M $MgCl_2$ in dH_2O
SOC-medium	2 g tryptone (Roth); 0.5 g yeast extract; 1 ml 1 M NaCl (Roth); 0.25 ml 1 M KCl (Roth); 1 ml 2 M Mg^{2+} (Roth); 1 ml 2M glucose (Roth) in 100 ml H_2O
LB^{Amp} medium	LB medium (10 g tryptone (Roth); 5 g yeast extract (Roth); 5 g NaCl (Roth); 100 µg/ml Ampicillin (Applichem)
LB^{Amp} plates	15 g agar (Roth) in 1L LB^{Amp} medium

4. Material and Methods

4.1. Cell lines

PhoenixE cells were maintained in DMEM + 10% FCS + Glutamine + Pen/Strep on cell culture dishes (Nunclon™Δ Surface, Nunc). HL60-TA-NFGR and HL60-TA-d.a.MKK6 cells were cultured in RPMI + 10% FCS + Glutamine + Pen/Strep in cell culture flasks (Nunclon™Δ Surface, Nunc). All cell lines and primary cells were maintained at 37°C in the 5% CO_2 incubator.

4.2. Granulocyte and monocyte differentiation

Granulocytes (G) and monocytes (Mo) were *in vitro* differentiated from $CD34^+$ stem cells isolated from cord blood of healthy donors [47]. After isolation the $CD34^+$ cells were expanded for 3 days in X-vivo + Glutamax + Pen/Strep medium supplemented with SCF (50 ng/ml), Flt3L (50 ng/ml), and TPO (50 ng/ml). For G differentiation $2x10^4$/ml cells were transferred into the granulocyte-mix (X-vivo + Glutamax + Pen/Strep medium supplemented with G-CSF (100 ng/ml) and SCF (20 ng/ml)) and cultured for additional 11 days. For Mo commitment, cells were cultured for 10 days in the monocyte-mix (X-vivo + Glutamax + Pen/Strep medium supplemented with M-CSF (100 ng/ml), IL-6 (20 ng/ml), SCF (20 ng/ml), and Flt3L (50 ng/ml)). All cytokines used were obtained from PeproTech.

4.3. Stimulation with pro-inflammatory cytokines

In vitro differentiated granulocytes, monocytes and HL60 cells were stimulated with different combination of pro-inflammatory cytokines (GM-CSF; GM-CSF/TNFα; GM-CSF/TNFα/IL-1β; TNFα/IL-1β) for 48 h (primary cells) or 72 h and 96 h (HL60 cell line). Following cytokines and final concentrations were used: GM-SCF (10 ng/ml), TNFα (25 ng/ml) and IL-1β (10 ng/ml). For control purpose HL60 cells were additionally stimulated with ATRA (1 μM) and VitD3 (25 ng/ml). GM-SCF, TNFα and IL-1β were obtained from PeproTech; ATRA and VitD3 were from Sigma.

4.4. Retroviral infection and gene transduction

For retroviral infection of HL60-TA-NFGR and HL60-TA-d.a.MKK6 cells with the control vector (pMCSV(puro) or HR-I-GFP) or the vector of interest (pMCSV(puro)-antisense cJun; HR-I-GFP-antisense cJun; HR-I-GFP-DN cJun), the PhoenixE (PhE) packaging cell line and a calcium-phosphate precipitation method were used. The infection procedure was performed according to the Nolan Lab protocol for retroviral transfection [www.stanford.edu/group/nolan]. For optimal transfection efficiency PhE cells were freshly plated onto a 6 cm dish 24 h prior to transfection to reach sub-confluent stage. Next, the transfection mix [10 µg DNA; 61 µl 2M $CaCl_2$; 430 µl bdH_2O; 500 ml 2xHBS] was prepared, vortexed for 10 sec and incubated for 15 min at room temperature (RT). During incubation time, 3 µl 50 mM Chloroquin (Sigma) were added to the cells for 10 min at RT followed by a drop wise addition of the transfection mix. The PhE cells were incubated with the DNA precipitates for 6 h at 37°C. Next, the medium was removed and a fresh medium (DMEM + 10% FCS + Glutamine + Pen/Strep) was added. After 48 h of incubation the first infection cycle was performed. The supernatant of transfected PhE cells was collected and the plates were subsequently supplemented with a fresh medium. Next, the supernatant was filtered (0.45 µm filter, IWAKI) and 500 µl/well were transferred on a 24-well-suspension plate (CellStar suspension culture plate, Greiner) for 3 h to 6 h at 37°C. Prior to the addition of the viral supernatant, the suspension plates were pre-coated with 300 µl/well RetroNectin (Takara Bio) overnight at 4°C followed by RetroNectin removal and blockage with 1 ml/well DMEM + 10% FCS + Glutamine + Pen/Strep for 15 min at RT. Upon expiration of the incubation period, 300 µl of the virus containing supernatant were removed and $1x10^5$ HL60-TA-NFGR or HL60-TA-d.a.MKK6 cells in 500 µl medium were added onto the virus pre-coated plate and incubated with the virus overnight. Then, the second infection cycle was performed. 500 µl medium was removed from the infected cells followed by the addition of 500 µl fresh viral supernatant collected from PhE cells and purified as described above. Six hours after infection, 500 µl medium was removed followed by an addition of 500 µl fresh RPMI + 10% FCS + Glutamine + Pen/Strep and an incubation at 37°C overnight. Next day, the cells were collected, washed three times with 1xPBS (Sigma) and resuspended at the desired cell density in RPMI + 10% FCS + Glutamine + Pen/Strep. In case of the pMSCV(puro) vector the cells were selected in the presence of puromycin (2µg/ml; Sigma) for 10 days.

4.5. Modulation of the d.a.MKK6 expression levels/p38MAPK activation

To induce the expression of d.a.MKK6 alone or in combination with the gene of interest (e.g. antisense cJun or dominant negative cJun), doxycycline (2 µg/ml; Sigma) was added for the time period of 48 h to HL60-TA-d.a.MKK6 cells.

To modulate the expression levels of d.a.MKK6 and thereby p38MAPK activation various concentrations of doxycycline (0.1 µg/ml, 0.25 µg/ml, 0.75 µg/ml, 2 µg/ml, and 4 µg/ml) were used.

To block the signal transduction downstream of MKK6 the p38MAPK inhibitor (SB203580, Calbiochem) and the JNK inhibitor (SP600125, Calbiochem) were used. The corresponding inhibitor was added at the final concentration of 10 µM to HL60-TA-d.a.MKK6 cells simultaneously with the d.a.MKK6 induction by doxycycline.

The applied Tet-on system allows to restrict the expression of d.a.MKK6 to a desired time period. Three experimental approaches were used to achieve transient induction of the MKK6-p38MAPK signalling cascade. (I) The "dox washing" method: after induction by doxycycline for indicated time periods (2 h, 4 h, 6 h, and 8 h) HL60-TA-d.a.MKK6 cells were washed twice with 1xPBS and resuspended in fresh medium. (II) Use of p38MAPK inhibitor: the p38MAPK inhibitor was added to HL60-TA-d.a.MKK6 cells after 6 h of d.a.MKK6 induction. (III) Combinatorial approach: 6 h after d.a.MKK6 induction HL60-TA-d.a.MKK6 cells were washed to remove the doxycycline followed by the addition of p38MAPK inhibitor.

4.6. Inhibition of the proteasome machinery

To analyse the mechanism that underlies the MKK6-triggered C/EBPα downregulation the proteasome inhibitor (MG132, PeptaNova) at 10 µM was added to the HL60-TA-d.a.MKK6 cells 6 h after the induction of d.a.MKK6.

4.7. SDS-PAGE and Western blot analysis

Cells were washed with 1xPBS, resuspended in the appropriate volume of 2xSampleBuffer ($1x10^5$ cells/10µl) and lysed by heating at 95°C for 5-10 min. Proteins were resolved by a 10% SDS-PAGE and transferred to a PVDF membrane (Immobilon Transfer Membranes;

Millipore) at 100 V for 90 min using a Bio-Rad Criterion Blotter (Bio-Rad). After the transfer the membranes were blocked with 5% dry milk in 1xPBST or 10% BSA in 1xTBST for 1 h at RT followed by the incubation with the primary antibody overnight at 4°C. Next, membranes were washed three times for 5 min with 1xPBST or 1xTBST followed by the addition of the horseradish peroxidase (HRP)–conjugated secondary antibody for 1-2 h at RT. Table 3 summarizes the primary and secondary antibodies used.

Table 3. List of primary and secondary antibodies

Antibody	**Dilution**	**Second step**	**Company**
P-MKK3/6 (Ser189/207)	1:1000 in 5%BSA/TBST	anti-rabbit-HRP	Cell Signalling
P-p38 MAPK (Thr180/Tyr182)	1:1000 in 5%BSA/TBST	anti-rabbit-HRP	Cell Signalling
P-SAPK/JNK (Thr183/Tyr185)	1:1000 in 5%BSA/TBST	anti-rabbit-HRP	Cell Signalling
p38	1:1000 in 5%BSA/TBST	anti-rabbit-HRP	Cell Signalling
MKK6	1:500 in 2.5%Milk/PBST	anti-rabbit-HRP	BioLegend
SAPK/JNK	1:1000 in 5%BSA/TBST	anti-rabbit-HRP	Cell Signalling
P-cJun (Ser63)	1:1000 in 5%BSA/TBST	anti-rabbit-HRP	Cell Signalling
Jun	1:750 in 2.5%Milk/PBST	anti-mouseHRP	BD Pharmingen
C/EBPα	1:250 in 2.5%Milk/PBST	anti-goat-HRP	Santa Cruz Biotechnology
KLF4	1:1000 in 2.5%Milk/PBST	anti-goat-HRP	Santa Cruz Biotechnology
actin	1.1000 in 2.5%Milk/PBST	anti-rabbit-HRP	Sigma-Aldrich
Secondary antibody	**Dilution**		**Company**
goat anti-rabbit IgG	1:2500		Pierce Biotechnology
goat anti-mouse IgG	1:5000		Pierce Biotechnology
donkey anti-goat IgG	1:1000		Santa Cruz

Secondary antibodies that were used for probing of membranes blocked with dry milk were diluted in 2.5% dry milk in 1xPBST. Dilution of secondary antibodies in 5% BSA in 1xTBST was used for BSA-blocked membranes. Membranes that were probed with more that one primary antibody were each time stripped with stripping buffer for 15 min at 60°C and re-blocked prior to the application of the subsequent antibody. Detection was performed with the chemiluminescent substrate SuperSignal WestDura Extended Duration

Substrate or the SuperSignal WestPico Rabbit IgG Detection Kit (Pierce Biotechnology). Lumi-Imager™ or the Fujifilm Las-4000 were used for data imaging. For densitometric analysis ImageJ was used.

4.8. Protein Dephosphorylation by Alkaline Phosphatase

To perform dephosphorylation, proteins had to be extracted using the Hunt Buffer. The cell pellet (2-4x10^6 cells) was resuspended in 100 µl Hunt Buffer, supplemented with the protease inhibitor (Roche) and the phosphatase inhibitor (Roche) and frozen in liquid nitrogen. Next, the sample was thawed at RT followed by re-freezing in liquid nitrogen. Finally the pellet was thawed at 37°C, frozen again and centrifuged for 30 min at 14000 rpm/4°C. Protein extracts were stored at -80°C. For protein dephosphorylation 50 µl protein extract were resuspended in 1 ml dephosphorylation buffer. 0.5 ml of the protein extract solution was kept untreated and used as a negative control while to the remaining extract (0.55 ml) 20 µl of calf intestinal alkaline phosphatase (CIAP; 1 U/µl; Fermentas) were added. To achieve dephosphorylation samples were incubated for 1 h at 37°C. For protein precipitation TCA (Roth) was added at the final concentration of 10%. The samples were incubated on ice for 30 min followed by centrifugation (10min/16000g/4°C). The pellet was washed once with 500 µl ice-cold acetone (Roth) and spun down (10min/16000g/4°C). Finally, the protein pellet was resuspended in 15 µl 2xSamle Buffer/Tris-Base and kept at -20°C for further use.

4.9. FACS staining

Cells were collected and washed twice with 1xPBS (centrifugation at 300 g/4 min/4°C), resuspended in the appropriate volume of 1xPBS to reach a concentration of 5x10^4-5x10^5 cells per 50 µl. To block unspecific binding 1/10 volume Beriglobin was added and incubated for 10-15 min on ice. 50 µl cell suspension were transferred into micronic tubes (Thermo Scientific) followed by the addition of 10 µl primary antibody (directly conjugated to the flurochrome or biotin conjugated) for 30 min at 4°C. Subsequently, cells were washed twice with 1xPBS/BSA/Azid and the second step antibody (Streptavidin-PerCP) was added and incubated for 30 min at 4°C. Next, the cells were either prepared for FACS by washing once with 1xPBS/BSA/Azid and once with 1xPBS or, in case of an

subsequent intracellular staining, first fixed by the addition of 100 µl Fixation Medium (An Der Grub) for 5 min at RT. After fixation, cells were washed and permeabilized by the addition of 100 µl Permeabilization Medium (An Der Grub) followed by an incubation with 10 µl of antibody directed against intercellular proteins for 30 min at 4°C.
The following murine monoclonal antibodies (mABs) were used: FITC-conjugated mAbs specific for CD15, CD86 (BD Pharmingen); phycoerythrin (PE)-conjugated mAbs specific for CD11b, (BD Pharmingen), LF (intracellular) (An Der Grub); Biotinylated mAbs specific for CD11b, NGFR (BD Pharmingen); allophycocyanin (APC)-conjugated mAbs specific for CD14 (Caltag Laboratories); second step streptavidin (SA)-PerCP (BD Pharmingen). All antibodies were pre-diluted 1:5 with 1xPBS/BSA/Azid in exception of APC-CD14 (1:30), LF (1:2), and SA-PerCP (1:100). The flow cytometric analysis was performed using a LSRII (BD Biosciences). Data were analyzed with CellQuest Pro software (BD Biosciences).

4.10. RNA isolation and cDNA generation

For RNA isolation the RNeasy Mini Kit (QIAGEN) was used. Cells were lysed in 350 µl RLT buffer (QUIGEN) supplemented with β-Mercaptoethanol (10µl per 1000µl RTL) by vortexing for 30 sec. Upon disruption samples could be stored at -80°C. For RNA isolation, 350 µl 70% EtOH were added and the sample was transferred to the RNeasy column and centrifuged for 15 sec at 8000 g. The column was washed with 350 µl RW1 buffer (15 sec/8000 g) followed by the addition of 80 µl of a DNase solution (10 µl DNase I stock solution; 70 µl RDD buffer) and incubation for 15 min at RT. Next, the column was washed with 350 µl RW1 buffer (15 sec/8000 g) followed by an additional washing with 500 µl RPE buffer (15 sec/8000 g) and PRE buffer (2 min/8000 g). To remove the residual fluid the column was further centrifuged at full speed for 1 min. For RNA elution, RNase-free water (20 µl) was used (1 min/8000 g). To increase the RNA yield the described elution procedure was repeated twice.
16.25 µl of total RNA were used for cDNA generation. First, 1 µl 25 mM oligo T20 primers (Fermentas) were added; for primer annealing the following PCR program was used: 5 min/70°C; 5 min/4°C. Next, 7.75 µl reverse transcription mix [5 µl RevertAid [TM] H Minus M-MuLV RT Buffer 5x (Fermentas); 1.25 µl 10 mM dNTP Mix (Fermentas); 0.5µl RiboLock[TM] RNase inhibitor (Fermentas); 1 µl ReveseAid[TM] H Minus M-MuLV RT (200 U/µl; Fermentas)] were added. The reverse transcription

reaction was performed at 42°C for 60 min followed by 15 min at 70°C and cooling to 4°C. The cDNA stock was stored at -20°C.

4.11. Real-time PCR

For real-time PCR analysis the SYBR Green detection system was used. 10 µl Power SYBR® Green PCR Master Mix (Applied Biosystem) were mixed with 2 µl cDNA (1:5 dilution with dH_2O), 0.5 µl 10 µM forward primer, 0.5 µl 10µM reverse primer, and 7 µl dH_2O. Table 4 summarizes the used primers.

