Supplemental Data

Monocytic cell differentiation from band-stage neutrophils under inflammatory conditions via M KK6 activation

René Köffel¹,⁸, Anastasia Meshcheryakova¹, Joanna Warszawska², Annika Henning¹, Karin Wagner⁶, Almut Jörgl¹, Daniela Gubi¹, Doris Moser³, Anastasiya Hladik⁵, Ulrike Hoffmann⁸, Michael B. Fischer⁴, Wim van den Berg⁷, Marije Koenders⁷, Clemens Scheinecker⁵, Bernhard Gesslbauer¹, Sylvia Knapp², Herbert Strobl¹,⁸

¹Institute of Immunology, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria; ²CEMM, Research Center for Molecular Medicine of the Austrian Academy of Sciences and Department of Internal Medicine I, Division of Infectious Diseases and Tropical Medicine, Medical University of Vienna, Vienna, Austria; ³Department of Cranio-, Maxillofacial and Oral Surgery, Medical University of Vienna, Austria; ⁴Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Vienna, Austria; ⁵Division of Rheumatology, Medicine III, Medical University of Vienna, Vienna, Austria; ⁶Center for Medical Research, Medical University Graz, Graz, Austria; ⁷Department of Rheumatology, Rheumatology Research and Advanced Therapeutics, Raboud University Nijmegen Medical Center, Nijmegen, The Netherlands; ⁸Institute of Pathophysiology and Immunology, Center of Molecular Medicine, Medical University Graz, Graz, Austria;

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Cytokines and reagents. Human stem cell factor (SCF), thrombopoietin (TPO), tumor necrosis factor alpha (TNFα), granulocyte colony-stimulating factor (G-CSF), IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-4, and macrophage colony-stimulating factor (M-CSF) were purchased from PeproTech (London, UK); fms-related tyrosine kinase 3 ligand (Flt3L) was obtained from Amgen (Seattle, WA); doxycycline (DOX) and PKH26 Red Fluorescent Cell Linker Kit were purchased from Sigma-Aldrich (Vienna, Austria), SB230580 and SP600125 were purchased from Calbiochem (San Diego, CA); MG132 was purchased from PeptaNova GmbH (Sandhausen, Germany).

Flow cytometry. For intracellular antigen detection, cells were processed with Fix&Perm Kit (An Der Grub, Austria). The following murine monoclonal antibodies (mAb) were used: FITC-conjugated mAbs specific for CD15, CD86 (BD Pharmingen); phycoerythrin (PE)-conjugated mAbs specific for CD11b, (BD Pharmingen), Lactoferrin (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 and SA-APC (BD Pharmingen). Isotype control mAbs were kindly provided by O. Majdic (Vienna, Austria).

Isolation of neutrophils and in vivo transdifferentiation. G-CSF mobilization in lys-EGFP mice (7- to 12-week-old) was performed by subcutaneous injections of human G-CSF (250 µg/kg; Neupogen®; Amgen Europe) on 4 consecutive days. At day 5, mice were sacrificed and blood was collected from the vena cava. Isolation of neutrophils was performed by density gradient centrifugation using Lymphoprep (Axis- Shield PoC AS, Oslo, Norway) according to the manufacturer’s protocol. After red blood cell lysis, neutrophils were cultured on tissue-culture dishes in a 37°C incubator with 5% CO₂ for 1 hour to deplete possible contaminating macrophages as already described (Sasmono et al., 2007). Nonadherent
neutrophils were then harvested from the supernatant and resuspended in sterile PBS (Sigma Aldrich). FACS analysis and histological analysis of isolated GFP* neutrophils confirmed their neutrophilic phenotype. To induce peritonitis, 7- to 12-week-old C57BL/6J mice were intraperitoneally injected with 2 mL sterile and aged 4% (w/v) thioglycollate (ThG) broth (Sigma Chemical Co., St. Louis, MO, USA). Four hours after ThG injection, 2 to 4x10^6 GFP*Ly6G^+F4/80^- granulocytes were transferred by intraperitoneal injection into the peritoneal cavity. At the indicated time points, mice were sacrificed (Ketamin/Rompun®) and peritoneal lavage was performed with 5 mL PBS. Typically, 2–4 x 10^7 cells were obtained per mouse. After red blood cell lysis, peritoneal cells were washed with staining buffer, incubated with mouse anti-CD16/32 (eBioscience) for 15 minutes to block Fc receptors. After washing, cells were stained with antibodies anti-Ly6G (Clone 1A8; Miltenyi Biotec), anti-F4/80, anti-B220 (Biolegend), anti-TCRβ (BD Biosciences), and CD11b (eBioscience) for 30 minutes and analyzed by flow cytometry. FACS analyses and sorting were carried out on BD FACSAria and LSRII cytometers. Data were analyzed with CellQuest Pro, FlowJo or FACS Diva software (all BD Biosciences). E.coli induced peritonitis was performed as described (Matt et al., 2013). 5x10^3 CFU E. coli 018:K1 were injected intraperitoneally into 7- to 12-week-old C57BL/6J mice. Peritoneal lavage and samples processing was performed as described above.