Table 4. Primers used for real-time PCR

cJun	F- 5' GAGGGGGTTACAAACTGCAA 3' R- 5' TCTCACAAACCTCCCTCCTG 3'
C/EBPα	F- 5' TTGTCACTGGTCAGCTCCAG 3' R- 5 'TGGACAAGAACAGCAACCAG 3'
KLF4	F- 5' GCCGCTCCATTACCAAGA 3' R- 5' GTGCCTTGAGATGGGAACTC 3'
GAPDH	F- 5' GAAATCCCATCACCATCTTCCAGG 3' R- 5' CGCGGCCATCACGCCACAGTTTCC 3'

All primers were designed using the Primer3 design tool and were obtained from MWG. The used primers spanned exon–intron boundaries, so they do not detect signals from possible co-amplified genomic DNA. Expression profiling was performed in a 96-well plate on the StepOne™ Real-Time PCR System (Applied Biosystems) using the following program: 10 min/95°C; [15 sek/95° C; 1 min/60°C] x 40 cycles and 15 sec/95°C; 1 min/60°C at the melting curve step. Based on melting curve analysis no primer dimers were generated during the applied 40 real-time PCR amplification cycles. For relative quantification, data were analyzed using the "Normalized Relative Quantification" method (LightCycler; Roche). Expression levels of target genes in cells were normalized to GAPDH and shown relative to unstimulated cells (time point 0).

4.12. Cloning of a dominant negative cJun into HR-I-GFP vector

A dominant negative form of cJun (DN cJun), where the most important phosphorylation sites at positions S63, S73, T91, and T93 are changed to A was obtained from Chen G. et al [66]. As DN cJun was provided in the HM6 vector it had to be re-cloned into a retroviral vector, which then could be used for retroviral infections. For this purpose, the Tet-inducible HR-I-GFP vector was chosen. Due to the absence of compatible restriction sites enclosing the insert in the donor vector (HM6) and the recipient vector (HR-I-GFP) the following cloning strategy was used: HM6-DN cJun was digested with HindIII [6 µl vector (1 µg/µl); 1 µl HindIII (10 U/µl); 6 µl Tango buffer 10x, and 1 µl dH_2O were mixed and incubated for 2 h at 37°C followed by enzyme inactivation for 15 min at 65°C]. For DNA cleanup form enzymatic reaction the linearized vector was separated by agarose gel electrophoresis; the fragment was purified using the QIAquick Gel Extraction Kit (QIAGEN). Next, the generated 5' extension was blunted with the use of the 5'-3' polymerase activity of the Klenow polymerase [17 µl gel purified, HindIII linearized HM6-DN cJun; 3 µl Klenow buffer 10x (Fermentas); 2 µl 1mM dNTPs (Fermentas); 3 µl Klenow Fragment (2 U/µl; Fermentas) and 5 µl dH_2O were incubated 30 min at 37°C followed by inactivation – 10 min/75°C]. As before, the purification from enzymes was performed by the usage of the QIAquick Gel Extraction Kit. Then an EcoRI digestion was performed [15 µl purified blunted HM6-DN cJun; 6 µl Tango buffer 10x; 3 µl EcoRI (10 U/µl; Fermentas); 6 µl dH_2O – digestion at 37°C for 2 h, inactivation at 65°C for 15 min] that gave rise to a DN cJun with the EcoRI end at the 3'site and an blunt end on the 5' site. Simultaneously with the generation of the DN cJun fragment, HR-I-GFP vector was digested with EcoRI in combination with an blunt end generating restriction enzyme - HpaI [1.4 µl HR-I-GFP vector (2.19 µg/µl); 1.5 µl EcoRI (10 U/µl); 3 µl HpaI (10 U/µl); 4µl Tango buffer 10x; 10 µl dH_2O – digestion at 37°C for 1 h, inactivation 65°C/15 min]. Finally, the ligation of the DN cJun into the HR-I-GFP vector was performed. To estimate the amounts of vector and insert, which will be used for ligation an analytic 1% agarosegel was run. Based on the intensities of the corresponding bands, 0.8 µl vector and 9 µl insert were used for ligation in the buffer containing 2 µl T4 DNA Ligase buffer 10x (Fermentas); 2 µl PEG 6000 (Fermentas), 1 µl T4 DNA Ligase (5 U/µl; Fermentas) and 5.2 µl dH_2O. The reaction was performed overnight at 16°C.

4.13. Cloning of cJun antisense into HR-I-GFP

A cJun antisense construct was kindly provided by Grondin et al [67]. Since the antisense cJun was obtained in the pMSCV (puro) vector that does not contain a detection marker, the insert had to be re-cloned into the green fluorescent protein (GFP) expressing, Tet-inducible HR-I-GFP vector. The antisense cJun was cut out from the pMSCV vector by EcoRI digestion [6 µl pMSCV-cJun antisense (1 µg/µl); 3 µl EcoRI (10 U/µl); 6 µl Tango buffer 10x; 15 µl dH_2O – digestion at 37°C for 2 h, inactivation 65°C/15 min]. Simultaneously, the digestion of the HR-I-GFP with EcoRI was performed as described above. Subsequently both linearized vector as well as the fragment were purified from 1% agarose gel and ligated [1 µl vector; 1 µl insert; 2 µl tango buffer 10x; 2 µl PEG 6000; 1 µl T4 DNA Ligase (5 U/µl); 13 µl dH_2O – overnight ligation at 16°C].

4.14. Agarose gel electrophoresis

Upon digestion with restriction enzymes the obtained fragment was loaded on a 1% agarose gel [1 g agarose (Seakem LE Agarose; Biozyme) was dissolved in 100 ml 1x TAE Buffer and 10 µl Ethidium Bromide (Roth) were added]. The gel was run using the Bio-Rad Sub-Cell GT system (Bio-Rad) in 1xTAE. The bands were visualized using the Lumi-Imager™.

4.15. Purification/DNA extraction from agarose gel

To extract the desire fragments out of the agarose gel the QIAquick Gel Extraction Kit (QIAGEN) was used. According to the protocol 3x volume of QG buffer was added to 1x volume of the excised gel fragment and dissolved at 50°C for 10 min. Next, 1x volume of isopropanol (Roth) was added. To bind DNA, sample was applied on the QIAquick column and centrifuged 1 min at 13000 rpm. To remove trace amounts of agarose the column was washed with 0.5 ml of QG buffer (1 min/13000 rpm). Additional washing was performed using 0.75 ml PE Buffer (prior to centrifugation the column membrane was incubated with the applied PE buffer for 5 min at RT). To remove residual fluid the column was centrifuged for 1 min at 13000 rpm followed by elution. Therefore 30 µl dH_2O

were added to the centre of the membrane, incubated for 1 min, and then centrifuged (1 min/13000 rpm).

4.16. Transformation of KCM competent cells

KCM competent cells were stored at -80°C. As the first step, KCM cells were thrown on ice for 10 min. In the meantime 20 µl 5x KCM buffer were mixed with 1 ul-10 µl DNA (ligation product) and filled up to 100 µl with dH_2O. The DNA mix (100 µl) was added to the competent cells, mixed gently, and incubated on ice for 20 min followed by incubation at RT for 10 min. Then, 900 µl SOC-medium were added and the mix was incubated for 40-60 min at 37°C/shaking. Finally, the cell suspension was transferred on LB^{+Amp} plates and grown overnight in the 37°C incubator.

4.17. Plasmid preparation

For the purification of low amounts of plasmid DNA the GeneJET^{TM} Plasmid Miniprep Kit (Fermentas) was used. A single colony was inoculated in 2 ml LB^{Amp} medium and incubated overnight at 37°C/shaking. Next, the bacterial culture was harvested by centrifugation at 8000 rpm for 2 min at RT. The pellet was resuspended in 250 µl Resuspension Solution; 250 µl Lysis Solution were added followed by mixing by inverting. In addition, 350 µl Neutralization Solution were added, mixed and centrifuged for 5 min at 13000 rpm. The supernatant was transferred to the GeneJET^{TM} spin column followed by centrifugation (1 min/13000 rpm). The column was washed twice by addition of 500 µl Wash Solution and centrifugation (1 min/13000 rpm). To remove residual solution the column was centrifuged (1 min/13000 rpm) followed by plasmid elution. Therefore 50 µl dH_2O were added onto the column membrane, incubated for 2 min at RT and centrifuged for 2 min at 13000 rpm.

To confirm the presence of the desire insert in the purified plasmid the insert was cut out by the usage of restriction enzymes, which were used for cloning. Furthermore, the right orientation of the cloned fragment was confirmed by additional digestion.

To purify high amounts of plasmid DNA the QIAfilter Plasmid Maxi Kit (QIAGEN) was used. As the first step a single colony was inoculated in 2 ml LB^{Amp} medium and a started culture was grown at 37°C/shaking for 8 hours. Next, the started culture was transferred in

100 ml LB^{Amp} medium and an overnight culture was grown. The bacterial cells were harvested by centrifugation (5000 g/15 min/4°C). The plasmid DNA purification was performed according to the protocol. The bacterial pellet was resuspended in 4 ml P1 Buffer, 4 ml P2 Buffer were added and mixed by inverting followed by an incubation period of 5 min at RT. Next, P3 Buffer (4 ml) was added, mixed and the lysate was applied into the QIAfilter Cartridge. During the subsequent incubation period (10 min at RT) the precipitate floated and formed a layer at the top of the solution thereby enabling successful filtration. In the meantime a QIAGEN-tip 500 column was equilibrated by applying 10 ml QBT Buffer. As next, the cell lysate was filtred trough the QIAfilter Cartridge into the equilibrated QUIAGEN-tip. The lysate then entered the column by gravity flow. The column was washed twice with 30 ml QC Buffer. Finally the plasmid DNA was eluted in 15 ml QF buffer. To precipitate the DNA 10.5 ml isopropanol were added followed by centrifugation (1 h/5000 g at 4°C). The DNA pellet was washed with 5 ml 70% EtOH and centrifuged (1 h/5000 g at 4°C). The pellet was air dried and dissolved in 200 µl dH_20. To determine the yield, DNA concentration was determined by spectrophotometry at 260 nm.

5. Results

5.1. Stimulation with pro-inflammatory cytokines redirects granulocytic cells to monocytes

Preliminary results of our lab showed that *in vitro* differentiated granulocytic cells (G) can be redirected to monocytes (Mo) by the overexpression of dominant active MKK6 (d.a.MKK6). Interestingly, stimulation with pro-inflammatory cytokines also shifts the cells into the monocytic direction (Köffel et al, unpublished data). In the following experiment different combinations of pro-inflammatory cytokines were tested for their ability to reprogram granulocytic cells to monocytes. $CD34^+$ cord blood stem cells were differentiated to granulocytes for 11 days in presence of G-CSF and SCF followed by 48 hour stimulation with different cytokine cocktails. The cells were thereby either incubated with GM-SCF alone, in combination with TNFα, or with a TNFα/IL1β mixture. In addition, stimulation was performed with GM-CSF/TNFα/IL-1β, a cytokine cocktail mimicking the pro-inflammatory milieu found in the inflamed joins of rheumatoid arthritis (RA) patients [68-71]. For evaluation of phenotypic changes $CD15^+/LF^+$ granulocytes were analyzed by FACS for the expression of the monocytic marker CD14. Furthermore, western blot analysis was performed to examine the activation status of the MAPKK MKK6.

As shown in Figure 6A, upon stimulation with pro-inflammatory cytokines the LF^+ granulocytes lost their LF expression and simultaneously showed an upregulation of the monocytic marker CD14. Stimulation with the GM-SCF/TNFα and the TNFα/IL1β combination showed a potent monocyte induction capacity (data not shown). However, the most prominent shift into the monocytic direction could be achieved in case of the GM-SCF/TNFα/IL-1β cytokine cocktail (Fig. 6A). Furthermore, stimulation with the GM-SCF/TNFα/IL-1β was shown to trigger MKK6 activation as revealed by western blot analysis (Fig 6B).

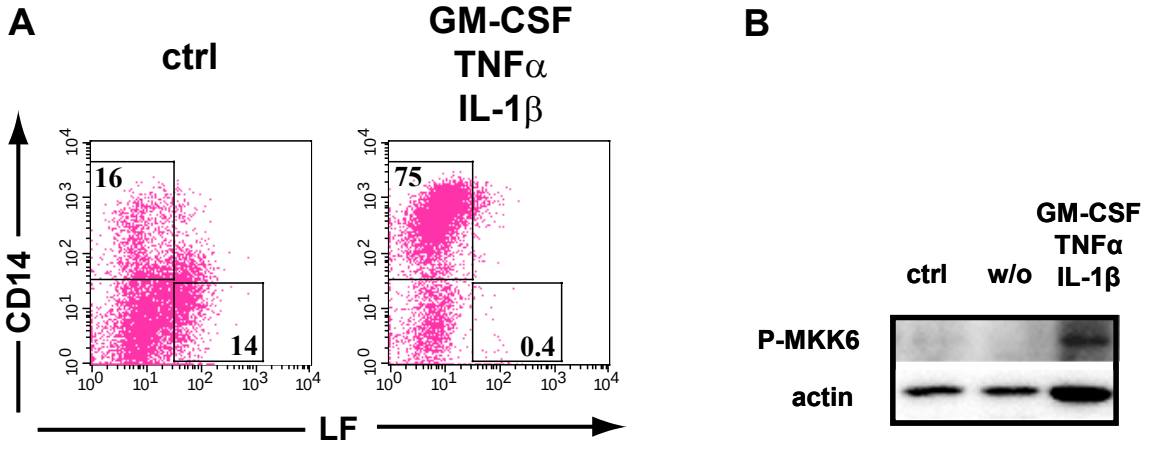

Figure 6. Pro-inflammatory cytokines trigger G to Mo lineage shift via MKK6 signalling cascade.
(A) Stimulation with pro-inflammatory cytokines redirects G to Mo lineage differentiation. *In vitro* differentiated granulocytes (day 11) were stimulated with the GM-CSF/TNFα/IL-1β cytokine cocktail for 48 h. Expression of granulocytic and monocytic markers was determined via FACS analysis. Cells showing the $CD15^+$ phenotype were further examined for CD14 versus LF expression. **(B) Pro-inflammatory cytokines trigger G to Mo switch via MKK6 activation.** *In vitro* differentiated granulocytic cells (ctrl) were stimulated with GM-CSF/TNFα/IL-1β (GM-CSF/TNFα/IL-1β) or left untreated (w/o) for 48h. MKK6 activation was determined by the P-MKK6 levels by western blot analysis. Re-probing with anti-actin antibodies served as loading control.

5.2. MKK6-dependent granulocyte to monocyte conversion is accompanied by the upregulation of cJun and downregulation of C/EBPα

Overexpression of d.a.MKK6 as well as stimulation with pro-inflammatory cytokines instructs granulocytic cells to acquire a monocytic phenotype. The phenotypic switch was found to be accompanied by changes in the expression levels of the critical transcriptional regulators of the monocytic and the granulocytic lineage, cJun and C/EBPα, respectively (Köffel et al, unpublished data). To further analyse the transcriptional changes that drive the G to Mo shift, *in vitro* differentiated granulocytes were stimulated with different combinations of pro-inflammatory cytokines for 48 hours and analyzed for the expression levels of the critical monocytic transcription factor cJun and the granulocyte promoting factor C/EBPα.

As already demonstrated by FACS analysis, stimulation with GM-SCF/TNFα/IL-1β induced the most prominent shift into the monocytic direction. In line with the FACS data (Fig. 6A) this cytokine cocktail showed the strongest effect on the protein level. A potent induction of the monocytic transcription factor cJun as well as a marked downregulation of the granulocytic factor C/EBPα could be observed (Fig. 7).

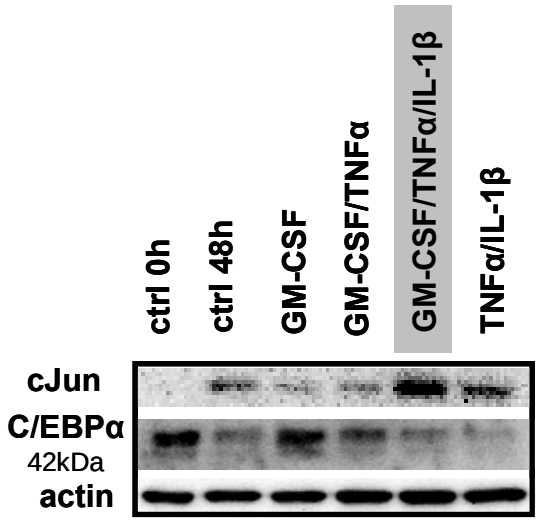

Figure 7. Stimulation with pro-inflammatory cytokines triggers the upregulation of cJun and downregulation of C/EBPα. $CD34^+$ progenitors were *in vitro* differentiated into granulocytic cells for 11 days followed by the stimulation with pro-inflammatory cytokines (GM-CSF; GM-CSF/TNFα; GM-CSF/TNFα/IL-1β; TNFα/IL-1β) for 48 h. Western blot analysis of cJun and C/EBPα levels. Re-probing with anti-actin antibodies served as loading control.