**Serum-induced arthritis.** Serum samples were collected from K/BxN mice, aged 1 to 3 months, and pooled (Monach et al., 2008). Wild-type male mice (8- to 9-week-old) were injected i.p. with 150 µL of serum on days 0 and 3. The development of arthritis was assessed by ankle thickening defined as the difference in the ankle thickness from the day 0 measure and grip strength. A clinical index was evaluated for each paw, according to the following criteria: 0 (normal) to 3, clear inflammation in the ankle or joint and grip strength decreasing from 0 (normal) to -3. The score of the mice was evaluated blindly. Mice on day 6 used for the study scored >1 ankle thickness and >-2 grip strength. Tissue from 4 swollen
paws per mouse was pooled, digested with collagenase (SIGMA-Aldrich), and processed for FACS analysis.

**Real-time RT-PCR.** All primers (supplemental Table 1) were designed using the Primer3 design tool. Expression profiling was performed in a 96-well plate on the StepOne™ Real-Time PCR System (Applied Biosystems). Based on melting curve analysis no primer dimers were generated during the applied 40 real-time PCR amplification cycles. Data were analyzed using StepOne software for relative quantification. Expression levels of target genes in cells were normalized to GAPDH.

**Global transcriptional profiling.** For whole transcriptome analysis 1ng total RNA was amplified using linear amplification with NuGen Ovation Pico WTA System V2, M01224v2 (NuGEN Technologies Inc; San Carlos, CA; Cat No. 3302-24) according to the manufacturer's manual. The SPIA cDNA was checked on the BioAnalyzer BA2100 (Agilent; Foster City, CA) using the RNA 6000 Nano LabChip (Agilent; Foster City, CA; Cat.No. 5065-4476). An examination of ~300ng generated cDNA showed a fragment size up to 4000nt (peak at 500nt) which was satisfying for further processing with the Encore Biotin Module, M01111v6 (NuGEN Technologies, Inc; San Carlos; Cat No. 4200). Hybridization of all samples to GeneChip Human 2.0 ST arrays was performed according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA, USA) at 45°C for 17 hours. Washing and staining (GeneChip® HT hybridization, Wash and Stain Kit; Affymetrix, Santa Clara, CA, USA) was done with the Affymetrix Genechip® fluidics station 450 according to the manual (protocol on fluidics station: FS450_0007). Arrays were scanned with the Affymetrix GeneChip scanner GCS3000. Labeling controls and hybridization controls were evaluated with Expression Console EC 1.1. Amplification, Hybridization and first analysis were carried out at the Division Core Facility for Molecular Biology at the Centre of Medical Research at the Medical University of Graz, Austria. Microarray data were analyzed with Partek Genomic Suite v6.6 software (Partek Inc, St Louis, MO, USA). Data pre-processing and filtering (Partek Software,
v.6.6): RMA (background correction, quantile normalization across all chips in the experiment, log2 transformation, median polish summarization); 1way Anova and 2way Anova. The import process of the .CEL files contained RMA normalization (robust multi-chip average) including background correction, quantile normalization across all arrays and median polished summarization based on log transformed expression values. Hierarchical clustering analysis and Principal Component Analysis were performed to compare the global expression profile for each sample and to check for outliers.