5.3. Promyelocytic HL60 cells undergo Mo differentiation in response to d.a.MKK6

To further analyze the molecular mechanisms of the MKK6-dependent granulocyte to monocyte conversion, promyelocytic HL60 cells were used. For this purpose a HL60 based cell line was chosen, which expresses a Tet-inducible d.a.MKK6-NGFR cassette upon induction by doxycycline (HL60-TA-d.a.MKK6). Cell surface NGFR expression was thereby used as a marker for successful d.a.MKK6 induction, as described previously [72]. Examination of the cell morphology revealed the acquirement of an adherent, inflammatory monocyte-like phenotype upon expression of d.a.MKK6 (Fig. 8A). In line with the morphological changes, an upregulation of the monocytic markers CD14 and CD11b as well as of the maturation marker CD86 could be observed upon expression of d.a.MKK6 in HL60 cells (Fig. 8B). Taken together, the morphological examination as well the analysis of surface marker expression demonstrated an MKK6-driven differentiation of promyelocytic HL60 into the monocytic lineage.

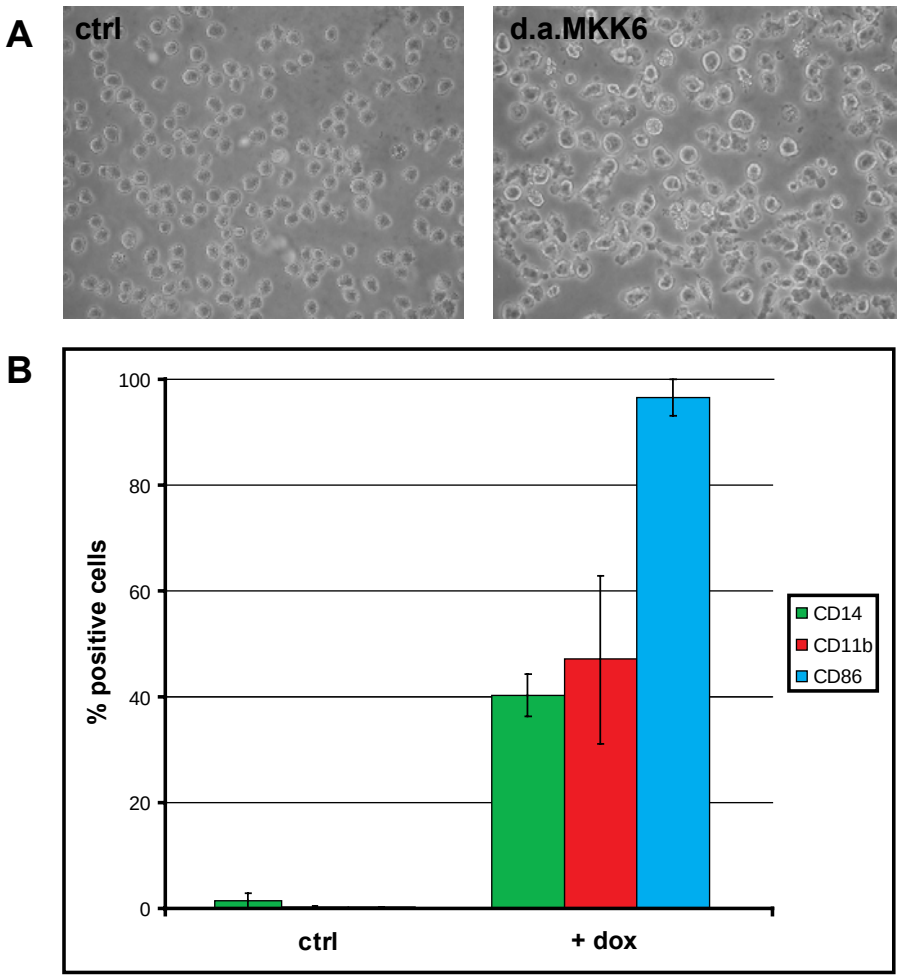

Figure 8. MKK6-triggered differentiation of HL60 cells.
(A) Promyelocytic HL60 cells acquire a monocytic phenotype upon d.a.MKK6 expression. (ctrl) unstimulated HL60-TA-d.a.MKK6 cells. (d.a.MKK6) HL60-TA-MKK6 cells expressing d.a.MKK6 for 48 h. **(B) Overexpression of d.a.MKK6 drives HL60 cells into the monocytic direction.** HL-60-TA-d.a.MKK6 cells were incubated with doxycycline for 48 h (+ dox) to trigger the d.a.MKK6 expression. Gated $NGFR^+$ cells were examined for the surface expression of CD14, CD11b, and CD86 by FACS. Data are shown as mean values ± S.D. of three independent experiments.

5.4. MKK6-dependent Mo conversion of HL60 cells is accompanied by a rapid induction of cJun and downregulation of C/EBPα

To examine whether the MKK6-driven transcriptional changes observed in primary cells (Fig. 7) similarly occur in the HL60-TA-d.a.MKK6 system, the expression levels of the transcription factors of interest, cJun and C/EBPα, were analysed in a time kinetic experiment. Therefore the expression of d.a.MKK6 was induced by doxycycline for 2 h, 4 h, 6 h, 8 h, 16 h, 24 h, and 48 h followed by western blot analysis.

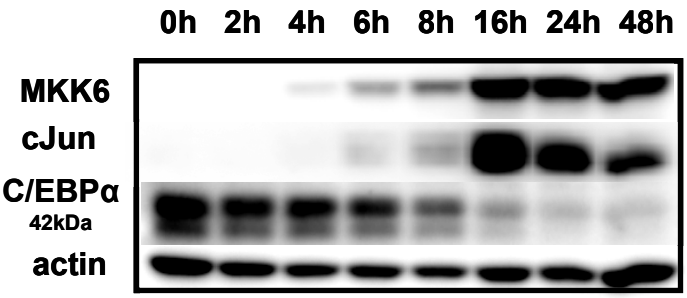

Figure 9. Expression of d.a.MKK6 in HL60 cells triggers a rapid cJun upregulation paralleled by C/EBPα downregulation. Expression of d.a.MKK6 was induced in HL60 cells for indicated time periods. cJun and C/EBPα protein levels were determined by western blot. Actin served as loading control.

Western blot analysis of HL60 cells revealed a rapid, time dependent cJun upregulation which is paralleled by C/EBPα downregulation upon d.a.MKK6 induction (Fig. 9). In line with the data observed for primary, *in vitro* differentiated granulocytic cells (Fig. 7, the shift to the monocytic direction was shown to be accompanied by the upregulation of cJun and downregulation of C/EBPα in the HL60-TA-d.a.MKK6 system. In conclusion, these data highlight the HL60 system as a valid model for further analysis of the molecular mechanisms of the MKK6-dependent G to Mo conversion.

5.5. The reciprocal expression pattern of cJun and C/EBPα on the protein level is also reflected on a transcriptional level

As the examination of cJun and C/EBPα expression on the protein levels revealed a reciprocal expression pattern (Fig. 9) we aimed to investigate whether the expression of these transcription factors was also regulated on the transcriptional level. To address this question, real-time RCR analysis was performed to examine cJun as well as C/EBPα mRNA levels at 2 h, 4 h, 6 h, 8 h, 16 h, 24 h, and 48 h post d.a.MKK6 induction.

Real-time PCR results revealed an upregulation of cJun mRNA, starting at 2 h and reaching a plateau at 6 h after d.a.MKK6 induction. In contrast, C/EBPα mRNA levels showed a strong, up to ten fold, downregulation starting at 2 h and reaching the minimal levels at 16 h post d.a.MKK6 triggering (Fig. 10). In conclusion, these data demonstrate two key players of G versus Mo lineage differentiation, C/EBPα and cJun, to be tightly regulated in response to MKK6 signalling on both the transcriptional as well as the protein level.

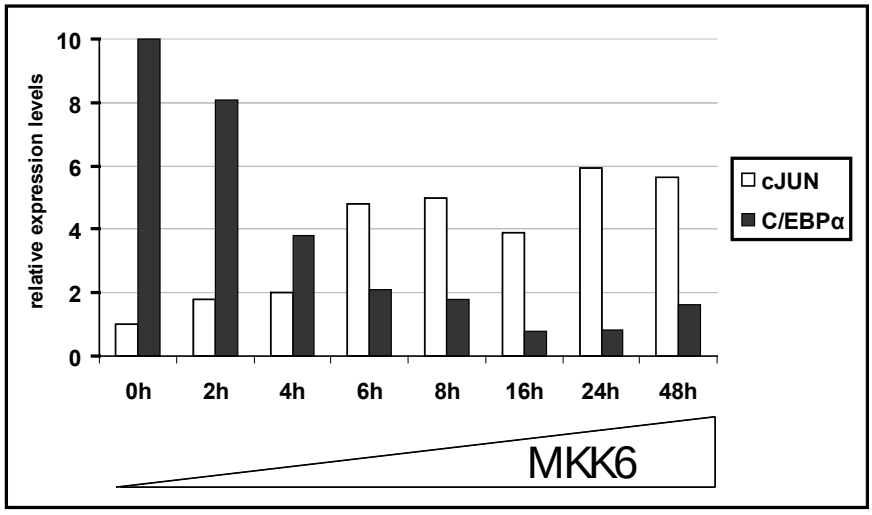

Figure 10. Transcriptional regulation of cJun and C/EBPα levels accompany the MKK6-dependent monocyte differentiation of HL60 cells. Real-time PCR analysis of cJun and C/EBPα mRNA levels in HL60 cells was performed at the indicated time points after d.a.MKK6 induction. Expression levels of target genes were normalized to GAPDH and shown relative to unstimulated cells (time point 0h). One representative result of two independent experiments is shown.

5.6. MKK6-dependent C/EBPα downregulation via proteasome dependent degradation

The MKK6-dependent granulocyte to monocyte shift is, as described above, associated with the downregulation of C/EBPα. Given that C/EBPα levels within cells are regulated via ubiquitinylation and subsequent proteasomal degradation [73] we made use of a proteasome inhibitor (MG132) [74] to investigate the mechanism that underlies the C/EBPα downregulation observed in our system. The expression of d.a.MKK6 was induced by doxycycline followed by the addition of the proteasome inhibitor at 6 hours post MKK6 induction. In parallel, for control purpose d.a.MKK6 expressing cells were cultured in the absence of inhibitor. Protein samples were collected from control HL60 cells (ctrl), as well as from MG132 treated HL60 cells (MG132) at 0 h, 6 h, 7 h, 8 h, 9 h, and 10 h. Figure 11A schematically illustrates the experimental setup.

In comparison to the control cells, where starting from 7 h after d.a.MKK6 induction a marked C/EBPα downregulation occurred, the protein levels remained unchanged in the presence of the proteasome inhibitor (Fig. 11B). As prolonged treatment with MG132 induces cell apoptosis, stabilization of C/EBPα levels could only monitored by short term

studies. In conclusion, the results demonstrate the MKK6-dependent C/EBPα downregulation to be based on proteasome dependent degradation.

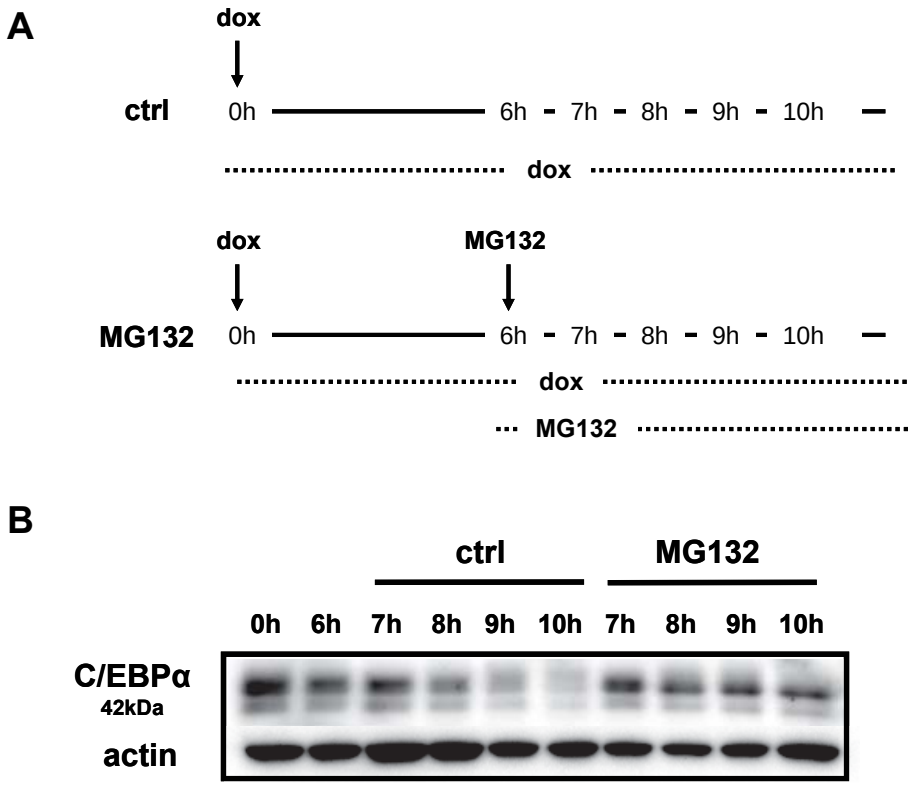

Figure 11. Inhibition of the proteasomal machinery by MG132.
(A) Examination of the proteasome-dependent C/EBPα degradation - schematical representation of the experimental setup. Expression of d.a.MKK6 was induced in HL60-TA-d.a.MKK6 cells by the addition of doxycycline (0h). 6h post d.a.MKK6 induction the proteasome inhibitor MG132 was added followed by an incubation period of 4 h. As a control in parallel d.a.MKK6 expressing HL60 cells were cultured in the absence of inhibitor. Protein samples were collected from control (ctrl) and MG132 treated cells (MG132) at indicated time points for western blot analysis. **(B) Stabilization of C/EBPα levels in the presence of the proteasome inhibitor.** At the time point of 6 h post d.a.MKK6 HL60 cells were treated with the proteasome inhibitor MG132 for the period of 4 h. As a control d.a.MKK6 expressing HL60 cells cultured in absence of the inhibitor we used. C/EBPα protein levels were examined by western blot analysis at 0 h, 6 h, 7 h, 8 h, 9 h, and 10 h. Re-probing with anti-actin antibodies was used as loading control.

5.7. MKK6-dependent cJun upregulation is accompanied by cJun phosphorylation

The monocytic transcription factor cJun was shown to be upregulated during MKK6-dependent granulocyte to monocyte conversion (Fig. 9). The activity of cJun is known to be regulated by posttranslational modifications such as phosphorylation [29]. Phosphorylation of Ser-63 and Ser-73 located within the N-terminal transactivation domain

potentiates the ability of cJun to activate transcription [30]. To examine whether cJun is phosphorylated in response to MKK6 during G to Mo shift we made use of an antibody that detects cJun only when phosphorylated on Ser-63. Expression of d.a.MKK6 was triggered in HL60 cells for 2 h, 4 h, 6 h, 8 h, and 48 h followed by western blot analysis.

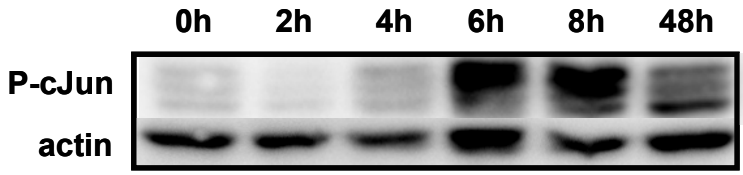

Figure 12. Phosphorylation of the N-terminal transactivation domain of cJun upon MKK6 induction. HL60 cells were examined for the P-cJun (anti-phospho Ser-63 cJun antibodies) levels 2 h, 4 h, 6 h, and 8 h post d.a.MKK6 induction. Actin served as loading control.

Phosphorylated forms of cJun could be detected at 6 h and 8 h. In contrast, at 48 h only a minor or non-phosphorylated form of this transcriptional factor was observed (Fig. 12). The results were obtained using an antibody directed against one of the main phosphorylation sites (Ser-63) located in the N-terminal transactivation domain. Phosphorylation of this domain at more than one residue give rise to multiple phosphorylation forms that appear as double or tripped bands, as seen in Figure 12.

In vitro protein dephosphorylation analysis was used as an additional strategy to validate the phosphorylation status of cJun in response to MKK6 signalling. Protein samples were collected from uninduced cells (0 h) or from cells stimulated for d.a.MKK6 induction (8 h). This was followed by *in vitro* dephosphorylation by the alkaline phosphatase (CIAP). As described in details in material and methods a special lyses and incubation buffer had to be used to allow the CIAP to be active. For control purpose, at each of the examined time points (0 h and 8 h), cells had to be treated under three different conditions: (i) collection in the normal sample buffer used for all WB analysis described above (Fig. 13; ctrl - SB); (ii) collection in the special lysis buffer without subsequent CIAP addition (Fig. 13; ctrl); (iii) collection in the special lysis buffer followed by incubation of the protein extracts with the alkaline phosphatase (Fig. 13; CIAP).

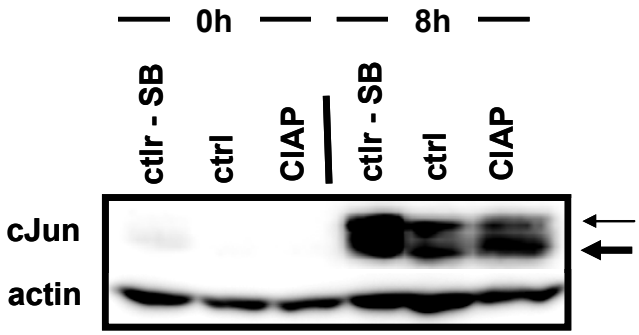

Figure 13. *In vitro* cJun dephosphorylation by CIAP.
Protein extracts were collected from uninduced HL60 cells or 8 hours upon d.a.MKK6 induction. *In vitro* protein dephosphorylation was performed by the usage of alkaline phosphatase (CIAP) as described in details in material and methods. cJun levels were determined via western blot using anti-cJun antibodies. Arrows indicate various phosphorylated forms. Re-probing with anti-actin antibodies served as loading control.

As expected, no cJun could be detected in uninduced cells. In contrast, in line with previous results (Fig. 9) cJun was upregulated upon MKK6 induction. As can be seen in Figure 13 (ctrl - SB and ctrl) at the 8 hour time point the cJun signal was represented by a double band. The upper band likely corresponds to the phosphorylated form of this transcription factor whereas the lower one presumably represents a cJun form lacking post-translational modifications. In the experiment in which the dephosphorylation was performed, the upper cJun band decreased, whereas the lower one increased simultaneously, thus demonstrating the conversion of phospho-cJun into non-phosphorylated cJun. (Fig. 13; CIAP).

In conclusion, the obtained data support that MKK6-induced cJun upregulation is accompanied by phosphorylation of the N-terminal transactivation domain. However, additional experiments are required to define the functional relevance of such post-translational cJun modifications in the context of G to Mo lineage shift.

5.8. Role of cJun in MKK6-dependent monocyte induction

To further investigate the molecular mechanism of the MKK6-dependent granulocyte to monocyte switch we aimed to examine the role of cJun within the conversion process.

As a strategy to interfere with cJun function, a dominant negative form of this transcription factor was used. Dominant negative cJun (DN cJun) was obtained from Chen G. et al [66] and represents a mutated form of cJun where the critical phosphorylation sites (S63, S73, T91, and T93) of the N-terminal transactivation domain [30;75] are changed to alanine. First, DN cJun had to be cloned in to a retroviral vector (Tet-inducible HR-I-GFP). Due to

the absence of compatible restriction sites the following cloning strategy was chosen, that is described in detail in material and methods. Briefly, the 5' end of DN cJun was blunted by the 5'-3' polymerase activity of Klenow polymerase; for the 3' end a sticky end generating enzyme was used. In parallel the HR-I-GFP vector was cut with a blunt end generating enzyme in combination with a sticky end generating enzyme, used before for the generation of the 3' of the DN cJun insert. Finally, DN cJun was cloned into the HR-I-GFP. The obtained construct (HR-I-GFP-DN cJun) was retrovirally transduced into HL60 bearing a Tet-inducible d.a.MKK6. This system allowed simultaneous induction of both d.a.MKK6 and DN cJun by the addition of doxycycline. FACS analysis was used to determine phenotypic changes. Furthermore, cJun and C/EBPα protein levels we examined by western blot.

As shown in Figure 14A, strong induction of the monocytic marker CD14 was observed in control HL60 cells, expressing d.a.MKK6. In contrast, upon d.a.MKK6 plus DN cJun co-induction HL60 cells showed a marked reduction of CD14 upregulation. CD11b, a marker of monocytes as well as granulocytes, showed only a tendency to downregulation in expression levels. The fact, that DN cJun was found to impede the MKK6-triggered G to Mo shift demonstrate the functional relevance of the post-translational phosphorylation of the N-terminal transactivation domain suggested to occur within the conversion process (Fig. 12). It furthermore highlights cJun as an indispensable key factor driving the lineage switch.

In addition to the examination of the DN cJun effect on the HL60 phenotype, we performed western blot analysis for cJun and C/EBPα. As depicted in Figure 14B, d.a.MKK6 expressing HL60 cells which were co-transduced with DN cJun showed strong expression of this transcription factor (DN cJun; MKK6; 4h and 48h) in comparison to the cJun levels in controls cells, transduced with the empty HR-I-GFP vector (HR; MKK6; 4h and 48h). Expression of DN cJun did not stabilize C/EBPα levels, as downregulation of this granulocyte promoting transcription factor still occurred in the presence of the DN cJun construct. In conclusion, these results indicate a cJun-independent mechanism responsible for the MKK6-triggered C/EBPα degradation that takes place during the switch into a monocytic cell.

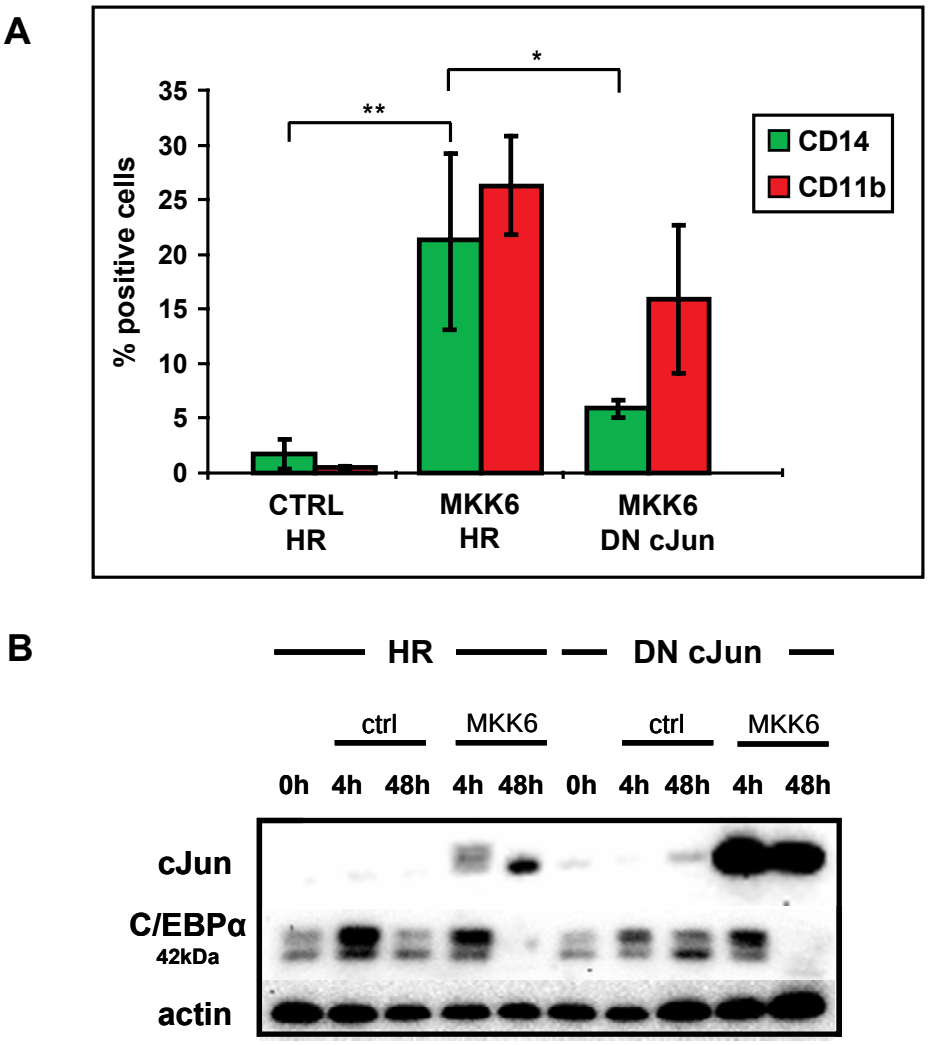

Figure 14. Expression of DN cJun in HL60-TA-d.a.MKK6 cells.
(A) DN cJun interferes with MKK6-dependent monocyte differentiation. DN cJun (HR-I-GFP-TA-DN cJun) was introduced in HL60-TA-d.a.MKK6 cells via retroviral infection followed by doxycycline-mediated, simultaneous induction of d.a.MKK6 and DN cJun for 48 h (MKK6; DN cJun). As control, d.a.MKK6 expressing HL60 cells (MKK6; HR) or control HL60 cells lacking the d.a.MKK6 construct (CTRL; HR) were transduced with empty HR-I-GFP vector. Expression levels of the monocytic markers CD14 and CD11b were examined by FACS. The values are means ± S.D. of three independent experiments. $p<0.05$ (*); $p< 0.005$ (**).
(B) Overexpression of DN cJun does not interfere with MKK6-dependent C/EBPα downregulation. HL60-TA-d.a.MKK6 cells were retrovirally transduced with empty control vector (HR) or dominant negative cJun (DN cJun). Protein levels of cJun and C/EBPα were analysed in uninduced cells (ctrl) or upon d.a.MKK6 induction (MKK6) at 4 h and 48 h time points. The same membrane was re-probed with anti-actin antibodies for loading control.

In addition to the DN cJun approach described above, as the initial strategy, we tried to interfere with MKK6-triggered cJun upregulation via cJun knockdown by an antisense construct. An antisense cJun was obtained form Grondin et al [67] and introduced into HL60-TA-d.a.MKK6 cells using the retroviral gene transduction system. As the construct was provided in the MSCV(puro) vector that does not contain a detection marker but includes a puromycin resistance element, HL60 cells after infection had to be selected in

presence of puromycin. At the end of the selection period, the expression of d.a.MKK6 was induced for 48 hours by addition of doxycycline. The cJun protein levels were examined by western blot. In parallel FACS analysis was used to determine the effect of cJun knockdown on the MKK6-dependent phenotypic shift.

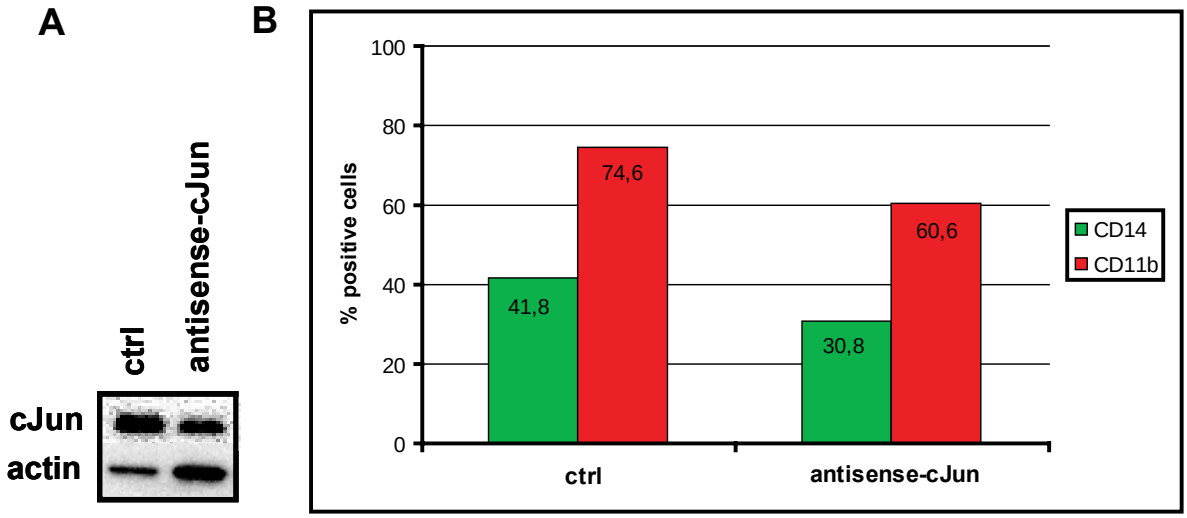

Figure 15. Expression of antisense cJun in HL60-TA-d.a.MKK6 cells.
(A) Antisense cJun inhibits MKK6-dependent cJun upregulation. cJun protein levels were examined in control HL60 cells (ctrl) and HL60 cells retrovirally transduced with MSCV(puro)-antisense cJun (antisense-cJun) at 8 h after d.a.MKK6 induction. Actin served as loading control. **(B) Antisense cJun interferes with MKK6-dependent monocyte induction.** HL60-TA-d.aMKK6 cells were retrovirally transduced with the MSCV(puro)-actisense cJun (antisense-cJun) followed by selection in the presence of puromycin. Expression levels of CD14 and CD11b were determined by FACS after 48 h of d.a.MKK6 induction.

As demonstrated in Figure 15A MKK6-triggered cJun upregulation was reduced in the presence of the antisense construct, confirming the success of the knockdown approach. FACS results demonstrated a moderate diminishment of MKK6-dependent upregulation of monocytic markers CD14 and CD11b in the presence of the antisense cJun construct (Fig. 15B).

Since the approach described above showed only a moderate effect we next cloned antisense cJun into another vector, the Tet-inducible HR-I-GFP. In contrast to the MSCV(puro) vector used before the HR-I-GFP vector contains GFP as a detection marker and allows co-induction of the introduced antisense cJun simultaneously with d.a.MKK6. As before, the retroviral system was used for gene transduction followed by doxycycline-mediated, simultaneous induction of both d.a.MKK6 and antisense cJun expression. However, in contrast to the expectation, significant abrogation of the MKK6-driven G to Mo shift could not be observed using the HR-I-GFP-antisense cJun construct. As one possible explanation we hypothesize that the strong cJun upregulation, triggered in

consequence of d.a.MKK6 overexpression, overtops the inhibitory capacity of the introduced antisense construct. Therefore, we decided to made use of another strategy to interfere with cJun and switched to the DN cJun approach, described above in detail.

5.9. MKK6-dependent granulocyte to monocyte conversion is accompanied by the induction of KLF4, a critical regulator of monocyte differentiation

KLF4, a member of the kruppel-like family of transcription factors was found to be highly expressed in monocytes. Furthermore, it was recently shown, that overexpression of KLF4 in promyelocytic HL60 cells induces monocyte differentiation measured by CD14 and CD11b upregulation [76]. Therefore, we were interested whether this critical regulator could be involved in the observed phenomena of MKK6-triggered G to Mo conversion. To address this question, HL60 cells expressing d.a.MKK6 as well as control cells (HL60-TA-NFGR) were examined for the KLF4 expression on the mRNA level by real-time PCR analysis as well as on protein level by western blot.