**Western blot.** For preparation of whole cell extracts, cells were washed with PBS, resuspended in sample buffer (0.5M Tris-HCl pH 6.8; 40% glycerol; 4% SDS; 5 µl/mL bromphenolblue; 5% beta-mercaptoethanol) and lysed by heating at 95°C for 10 minutes. Proteins resolved by SDS-PAGE were transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA). Membranes were probed with anti-MKK6 (BioLegend; San Diego, CA), anti-phosphorylated MKK6, anti-phosphorylated or total p38 MAPK and anti-phosphorylated c-Jun (Cell Signaling Technology, Beverly, MA), anti-c-Jun (BD Pharmingen), anti-C/EBPα, anti-MafB, anti-Actin (Sigma-Aldrich, Vienna, Austria), followed by horseradish peroxide-conjugated goat anti-rabbit or goat anti-mouse IgG (H+L) antibodies (Pierce Biotechnology, Rockford, IL). Protein detection was performed with the chemiluminescent substrate SuperSignal WestPico (Pierce Biotechnology) on a luminescent image analyzer (LAS-4000; FUJIFILM).

**Osteoclast generation.** In brief, neutrophils isolated from G-CSF mobilized donors were maintained in complete RPMI and stimulated with pro-inflammatory cytokines (GM-CSF 10 ng/mL, TNF-α 25 ng/mL, IL-1β 10 ng/mL) or left untreated (G-CSF 100 ng/mL) for 4 to 5 days. Afterwards cells were collected, washed and further cultured under osteoclast-promoting conditions. Osteoclast formation and osteoclastic potential were assessed by tartrate-resistant acid phosphatase (TRAP) staining and pit forming assays as essentially described.(Quinn et al., 1998) High resolution images of cortical bovine bone discs were
generated with a scanning electron microscope (JSM 6310, Jeol Ltd.®, Japan) at an acceleration voltage of 15 kV.

**Immunohistochemistry.** Cytospins with typically 2 x 10^4 cells were prepared and subsequently stained with May-Grünwald-Giemsa solutions according to the manufacturer’s protocol (Merck KGaA). Actin was revealed by rhodamine-phalloidin staining (Molecular Probes). Nuclei were stained with DAPI, and slides were mounted using mounting medium (Dako). Pictures were taken using Eclipse 80i microscope (Nikon) and Lucia G software (Laboratory Imaging).

**Oxidative burst.** Detection of ROS in neutrophilic granulocytes was performed as essentially described using 2',7'-dichlorodihydrofluorescein diacetate (DCF-FA; Molecular probes®) (Bluml et al., 2008; Yang et al., 2010). In brief, CD34^+^ cells were induced to differentiate for 11 days into neutrophilic granulocytes in serum-free cultures supplemented with G-CSF and SCF. Day 11 cells (1x10^6/mL) were loaded with DCF-DA (0.5 mg/mL) at 37°C for 5 min. Control samples were kept on ice. After addition of PMA (10ng/mL) and Ionomycin (1µM) cells were placed in a pre-warmed waterbath (37°C) for 30 min. Stimulation was stopped by adding ice-cold 0.5% BSA/ PBS and, after centrifugation, green fluorescence was immediately measured by FACS.