Real-time PCR analysis revealed that KLF4 mRNA was upregulated up to 5-fold after 48 hours of d.a.MKK6 expression. In contrast, KLF4 expression levels in controls cells lacking d.a.MKK6 remained unchanged (Fig. 16A). In line with the real-time PCR results marked upregulation of KLF4 protein levels could be detected in HL60 cells expressing d.a.MKK6 (MKK6), whereas control cells (NGFR) showed stable KLF4 expression (Fig. 16B). As KLF4 induction could only be observed at the latest time point of 48 hours, it seems to lag behind the cJun and C/EBPα expressional changes that were shown to occur shortly upon MKK6 induction (Fig. 10). Based on these findings KLF4 upregulation is probably attributed to secondary effects of d.a.MKK6 signalling and seems not to be linked to the first wave of MKK6-dependent transcriptional changes.

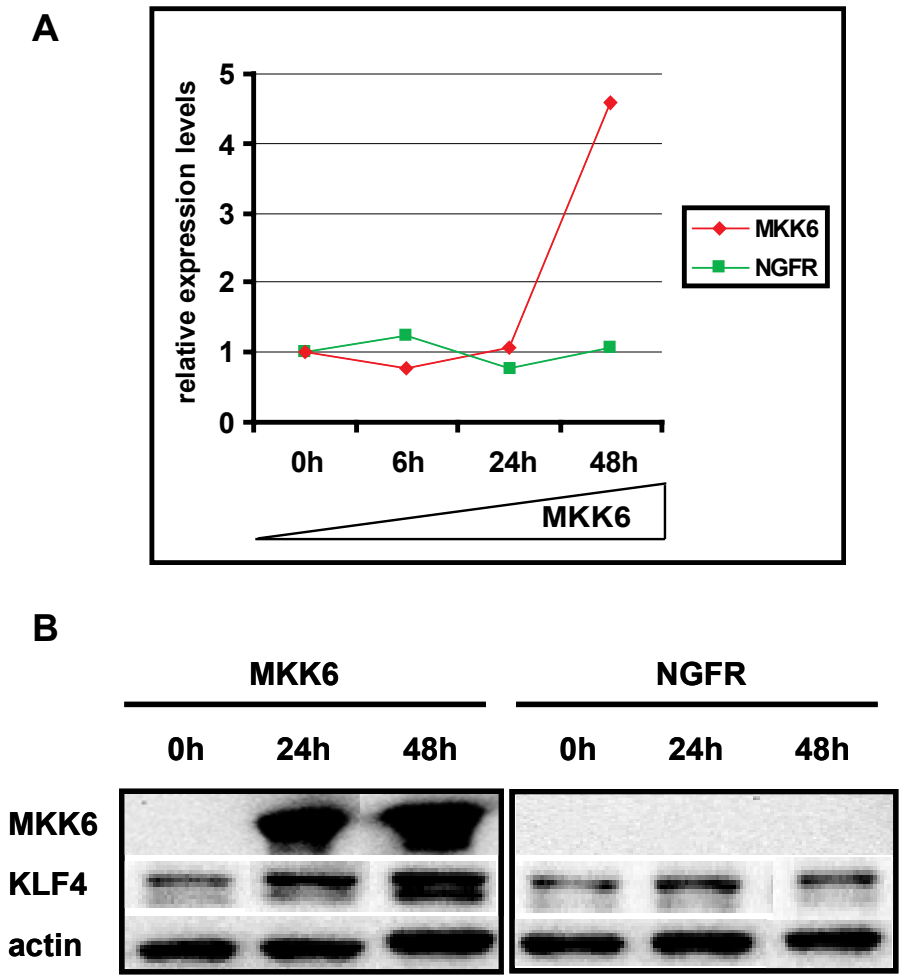

Figure 16. MKK6-dependent KLF4 induction.
(A) MKK6-triggered induction of KLF4 mRNA levels. HL-60 cells expressing d.a.MKK6 (MKK6) or control cells (NGFR) were examined for KLF4 mRNA levels by real-time PCR. Expression level of the target gene was normalized to GAPDH and shown relative to unstimulated cells (0h). A representative result of two independent experiments is shown. **(B) MKK6-dependent KLF4 upregulation on the protein level.** KLF4 protein levels were determined in HL-60 cells expressing d.a.MKK6 (MKK6) as well as control cells (NGFR) at different time points (0 h, 24 h and 48 h). Actin served as a loading control.

5.10. The transcriptional changes downstream of MKK6 are dependent on the p38MAPK signalling

Overexpression of d.a.MKK6 in the promyelocytic HL60 cells was shown to induce upregulation of cJun accompanied by its phosphorylation, and the downregulation of C/EBPα. (Fig. 9 and Fig. 12). Phosphorylation of the transactivation domain of cJun is known to be mediated by the cJun N-terminal kinase (JNK) [77]. In addition to the classical JNK pathway leading to the phosphorylation of this transcription factor resent

studies identified p38MAPK as a kinase directly phosphorylating cJun [78]. To examine which signalling pathway, downstream of MKK6, is responsible for the transcriptional changes within the observed monocyte lineage induction of HL60 cells we had a look at the activation status of both kinases. As it is known that p38MAPK as well as JNK are activated by phosphorylation [79] we analyzed the phosphorylation level of p38MAPK and JNK upon d.a.MKK6 induction in a time kinetic experiment.

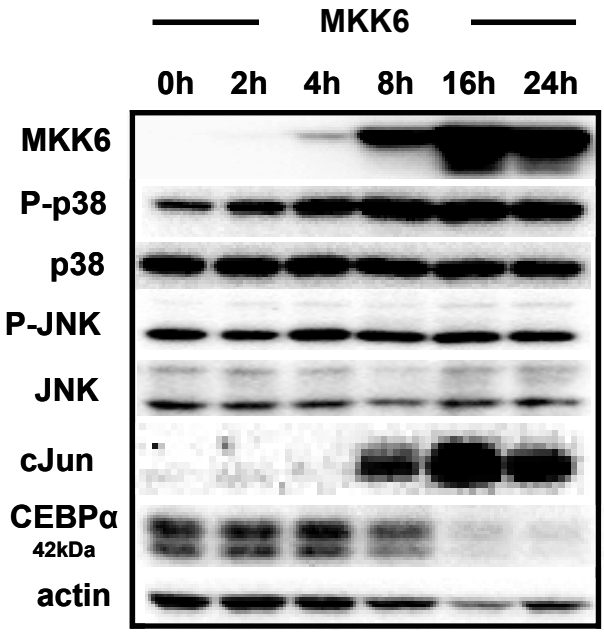

Figure 17. p38MAPK induced downstream of MKK6.
Time kinetic analysis of protein levels upon d.a.MKK6 induction in HL60 cells. p38MAPK and JNK activation was examined by the determination of phospho-p38 (P-p38) and phospho-JNK (P-JNK) levels, respectively. In addition cJun and C/EBPα protein levels were examined. Actin was used for loading control.

Expression of d.a.MKK6 was found to induce strong p38MAPK activation as the phosphorylation level of p38MAPK was markedly increased starting from the 4 h time point (Fig. 17). In contrast, JNK phosphorylation levels remained unchanged (Fig. 17).

Next, to further examine the signal transduction downstream of MKK6 we made use of the specific p38MAPK inhibitor SB203580 [80] as well as the specific JNK inhibitor SP600125 [81]. The inhibitors were added to the HL60 cells simultaneously with the induction of d.a.MKK6; protein expression levels of cJun and C/EBPα were examined in a time kinetic experiment by western blot. In parallel, HL60 cells cultured in presence of the p38MAPK inhibitor were analyzed for the CD14, CD11b and CD86 induction by FACS at the 48 hour time point.

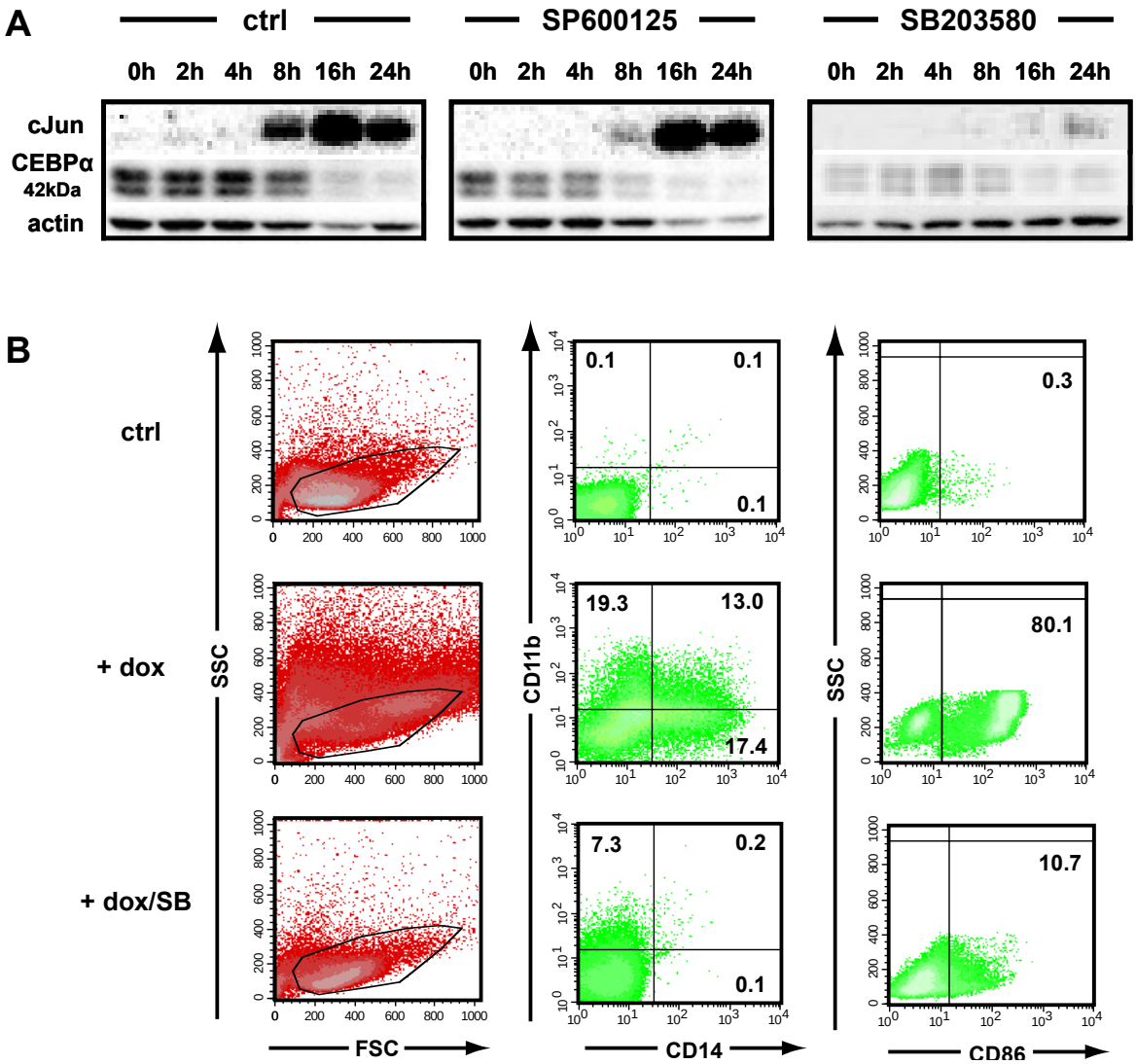

Figure 18. p38MAPK acts as the downstream effector of MKK6, diving G to Mo switch.

(A) MKK6-dependent transcriptional changes are abrogated in presence of the p38MAPK inhibitor. The JNK inhibitor (SP600125) or the p38MAPK inhibitor (SB203580) was added to the HL60-TA-d.a.MKK6 cells simultaneously with d.a.MKK6 induction by doxycycline. Expression levels of cJun and C/EBPα were determined in a time kinetic experiment by western blot analysis. The same membrane was re-probed with anti-actin antibodies for loading control. **(B) The MKK6-dependent induction of a monocytic phenotype is abolished in the presence of the p38MAPK inhibitor.** Expression of d.a.MKK6 was induced in HL60-TA-d.a.MKK6 cells simultaneously with the addition of the p38MAPK inhibitor (SB203580). CD14/CD11b and CD86 expression levels were determined by FACS at the 48 h time point.

As shown in Figure 18A, addition of the JNK inhibitor did not interfere with MKK6-dependent transcriptional changes, in terms of cJun upregulation and C/EBPα downregulation. In contrast, the p38MAPK inhibitor showed a strong inhibitory effect. It abolished the MKK6-dependent cJun upregulation and, when normalized to the actin levels, showed to some extend stabilization of C/EBPα levels. In line with the results obtained on the protein level, FACS analysis revealed an abrogation of the MKK6-dependent phenotypic changes in the presence of p38MAPK inhibitor. A marked

upregulation of CD14/CD11b (13% $CD14^+CD11b^+$; 19.3% $CD11b^+$; 17.4% $CD14^+$) and CD86 (80.1%) could be observed in control cells after d.a.MKK6 triggering. In contrast, HL60 cells treated with the p38MAPK inhibitor showed only a slight induction of these marker molecules (0.2% $CD14^+CD11b^+$; 7.3% $CD11b^+$; 0.1% $CD14^+$; 10.7% $CD86^+$) (Fig. 18B).

Taken together the obtained results demonstrate p38MAPK as the downstream effector of MKK6 driving G to Mo conversion.

5.11. Physiological levels of p38MAPK activation are sufficient for the MKK6-dependent monocyte induction

To further investigate the molecular mechanism driving the granulocyte to monocyte lineage switch we aimed to examine which MKK6 expression and p38MAPK activation levels are sufficient to trigger a transcriptional program that is able to drive the cells into the monocytic direction. For this purpose HL60-TA-d.a.MKK6 cells were stimulated with various doxycycline concentrations ranging from 0.1 µg/ml up to 4 µg/ml. The acquirement of a monocytic phenotype was determined by FACS analysis. In parallel the MKK6-dependent transcriptional changes were analysed in a time kinetic experiment.

As demonstrated by FACS, independently of the doxycycline concentration used for the initial d.a.MKK6 induction the HL60 cells showed a comparable upregulation of the monocytic markers CD14 and CD11b as well as the maturation marker CD86 (Fig. 19A).

Similarly, on the protein level, a comparable MKK6 induction as well as p38MAPK activation was observed for the diverse doxycycline concentration (Fig. 19B). In line with similar p38MAPK phosphorylation pattern, a conformable cJun upregulation could be seen for all used doxycycline concentrations (Fig 19B). To quantify the effect of the doxycycline titration a densitometric analysis of the western blot results was performed. The ratio of phospho-p38 to p38 (P-p38/p38), representing the p38MAPK activation status was calculated for each individual time point. The obtained results were displayed by a graphical representation (Fig. 19C).