**Phagocytosis assay.** Phagocytosis assay was performed using Phagotest kit (Glycotope Biotechnology, Germany) according to manufacture protocol. In brief, sorted monocytes, neutrophils, and CD14^hi^ monocytic cells (5 x10^5^ each) were incubated with FITC-E.coli for 30 min at 37°C and on ice as a control. Afterwards, cells were washed and surface bound fluorescent signal was quenched by adding quench solution before analysis according to manufacture’s protocol. In addition, microscopic examination confirmed intracellular FITC-E.coli signals. Analysis of phagocytic capacity was performed by FACS analysis.
Supplemental Table 1. RT-PCR primer

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Supplemental Figure 1. (A) Day 11 generated neutrophils exhibit robust oxidative burst activity. G-CSF dependent primary neutrophils were generated from CD34+ cells in vitro. Day 11 generated cells were stimulated with PMA/Ionomycin for 30 min and assessed for oxidative burst activity (thick line). Unstimulated control is represented by filled histogram. Data are representative of four independent experiments. (B) D.a.MKK6 expression in monocytic cells fails to increase CD14 or CD11b expression levels. CD34+ cells transduced with TA/d.a.MKK6 were differentiated into monocytes (M-CSF, 100 ng/mL; IL-6, 20 ng/mL; SCF, 20 ng/mL; Flt3L 50 ng/mL) for 8 days. At day 8, cells were stimulated with DOX (2 µg/mL) to induce d.a.MKK6 (MKK6) or empty vector control (CTRL) for 48 hours and analyzed by FACS for CD14 and CD11b expression. (C) Protein expression levels of neutrophil (C/EBPα, Gfi-1) and monocyte (c-Jun, MafB) associated transcription factors by FACS-sorted d.a.MKK6 induced in vitro generated neutrophilic granulocytes. NGFR+ d.a.MKK6 induced or empty vector control cells were isolated using FACS sorting after 24 hours. Protein levels were assessed by western blot. (D) Stabilization of C/EBPα protein expression levels by MG132 in d.a.MKK6 expressing HL60 cells interferes with Egr-1 mRNA induction, whereas Gfi-1 mRNA downregulation is unaffected. (E) d.n.c-Jun fails to stabilize C/EBPα expression levels in MKK6-activated HL60 cells. HL60 cells co-expressing d.a.MKK6 (MKK6) and d.n. c-Jun (d.n.c-Jun) were analyzed by western blot for C/EBPα. (F) Cell numbers of d.a.MKK6-activated HL60 cells. HL60 cells expressing d.a.MKK6 (MKK6) or empty vector control (CTRL) were induced by DOX and cell numbers were determined 48 hours later.
Supplemental Figure 2. (A) Stimulation of primary G-CSF-induced neutrophilic granulocytes. CD34+ cells were differentiated under serum free conditions to neutrophilic granulocytes with G-CSF and SCF for 11 days. At day 11 cells were washed and stimulated with G-CSF (100 ng/mL) or pro-inflammatory cytokines (GM-CSF, 10 ng/mL; TNFα, 25 ng/mL; IL-1β, 10 ng/mL). After 72 hours cells were processed for FACS analysis. Representative dot plots show CD14 versus Lactoferrin (LF) expression. (B) D.a.MKK6-activated HL60 cells show upregulation of CD86. HL60 cells, stably transfected with d.a.MKK6, were stimulated with (MKK6) or without (CTRL) DOX for 48 hours. Cells were then analyzed for expression of CD86 by FACS. Bar diagram represents mean values ± SD of six independent experiments. (C) D.a.MKK6-activated HL60 cells show enhanced immunostimulatory capacity. D.a.MKK6-activated HL60 cells were g-irradiated (20 Gy) and graded numbers of these stimulator cells were co-cultured with a constant number of 1 x 10^5 purified allogeneic T cells in RPMI medium containing 10% FCS using U-bottom, 96 well tissue culture plates (Nalge Europe, Brussels, Belgium). Cultures were pulsed at day 6 for 18 hours with 1 μCi [0.037 MBq]/well methyl-3H thymidine (Amersham, Buckinghamshire, UK). Incorporated radioactivity was measured using a 1450 microbeta plate reader (Wallac-Trilux Instrument; Life Science, Vienna, Austria). Mean values ± SD of three independent experiments.