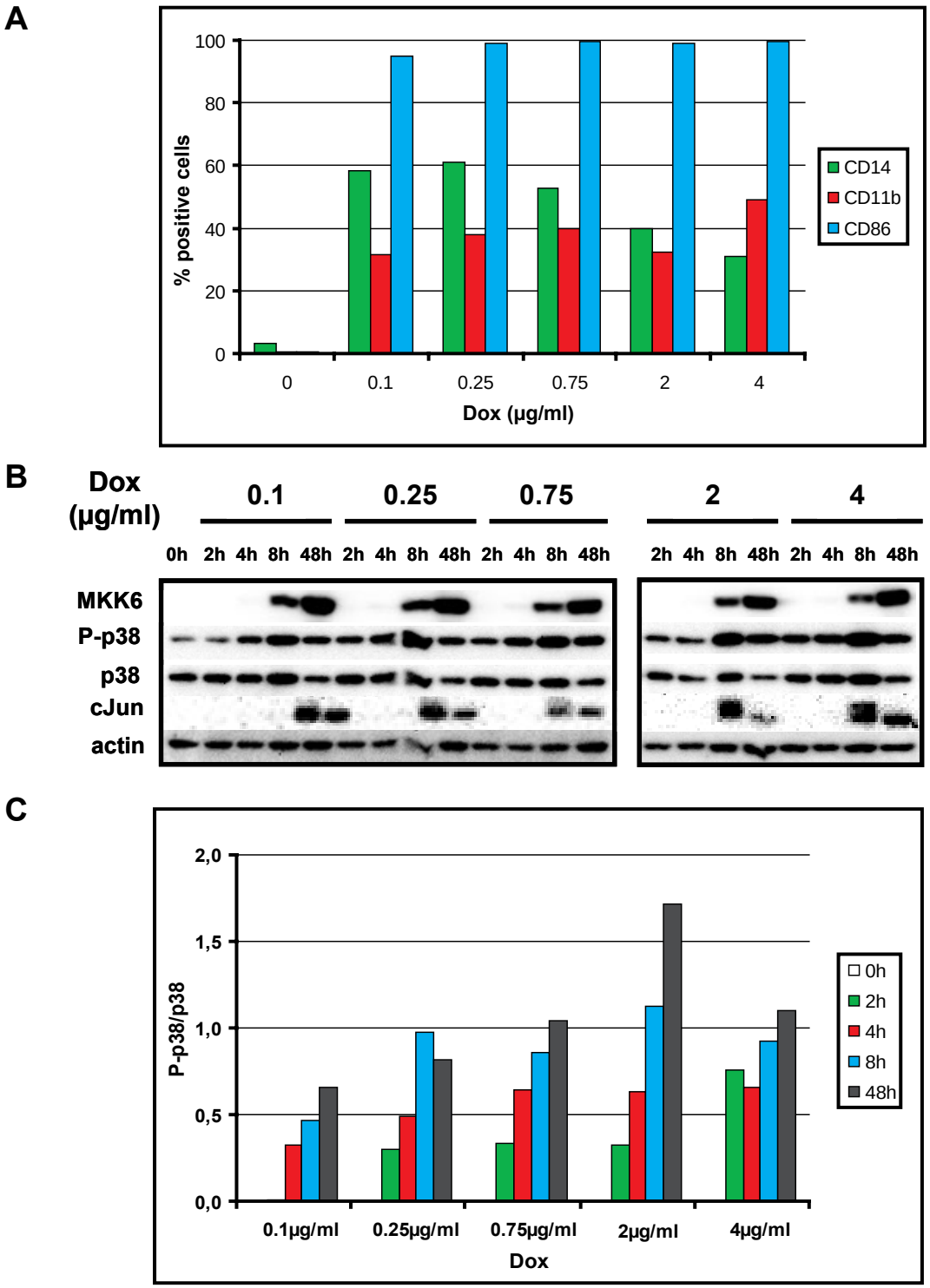

Figure 19. Modulation of MKK6 expression levels/p38MAPK activation.
(A) Comparable induction of monocytic markers CD14 and CD11b as well as maturation marker CD86 for various doxycycline concentrations used for d.a.MKK6 induction. Titration of doxycycline (0.1 µg/ml, 0.25 µg/ml, 0.75 µg/ml, 2 µg/ml, and 4 µg/ml) used for the d.a.MKK6 induction in HL60 cells. Expression levels of the cell surface markers CD14, CD11b and CD86 were determined by FACS at the 48 h time point. **(B) Comparable levels of MKK6 induction, p38MAPK activation as well as cJun upregulation upon d.a.MKK6 induction with various doxycycline concentrations.** Titration of the doxycycline concentration (0.1 µg/ml, 0.25 µg/ml, 0.75 µg/ml, 2 µg/ml, and 4 µg/ml) used for the d.a.MKK6 induction in HL60 cells. p38MAPK activation, determined by phospho-p38 (P-p38) levels, and cJun induction was analyzed in a time kinetic experiment via western blot. Actin served as loading control. **(C) Comparable p38MAPK phosphorylation levels after d.a.MKK6 induction with diverse doxycycline concentration.** Graphical representation of the densitometric quantification of the western blot obtained from the doxycycline titration experiment. The P-p38/p38 ratio represents the activation status of p38MAPK.

D

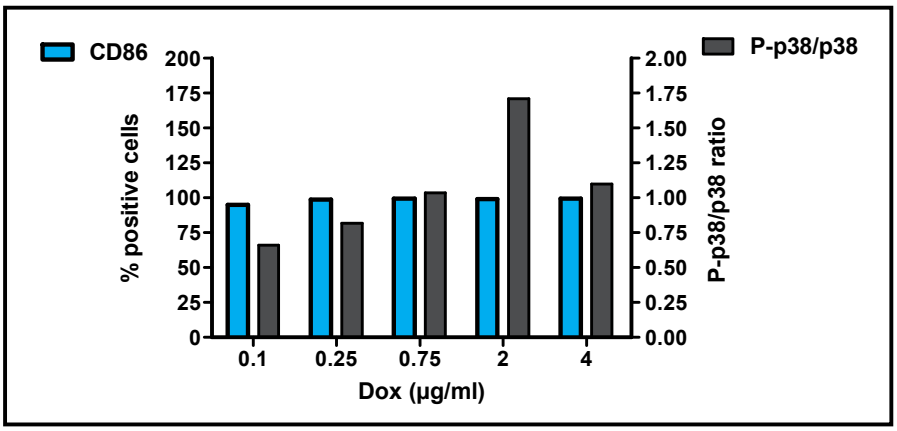

Figure 19 (continued). Modulation of MKK6 expression levels/p38MAPK activation.

(D) Non-saturated p38MAPK phosphorylation levels are sufficient to induce CD86 upregulation. Graphical representation of the p38MAPK activation levels in correlation with induction of CD86. Densitometric analysis of the western blot signals at the corresponding 48 h time points was used to calculate the P-p38/p38 ratio. The corresponding surface expression levels of CD86 were determined via FACS analysis 48 h upon d.a.MKK6 induction.

As depicted in Figure 19C, time kinetic analysis of the p38MAPK activation levels confirmed for all used doxycycline concentrations a similar, time dependent pattern of p38MAPK phosphorylation. Furthermore, the p38MAPK activation was shown to reach the plateau close to 100 % starting at the 8 h time point. An exception occurred in the case of the lowest doxycycline concentration (0.1µg/ml) where the p38MAPK phosphorylation did not exceed the level of 65 % during the time course analysis.

An additional representation of the doxycycline titration analysis is depicted in Figure 19D, where the correlation of P-p38/p38 to the induction of the monocytic maturation marker CD86 is illustrated. Although varying levels of p38MAPK phosphorylation were observed upon stimulation with various doxycycline concentrations a comparable CD86 upregulation could be monitored (Fig. 19D). The most interesting example is the lowest doxycycline concentration (0.1 µg/ml) used for d.a.MKK6 induction: despite of the fact that a complete p38MAPK phosphorylation could not be achieved the HL60 cells showed a monocyte induction capacity (Fig. 19A), as well as the upregulation of the maturation marker CD86 comparable to the results obtained with 2 µg/ml doxycycline (Fig. 19D).

In summary, the results demonstrate, despite of the tremendously high MKK6 levels emerging due to the d.a.MKK6 overexpression a time-dependent pattern of p38MAPK phosphorylation resulting in an exhaustive p38MAPK phosphorylation only in case of longer lasting MKK6 signalling. Furthermore, non-saturated phosphorylation levels were found to be sufficient to trigger maximal induction of a monocytic phenotype. In conclusion, these finding suggest moderate p38MAPK phosphorylation levels, mimicking the physiological situation, to be sufficient to drive the phenotypic switch.

5.12. Short term MKK6 induction is sufficient to redirect granulocytic cells to monocytes

The next question we asked was whether it is necessary to overexpress d.a.MKK6 and thereby induce cJun upregulation and C/EBPα downregulation continuously during the granulocyte to monocyte conversion or if a transient MKK6 induction is sufficient to trigger a transcriptional program that then drives the cell into the monocytic direction. Two experimental approaches were used to address this question. First, to restrict the d.a.MKK6 expression to the desired time period, we made use of the "dox washing" method. HL60-TA-d.a.MKK6 cells were transiently stimulated with doxycycline (2 h, 4 h, 6 h, and 8 h) followed by cell washing and subsequent culturing in fresh medium without doxycycline for up to 48 h (Fig. 20A). To examine the effects of such short term MKK6 induction on the MKK6-dependent transcriptional changes, protein samples were collected at the indicated time points as well as at the endpoint of 48 hours.

In line with previous data (Fig. 9) continuous doxycycline stimulation triggers time-dependent MKK6 overexpression, leading to upregulation of cJun and downregulation of C/EBPα (Fig. 20B, corresponding time points are depicted in red). In contrast, removal of doxycycline either at the 2 h, 4 h, 6 h, and 8 h time point resulted in a strong decline of the MKK6 levels detected at the corresponding 48 hour time points. As a consequence the MKK6-triggered cJun upregulation declined as well, reaching basal expression levels. In contrast, the downregulation of C/EBPα was not reversible in case of doxycycline removal (Fig. 20B). Taken together, the results highlight the "dox washing" approach as a suitable method for the restriction of the d.a.MKK6 signal transduction to a desired time period.

Based on this finding the "dox wash" method was used to investigate whether a short term MKK6 induction would be sufficient to drive the promyelocytic HL60 cells into the monocytic direction. HL60-TA-d.a.MKK6 cells were induced by doxycycline followed by "dox washing " after 4 h and 6 h of induction. The acquirement of the monocytic phenotype was monitored by FACS by gating on $NGFR^+$ cells at the endpoint of the subsequent culture period of 48 hours.

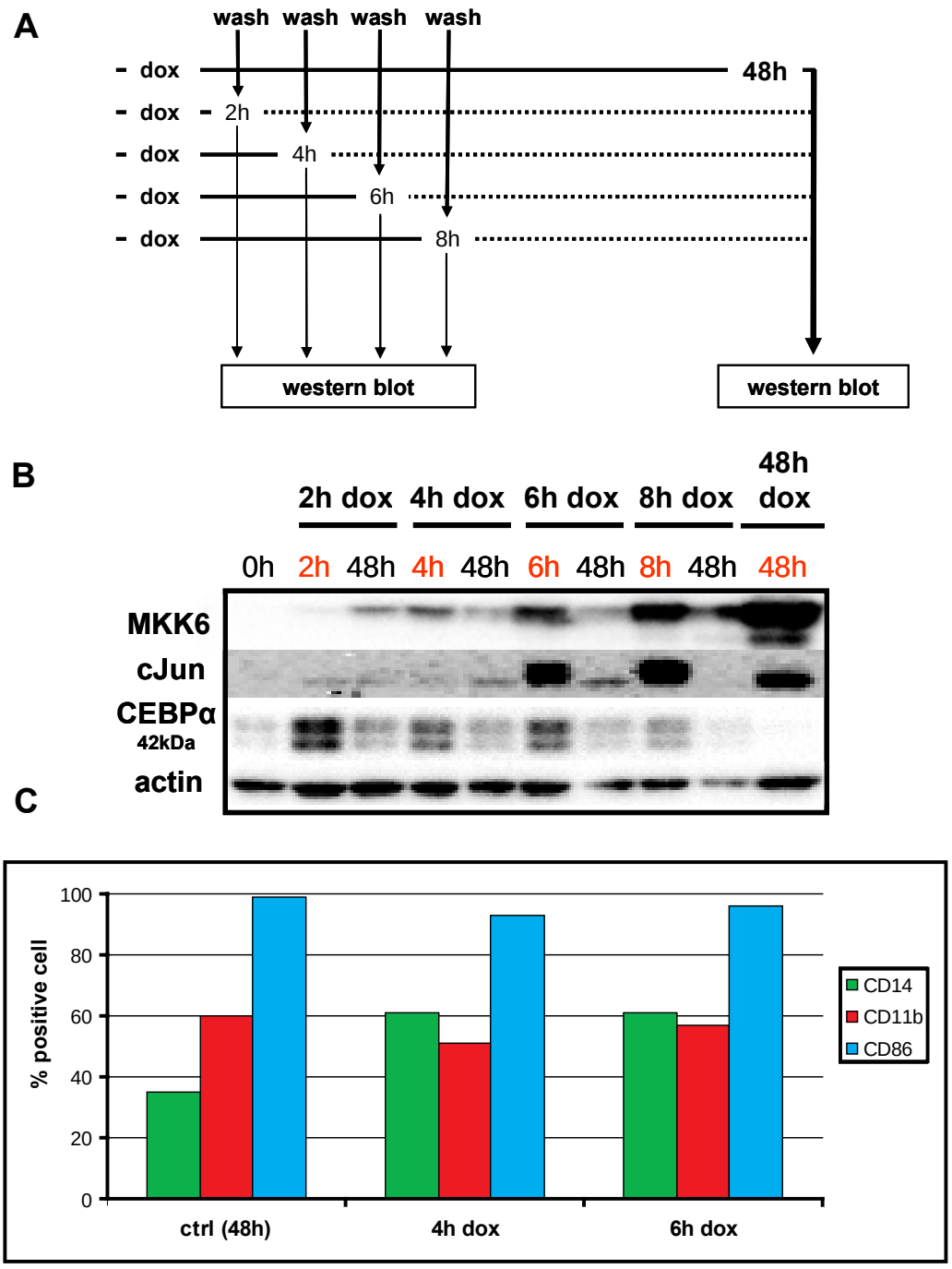

Figure 20. Restriction of the MKK6-p38MAPK signalling cascade.
(A) The "dox washing " approach – schematical representation of the experimental setup. Control cells were continuously simulated with doxycycline over the culture period of 48 h. In the "dox washing" approaches the cells were washed 2 h, 4 h, 6 h, and 8 h after d.a.MKK6 induction followed by subsequent culturing in a fresh medium without doxycycline till the endpoint of 48 h. Protein samples were collected at the indicated time points and at the endpoint of 48 h for western blot analysis. **(B) Restriction of the d.a.MKK6 expression by the "dox washing" approach.** HL60-TA-d.a.MKK6 cells were transiently stimulated with doxycycline for 2 h, 4 h, 6 h, and 8 h (time points indicated in red), washed and further cultured in a fresh medium for additional 48 h. Protein samples were collected at the corresponding time points and at the endpoint of 48 h. MKK6, cJun and C/EBPα levels were examined via western blot. Re-probing with anti-actin antibodies served as loading control. **(C) Short term MKK6 signalling is sufficient to redirect granulocytic cells to monocytes.** The "dox washing" approach was used for HL60-TA-d.a.MKK6 cells to restrict the d.a.MKK6 induction to the desire time period (4h dox and 6h dox). For control sustained doxycycline stimulation was performed (ctrl (48h)). The upregulation of CD14, CD11b and CD86 was determined by FACS analysis of $NGFR^+$ gated cells at the 48 h time point.

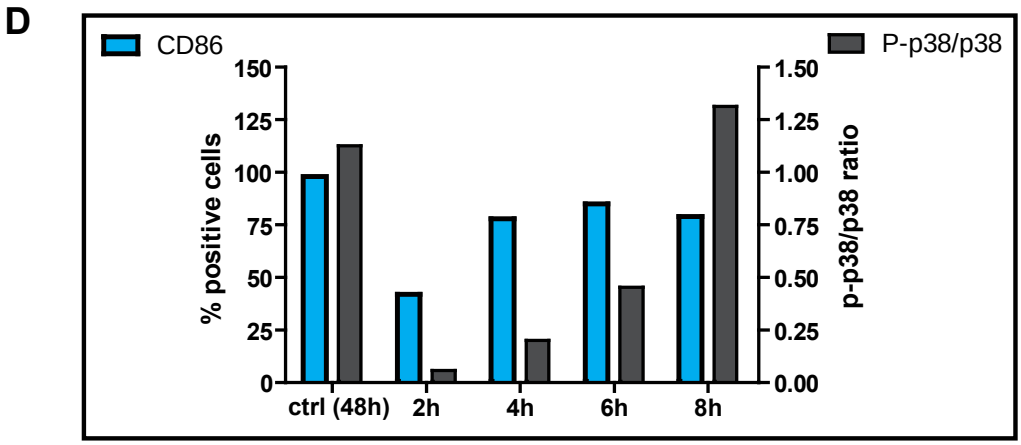

Figure 20 (continued). Restriction of the MKK6-p38MAPK signalling cascade.
(D) Low levels of p38MAPK activation are sufficient to trigger a strong CD86 induction. Graphical representation of the p38MAPK activation in correlation with induction of CD86. HL60-TA-d.a.MKK6 cells were transiently stimulated with doxycycline for 2 h, 4 h, 6 h, and 8 h, washed and further cultured in medium without doxycycline for additional 48 h. Sustained doxycycline stimulation was used as control (ctrl (48h)). The CD86 expression levels were determined via FACS at the endpoint of 48 h. TheP-p38/p38 ratio was calculated based on the densitometric analysis of the corresponding western blot signals.