Supplemental Figure 3. Cell morphology of neutrophils isolated from G-CSF mobilized donors. Neutrophils were isolated from G-CSF mobilized donors at day 4 after Neupogen™ treatment. Afterwards cytospins were prepared and cell morphology were analyzed by May-Grünwald-Giems staining (bar 10µm). Cells from four different donors are shown.
Supplemental Figure 4. Characterization of neutrophil-derived CD14^+ monocytic cells.
(A) Neutrophil-derived CD14^+ monocytic cells (CD14^+; day 5 after stimulation with GM-CSF (10 ng/mL) / TNFα (25 ng/mL) / IL-1β (10 ng/mL), normal blood monocytes (Mo), and neutrophils (PMN) were assessed for their phagocytosis capacity of *E.coli*-FITC. One representative experiment of three independent donors is shown. (B) Freshly isolated neutrophils from G-CSF mobilized donors were FACS sorted for CD15^+CD54^- versus CD15^+CD54^+ cells. Afterwards, sorted cells were stimulated with GM-CSF (10 ng/mL) plus TNFα (25 ng/mL), and IL-1β (10 ng/mL) for 5 days. At day 4, cells were analyzed for the expression of CD14 versus CD54. Representative dot plots of 3 independent donors are shown. (C) Giemsa staining of freshly isolated FACS sorted CD15^+CD54^- neutrophils.
Supplemental Figure 5. Ly6G⁺F4/80⁺ monocyte-like cell populations arise in ThG-induced peritonitis (A), bacterial infection (B), and in the K/BxN mouse model of rheumatoid arthritis (C). (A, B) Leukocytes from peritoneal lavage fluid (PLF) of C57BL/6 mice were examined for surface expression of Ly6G, CD11b, and F4/80. Gated Ly6G⁺ cells analyzed for CD11b versus F4/80 expression in ThG-induced peritonitis at day 1 (A) or 17 hours after i.p. injections of *E. coli* (B) are shown. (C) Cells derived from arthritic joints were isolated at day 6 after K/BxN serum induction of C57BL/6 mice. Joint derived cells were stained with Ly6G, CD11b, and F4/80. Gated Ly6G⁺ cells are analyzed for CD11b versus F4/80 expression. Bars represent percentages of Ly6G⁺ neutrophils, Ly6G⁺F4/80⁺ monocytes-like cells, and F4/80⁺ monocytes/macrophages among total cells ((A) n=5; (B) n=5; (C) n=3)).
Supplemental Figure 6. Morphology of purified GFP^LY6G^F4/80^ neutrophils used for cell transfer experiments. May-Grünwald-Giemsa stained microscopic images of GFP^LY6G^F4/80^ neutrophils are shown (bar 10 µm).
Supplemental Figure 7. Hypothetical molecular model of neutrophil to monocyte phenotypic conversion. Neutrophils express high levels of C/EBPα resulting in the repression of the monocyte-promoting transcription factor c-Jun. Neutrophils from the bone marrow reserve pool are mobilized into peripheral blood in response to systemic inflammatory signals or as a result of G-CSF treatment. Inflammatory lesion-associated microenvironmental signals induce MKK6 activation in neutrophils attracted to lesional sites. MKK6 activation induces p38 phosphorylation resulting in proteasomal degradation of C/EBPα, which in turn allows c-Jun expression. Additionally, c-Jun is phosphorylated by MKK6-p38 signaling at the N-terminal transactivation domain, representing a critical prerequisite for the establishment of the monocyte-like phenotype. MKK6-p38 inducible neutrophil-derived CD14hi-c-Jun+ monocytes/macrophages express additional monocyte-affiliated transcription factors. These neutrophil-derived inflammatory monocytes/macrophages may further differentiate to macrophages, osteoclasts or myeloid dendritic cells depending on the local microenvironment.
Supplemental References