As demonstrated by FACS results, short term d.a.MKK6 expression (4 h or 6 h) triggered the upregulation of the monocytic markers CD14 and CD11b as well the maturation marker CD86 at the comparable levels to control cells where d.a.MKK6 induction sustained over a time period of 48 hours (Fig. 20C). Similar results were obtained when examining the total cell population (data not shown). In conclusion, the obtained data indicate that short term MKK6 signalling is sufficient to initiate a transcriptional program that then drives the cell into the monocytic direction.

The correlation of the p38MAPK phosphorylation levels to the MKK6-triggered CD86 induction was analyzed for the doxycycline titration experiments described above (Fig. 19D). The results demonstrated a non-saturated p38MAPK phosphorylation to be sufficient to trigger a strong CD86 upregulation. The used doxycycline titration method represents an approach that allowed us to modulate the MKK6 signalling strength. In addition to that the “dox washing” method was used as a further strategy for the MKK6 signal transduction restriction. To analyse the effects of the short term MKK6 induction on the activation of the downstream effector p38MAPK, the p38MAPK activation status was determined by the calculation of the P-p38/p38 ratio. The obtained results were correlated to the corresponding CD86 expression levels and depicted in a graphical representation (Fig. 20D). As shown in Figure 20D, short term pre-triggering of d.a.MKK6 for 2 h resulted in moderate CD86 induction and minor p38MAPK activation. In contrast, 4 h of d.a.MKK6 induction were already sufficient to induce a strong CD86 upregulation, although there was still a weak p38MAPK activation as depicted by the corresponding

P-p38/p38 value. Similar results were obtained for the 6 h time point. CD86 induction levels were comparable to the control cells, when d.a.MKK6 signalling sustained over 48 hours (Fig. 20D; ctrl 48h), despite an incomplete p38MAPK activation of about 50%. An exhausting p38MAPK phosphorylation could only be achieved when the MKK6 was pre-triggered for 8 h. The upregulation of the maturation marker CD86 in that case did not exceed the value of a short term induction for 4 h or 6 h.

In summary, the obtained data demonstrate a correlation between the duration of the MKK6 signalling and the phosphorylation level of the downstream effector p38MAPK. Furthermore it once again highlights that low levels of p38MAPK activation, likely mimicking the physiological situation, are already sufficient to trigger the transcriptional program that redirects granulocytic cells to monocytes. Taken together, the results manifest the used HL60-TA-d.a.MKK6 system as a suitable *in vitro* model for the analysis of the MKK6-dependent granulocyte to monocyte switch as it recapitulates the key effects of signal transduction downstream of MKK6.

In addition to the "dox washing" approach described above the MKK6 signalling cascade can be restricted for a desired time period by the inhibition of the downstream effector p38MAPK. For this purpose the p38MAPK inhibitor (SB203580) was used. It was added to the HL60 cells 6 hours after d.a.MKK6 induction. Furthermore, a combinatorial approach was performed where at the time point of interest, 6 hours upon d.a.MKK6 induction, the cells were first washed to remove the doxycycline followed by the subsequent addition of the p38MAPK inhibitor. Phenotypic changes were analyzed by FACS at the endpoint of 48 hours.

Control cells, which were continuously stimulated with doxycycline over a time period of 48 hours (Fig. 21; + dox 48h (ctrl)), as anticipated, showed a strong upregulation of the monocytic markers CD14 and CD11b as well as the maturation marker CD86. A comparable conversion into a monocytic cell, as already shown before (Fig. 20C) could be observed in the approach where the "dox washing" was performed (Fig. 21; dox wash 6h). Restriction of signal transduction downstream of MKK6 by the p38MAPK inhibitor resulted in minor CD14 upregulation, in comparison to the control cells or the "dox washing" approach. However, the cells showed strong CD11b and CD86 induction, overall indicating a comparable shift into the monocytic direction (Fig. 21; SB 6h). Combinatorial approach revealed similar results regarding the markers expression. For control purposes an experiment was performed where the p38MAPK inhibitor was added simultaneously

with the d.a.MKK6 induction at the time point of 0 hours. In comparison to the approaches described above, those cells demonstrated only a minor upregulation of CD14, CD11b and CD86 (Fig 21; SB (ctrl)). One possible explanation for the slight induction of monocytic markers could be based on an incomplete inhibition of p38MAPK by the specific inhibitor within the period of 48 hours.

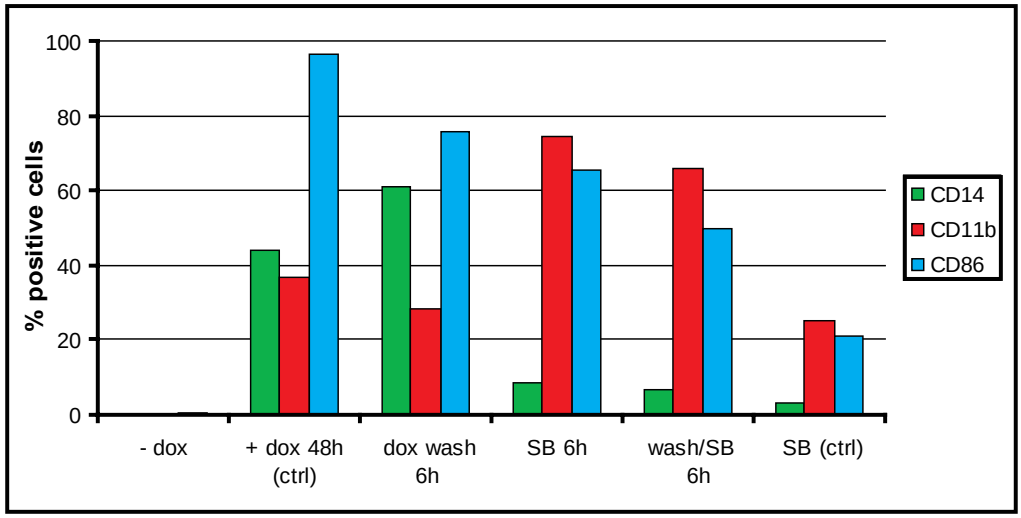

Figure 21. Short term pre-triggering of the MKK6 signalling cascade is sufficient to drive monocyte differentiation. The MKK6-p38MAPK signal transduction was restricted to 6 h either by the "dox washing" method (dox wash 6h), the addition of the p38MAPK inhibitor SB203580 (SB 6h) or a combinatorial approach (wash/SB 6h). Sustained doxycycline alone (dox 48h (ctrl)) or in combination SB203580 (SB (ctrl)) were used as controls. $NGFR^+$ gated cells were examined for the CD14, CD11b and CD86 expression levels at the end of the culture period (48 h) by FACS.

Taken together, the obtained results further indicate a short term induction of the MKK6-p38MAPK signalling cascade to be sufficient to trigger a monocyte differentiating transcriptional program able to redirect the differentiation of granulocytic cell to the monocytic lineage.

5.13. Stimulation with pro-inflammatory cytokines drives HL60 differentiation into the monocytic direction

HL60-TA-d.a.MKK6 cells were used in numerous studies to examine the molecular mechanism of the MKK6-dependent granulocyte to monocyte conversion. They represent a suitable cell line model mimicking the MKK6-dependent phenotypic G to Mo switch observed in primary cells. Reprogramming of the *in vitro* differentiated granulocytes into monocytic cells can either be achieved by d.a.MKK6 overexpression or by the stimulation with pro-inflammatory cytokines. However, for HL60 cells, thus far, only the overexpression approach was used to induce monocyte differentiation. Therefore, we

aimed to investigate whether a stimulation with the GM-CSF/TNFα/IL-1β combination of pro-inflammatory cytokines, which was shown to have the most potent monocyte induction capacity in primary derived granulocytic cells, would similarly drive the HL60 cells into the monocytic direction. To address this question, HL60 cells were cultivated in the presence of GM-CSF, TNFα and IL-1β for 5 days, since a 48 hour stimulation used for primary cells showed only minor effects (data not shown). As a control HL60 cells were in addition stimulation with VitD3 or ATRA, which are known to induce monocyte and granulocyte differentiation, respectively [82]. Induction of CD14, CD11b and CD86 was determined by FACS. Additionally, the expression levels of the transcription factors of interest, cJun and C/EBPα, were monitored by western blot.

As expected, stimulation with VitD3 triggered a potent shift into the monocytic direction as demonstrated by the strong induction of $CD14^+/CD11b^+$ double positive HL60 cells (81.7%) as well as by the upregulation of the maturation marker CD86 (30.2%). In contrast, HL60 cells cultured in presence of the granulopoiesis inducing agent ATRA [83] were directed into the granulocytic lineage as shown by the upregulation of CD11b (73%). In comparison to the monocyte induction, monitoring of granulocytic differentiation is not as well defined for HL60 cells, since they do not express the typical granulocyte markers, e.g. lactoferrin [82]. Therefore, only strong upregulation of CD11b, an integrin important for the adhesion of neutrophils and monocytes and for phagocytosis [84;85], can be used as a marker for granulocyte differentiation of HL60 cells. Results obtained for the stimulation with GM-CSF/TNFα/IL-1β suggest the induction of a monocytic phenotype, as demonstrated by CD11b (16.5%) and CD86 (7.1%) upregulation in combination with a slight shift into the $CD14^+$ direction (Fig. 22A).

In addition to the monitoring of phenotypic changes, assayed by surface marker expression, HL60 cells were examined for cJun and C/EBPα expression levels. Control cells expressing d.a.MKK6 showed the anticipated time-dependent upregulation of cJun paralleled by the downregulation of C/EBPα (Fig. 22B). In line with the data described above (Fig. 12), at the 8 h as well as 72 h time point the cJun signal was represented by a triple band, suggesting a MKK6-triggered phosphorylation of the N-terminal transactivation domain. Similar results were obtained for the stimulation with the monocyte inducing agent VitD3, where a comparable cJun upregulation likely paralleled by phosphorylation as well as the C/EBPα degradation were observed. In contrast, examination of HL60 cells cultured in presence of ATRA, which were found to be differentiated into granulocytes (Fig. 22A), revealed a C/EBPα downregulation, despite the

absence of a cJun induction (Fig.22B). The transcriptional changes observed for HL60 cells stimulated with the GM-CSF/TNFα/IL-1β mixture of pro-inflammatory cytokines were in line with phenotypic shift estimated by FACS. A time-dependent cJun upregulation, paralleled by C/EBPα downregulation could be detected. Western blot results furthermore suggest a cJun phosphorylation, indicated by the appearance of a triple cJun band at the 72 h time point (Fig. 22B).

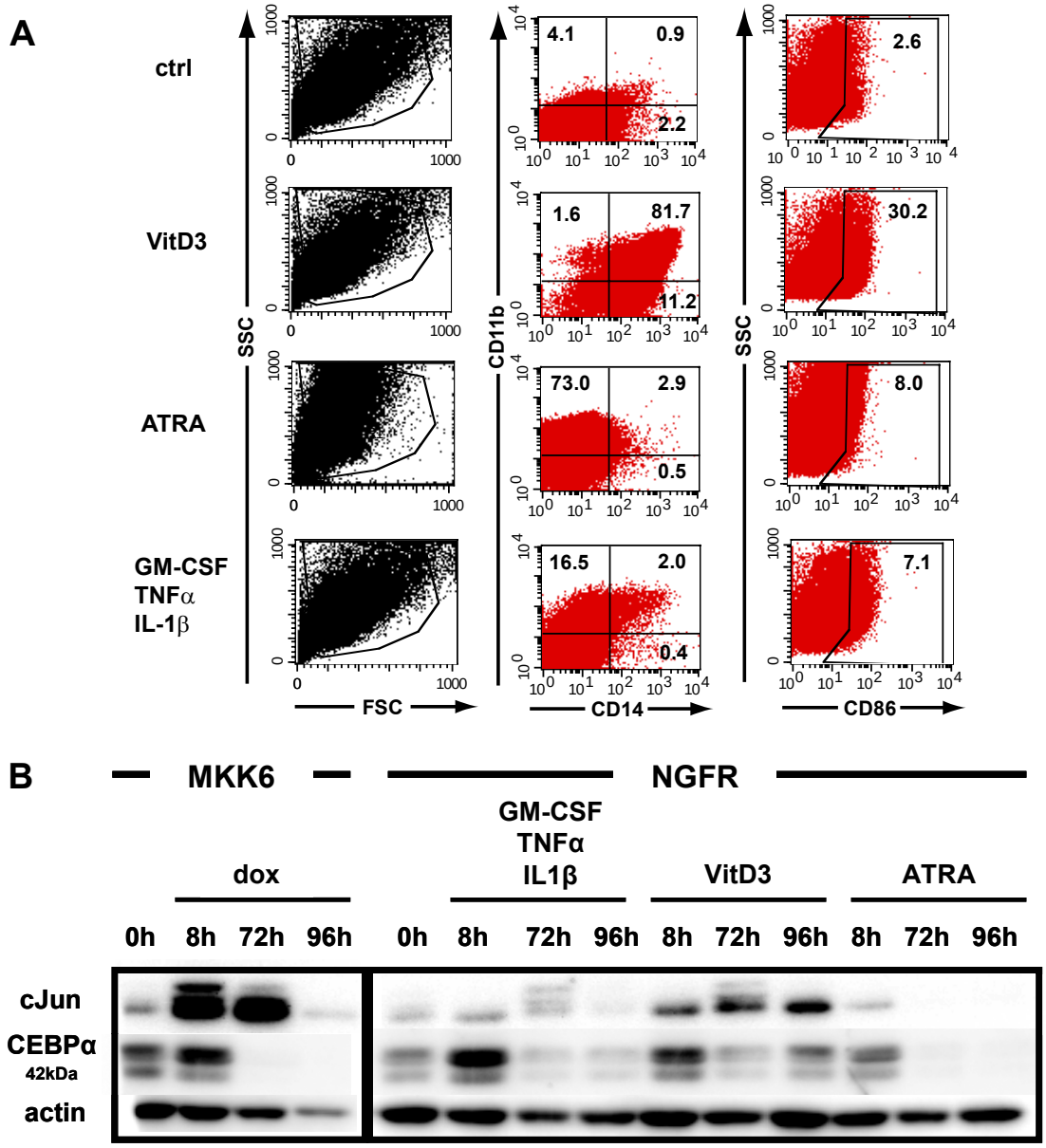

Figure 22. Stimulation of HL60 cells with pro-inflammatory cytokines.
(A) HL60 cells undergo monocyte differentiation upon stimulation with pro-inflammatory cytokines. HL60 cells were cultured in the presence of VitD3, ATRA or a combination of pro-inflammatory cytokines (GM-CSF/TNFα/IL-1β) for 5 days. Cells were examined for the CD14/CD11b expression as well as CD86 induction by FACS. **(B) Stimulation with GM-CSF/TNFα/IL-1β triggers the upregulation of cJun and the downregulation of C/EBPα.** HL60 cells were stimulated with VitD3, ATRA, or a combination of pro-inflammatory cytokines (GM-CSF/TNFα/IL-1β) for 5 days. In parallel d.a.MKK6 was express in HL60-TA-d.a.MKK6 cells up to 5 days. cJun and C/EBPα levels were examined via western blot analysis. Actin served as loading control.

In conclusion, the obtained data demonstrate that similarly to $CD34^+$ derived granulocytic cells the promyelocytic HL60 cells can be directed into the monocytic lineage by the stimulation with pro-inflammatory cytokines whereby in both cases the shift into the monocytic direction is accompanied by a reciprocal expression pattern of the key transcription factors cJun and C/EBPα. These findings further highlight the HL60-TA-d.a.MKK6 system as a suitable model for the analysis of the MKK6-dependent granulocyte to monocyte switch.

6. Discussion

Myeloid cells arise from multipotential hematopoietic stem cells (HSCs) that have the ability to differentiate into multiple cell types as well as to replenish the pluripotent population. The process of myelopoietic lineage decision is thereby tightly regulated by the differential expression of a complex network of subset specific transcription factors. However, there is no single transcription factor that is expressed exclusively by myeloid cells and that, alone, acts as a master regulator of myeloid fate choice. Rather, myeloid gene expression is thought to be controlled by the combinatorial effects of several key transcription factors. Traditionally, haematopoiesis has been viewed as linear and hierarchical, but there is increasing evidence of plasticity during myelopoietic commitment. Changes in the expression levels and timing of transcription factors were shown to be able to trigger lineage conversion of committed cells, indicating that the regulation of transcription factors might be primarily critical for maintaining hierarchical hematopoietic development [10;86].

The herein applied *in vitro* differentiation system allows the generation of a variety of myelopoietic cells subsets from $CD34^+$ cord blood stem cells. The commitment to a distinct lineage is driven by specific cytokines applied during the differentiation process. The validation of the in *vitro* generated myelopoietic cells of interest, granulocytes and monocytes, used in this study revealed the lineage characteristic morphology and the expression of the subset-specific surface markers depicted in Figure 4. In addition to the described *in vitro* differentiation strategy we made use of a retroviral Tet-on gene transduction system to identify a signalling cascade able to reprogram committed granulocytic cells. Usage of the Tet-on method allows a stable introduction of candidate signalling compounds into the $CD34^+$ progenitors followed by the induction of the gene of interest either at desired time point within the differentiation process or at the end of the commitment period.

Our data provides evidence that MKK6-p38MAPK signalling is able to reprogram committed granulocytic cells and redirect them to the monocytic lineage. In line with the results obtained from primary *in vitro* differentiated cells, signal transduction downstream of MKK6 similarly drives the differentiation of a promyelocytic HL60 cell line to the monocytic direction. Based on these findings, HL60-TA-d.a.MKK6 cells were chosen as a

suitable model for further investigations of the molecular mechanisms underlying the MKK6-dependent conversion process. The shift into a monocytic cell was found to be accompanied by the upregulation of the monocytic transcription factor cJun in parallel with a downregulation of the granulocyte promoting factor C/EBPα. As revealed by real-time PCR results and western blot analysis the reciprocal expression pattern appeared on the transcriptional as well as the protein levels. Time course analysis manifested the MKK6-triggered changes in cJun and C/EBPα expression levels as primary transcriptional events, initiating G to Mo conversion. As it is known that the C/EBPα levels within cells are regulated via ubiquitinylation and subsequent proteasomal degradation [73] we asked whether the observed downregulation was based on a proteasomal degradation. To address this question we made use of an inhibitor of the proteasomal machinery. The obtained results showed a stabilization of C/EBPα protein levels in the presence of the proteasome inhibitor, indicating the MKK6-triggered C/EBPα downregulation to be based on a proteasome dependent degradation. Further investigations of the additional transcription factor that was found to be induced in a MKK6-dependent manner, cJun, suggested a phosphorylation of its N-terminal transactivation domain to occur within G to Mo conversion. To get insight into the functional relevance of such posttranslational cJun modification we transduced HL60-TA-d.a.MKK6 cells with a dominant negative form of this transcriptional factor (DN cJun). DN cJun was found to impede the MKK6-triggered G to Mo shift highlighting cJun as a key player driving G to Mo lineage conversion. The classical view on the signal transduction of mitogen-activated protein kinase pathways suggests cJun N-terminal kinase JNK as the MAPK mediating cJun phosphorylation [87]. Interestingly, our study in granulopoietic cells indicated a cJun phosphorylation to be mediated by a different member of the MAPK family, p38MAPK, acting as the downstream effector of MKK6. The MKK6-p38MAPK triggered phosphorylation thereby showed a time dependent character with exhausting cJun phosphorylation levels coming up at the 8 hour time point after MKK6 induction followed by a subsequent decline of phospho-cJun levels in case of longer lasting signal transduction periods. One could hypothesize, that these changes in the phosphorylation status of cJun might reflect an alteration of the composition of the p38 isoforms found to be expressed at a particular stage of the MKK6-p38MAPK driven conversion process. As demonstrated by Pramanik et al [27] the p38MAPK family members have opposing effects on the regulation of both cJun expression and activation. P38β was shown to potentiate the transcriptional activity of cJun mediating the phosphorylation of its N-terminal domain, whereas the γ and δ isoforms

predominantly suppress the cJun *trans*-activation by MKK6 [27]. Based on these findings one could hypothesize that the MKK6-driven changes of the transcriptional program that underlie G to Mo lineage conversion might include an alteration in the p38α, β, γ, and δ expression levels. A shift in the p38 isoform expression profile could in turn affect the signal transduction downstream of MKK6-p38MAPK converting an activating p38 signal into a suppressive. In support, monocytes and granulocytes found in RA patients showed a diverse isoform expression pattern [42]. In conclusion, the intriguing findings regarding the opposing functions of the p38 isoforms open a new direction for our prospective investigations. Further examinations of the MKK6-p38MAPK triggered modulations of the transitional program identified KLF4, a known critical regulator of monocytic differentiation to be induced in a MKK6-dependent manner. The results demonstrated a KLF4 upregulation to occur on the transcriptional as well as the protein levels. Time course analysis suggested the observed KLF4 induction to attribute to secondary effects of MKK6-triggered transcriptional changes. Beside the identification of key transcriptional factors driving G to Mo lineage conversion we focused on a more detailed analysis of the MKK6-p38MAPK signalling cascade. Short term induction of MKK6-p38MAPK was found to be sufficient to trigger the transcriptional signature described above that in turn reprograms granulocytic cells and drives the differentiation into the monocytic lineage. Furthermore, the data demonstrated low levels of p38MAPK activation, likely mimicking the physiological situation, to be sufficient to trigger a monocyte differentiating program, thus redirecting granulocytic cells to monocytes. Interestingly, recent studies identified an alternative, MAPKK independent mechanisms triggering p38MAPK activation - the TAB1-dependent autophosphorylation. TAB1, an adaptor protein classically involved in the signal transduction of the MAPKKK TAK1, was found to directly interact with p38MAPK inducing the activation of this MAPK by an autocatalytic mechanism. Ge at al further identified external stimuli, e.g. TNFα, to be capable to trigger the TAB1-induced p38MAPK phosphorylation [88;89]. Based on these novel findings one could hypothesize, that under certain circumstances such p38MAPK autoactivation mechanism may participate in the signalling network driving the p38MAPK-dependent G to Mo conversion.

In summary, a detailed analysis of the basic molecular mechanisms driving G to Mo lineage switch was performed within the study. Based on the obtained results we could identify key factors mediating the conversion process and gain further insight into the

regulatory mechanisms guiding the MKK6-triggered modulations of the transcriptional network. Figure 23 summarizes the main findings observed during the study and schematically illustrates the signalling cascade driving G to Mo conversion.

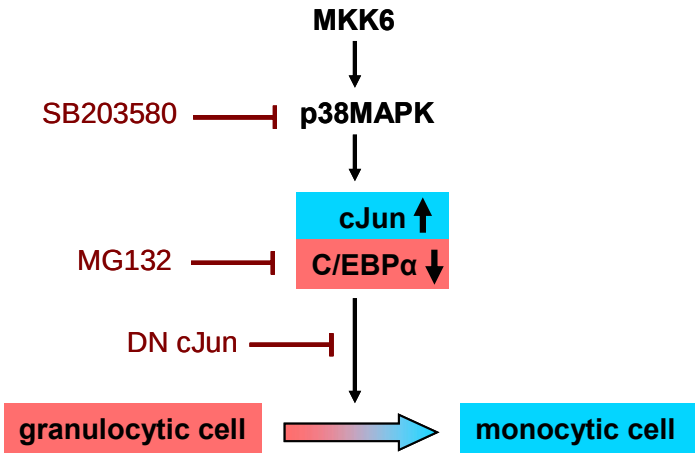

Figure 23. Granulocyte to monocyte lineage conversion – the signalling cascade. Activation of p38MAPK by MKK6 triggers the upregulation of cJun and downregulation of C/EBPα, transcriptional changes underling G to Mo lineage shift. The conversion process can be interfered with by overexpression of the dominant negative form of cJun (DN cJun). Furthermore, inhibition of signal transduction downstream of p38MAPK by the specific inhibitor (SB203580) abolishes the MKK6-dependent transcriptional changes. In addition, stabilization of C/EBPα levels can be achieved in the presence of the proteasome inhibitor (MG132).

In addition to the analysis of the molecular mechanisms of the granulocyte to monocyte conversion we aimed to investigate external stimuli able to trigger the described lineage switch. The examined p38MAPK signalling cascade is known to be a common pathway for the transduction of inflammatory stress signals [90]. Based on this knowledge we tested several combinations of pro-inflammatory cytokines for their ability to reprogram granulocytic cells to monocytes. The most prominent shift into the monocytic direction could be achieved by a cocktail of GM-CSF/TNFα/IL-1β. These cytokines are very prominent in the pro-inflammatory milieu found in inflamed joints of rheumatoid arthritis (RA) patients [68-71].

RA is a chronic inflammatory autoimmune disease associated with the destruction of affected joints. The inflamed synovium consists of diverse cell populations, including monocytes/macrophages, granulocytes, synovial fibroblasts, B cells, and T cells. All populations contribute in their own way to the promotion and perpetuation of the disease

[91]. Classically, granulocytes are thought to be among the first cells to arrive at site of inflammation. As the primary effector cells of the innate immune response they contribute to the progression of inflammatory process by the induction of the respiratory burst. Beside the release of the granule content, activated granulocytes are capable of producing pro-inflammatory cytokines, central to the promotion of inflammation [48;92]. Numerous studies highlights granulocytes as the main participants in the induction as well as perpetuation of chronic inflammatory diseases [92]. As an example, granulocyte depletion was shown to block RA induction in K/BxN mouse RA model, highlighting the indispensable role of granulocytes for disease initiation [93]. In humans, granulocytes were found to invade the synovial fluid of patients with RA, during acute inflammation and in flare of the disease, thus contributing to the joint destruction through the release of granule enzymes and reactive oxygen metabolites [52;94;95]. Joint inflammation is thereby characterized by the presence of large quantities of immune complexes and high levels of pro-inflammatory cytokines, whereby TNFα and IL-1 were found to be the major cytokines driving the pathogenesis of RA [96]. Our study highlights such pro-inflammatory cytokines as a main stimulus triggering the conversion of granulocytic cells into monocytes. Although the occurrence of such lineage shift was not shown *in vivo* so far, based on our *in vitro* findings we raised the question for the impact of the observed G to Mo switch in the course of chronic disease progression.

Numerous studies revealed that in addition to granulocytes both monocytes and synovial macrophages could be considered as critical players in RA. These cells are involved in the initiation and perpetuation of inflammation, leukocyte adhesion and migration, matrix degradation and angiogenesis by secretion of chemokines, cytokines, growth factors, proteases and other mediators [97]. A further contribution to disease progression is mediated by the bone destructive activity of monocytes, which were shown to undergo osteoclast differentiation upon contact with the appropriate signals. Synovial fibroblast-like cells and activated T cells found in synovial membrane appear as the most important cell types, providing the necessary signals for osteoclasts induction [98;99]. The key molecules driving the osteoclast differentiation was found to be the receptor activator of nuclear factor (NF)κB ligand (RANKL), expressed locally in the synovial tissue [100]. Another key mediator for osteoclast formation is TNFα. It is not only an inducer of RANKL expression but also directly enhances the differentiation and activation of osteoclasts through the interaction with the TNFα-receptor type 1. The concomitant presence of TNF thus potentiates the effect of RANKL and boosts osteoclast formation. Signalling through

TNFα-receptor type 1 involves mitogen-activated protein kinases and NFkB [101]. Of particular note, activation of p38MAPK pathway was thereby shown to play a pivotal role in the TNF-induced osteoclast differentiation [102].

Our study analyzed the potential of pro-inflammatory cytokines, prominent in the synovial fluid, as a trigger for the granulocyte to monocyte conversion, suggesting the described lineage shift as a novel mechanism participating in the progression of RA pathogenesis. However, validations in mouse model are required to evaluate the *in vivo* relevance of the observed phenomenon. So far, based on the findings obtained from primary, *in vitro* differentiated cells we hypothesize the MKK6-p38MAPK dependent G to Mo lineage conversion to contribute to the perpetuation of RA progression by at least two mechanisms. On the one hand, the switch into the monocytic lineage generates a cell type responsible for the prolonged and excessive production of pro-inflammatory cytokines, central to the pathogenesis of inflammatory bone and joint destruction. On the other hand, the redirection into the monocytic cell subset enlarges the pool of monocytes capable to undergo osteoclast differentiation in the synovial inflammatory tissue. The local accumulations of osteoclasts in turn triggers bone erosions characteristically found in the inflamed joints of RA patients (Fig. 24).

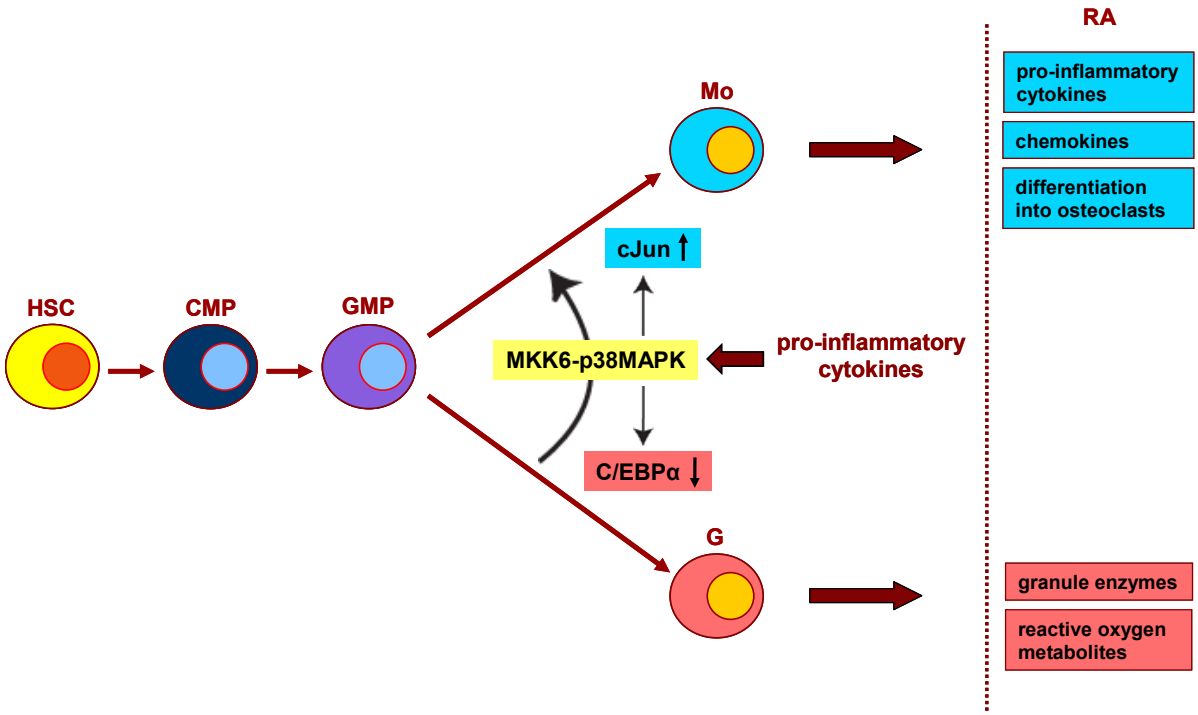

Figure 24. MKK6-p38MAPK-dependent G to Mo conversion – a model for the contribution to RA progression. Stimulation with pro-inflammatory cytokines triggers the MKK6-p38MAPK signalling cascade, which in turn drives the upregulation of the critical monocytic transcription factor cJun and the downregulation of the granulocytic factor C/EBPα, thus, promoting G to Mo conversion. The impact of Mo and G for RA progression is illustrated.

In conclusion, identification of the phenomenon of MKK6-p38MAPK dependent granulocyte to monocyte switch might provide new insights in our understanding of the nature of chronic inflammatory diseases. In addition to RA, which was taken in our study as a prototype of such an disorder, a variety of common diseases are known to be driven by chronic inflammation, including the most prominent ones such as inflammatory bowel disease, atherosclerosis and psoriasis [103-105]. As chronic inflammation is known to accompany the development and/or progression of autoimmune diseases it would be intriguingly to further extend the list by well known, severe autoimmune disorders including multiple sclerosis, type 1 diabetes mellitus, and systemic lupus erythematosus [106-108].

Beside the presumed impact of G to Mo conversion on the initiation and/or perpetuation of chronic inflammatory diseases as well as autoimmune disorders we propose the observed lineage switch as a novel mechanism participating in the progression of inflammatory processes in general and, therefore, open new perspectives for further investigations. Central to them is the validation of the *in vivo* relevance of the observed phenomena. RA mouse models could be used for the initial approval of the occurrence of such G to Mo switch *in vivo*. In case of promising results the study could further be extended to a variety of additional, inflammation-driven disease models.

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