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Transcriptional effects of Colony-stimulating factor-1 in mouse macrophages

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ABSTRACT

Colony-stimulating factor-1 (CSF-1) is a major regulator of macrophage development. CSF-1-dependent signalling has been implicated in proliferation, survival, and differentiation of mononuclear phagocytes, however, relatively little is known about the effects of CSF-1 on macrophage gene transcription and on CSF-1-responsive gene promoters. We used a combination of transcription profiling and *in silico* motif search to characterize genes that are regulated in mature bone marrow-derived macrophages cultured in the presence or absence of CSF-1. The expression of many known differentiation-associated macrophage markers was not significantly affected in the absence of CSF-1. Genes repressed by CSF-1 comprised a considerable number of granulocyte-specific genes. The respective gene promoters; however, were not significantly enriched for specific DNA patterns, suggesting that these genes are regulated by promoter-distal elements or at a post-transcriptional level. Genes downregulated upon CSF-1 deprivation showed a highly significant association with cell division which is in line with the known role of CSF-1 as a proliferation stimulus for mouse macrophages. Interestingly, three DNA patterns were significantly co-enriched in CSF-1-dependent gene promoters, including motifs related to NFY, CHR, and E2F sites. These motifs showed a strong positional preference on target promoters at −60, −30 and 0 bp upstream of the transcription start site, and define the common promoter structure of CSF-1-responsive genes.

Introduction

Colony-stimulating factor 1 (CSF-1) is the key cytokine controlling differentiation, proliferation, and survival of macrophages (Hume and Gordon 1983). Mice homozygous for an inactivating mutation of the *Csf1* gene ($Csf1^{op}/Csf1^{op}$) show marked deficiency in monocytes, osteoclasts and tissue macrophages (Dai et al. 2004; Cecchini et al. 1997), highlighting the essential function of CSF-1 for mononuclear phagocytes. Apart from lineage commitment, CSF-1 has also been implicated in macrophage immune functions. In mature macrophages, CSF-1 synergizes with LPS for IL-6 production (Sweet et al. 2002), thus promoting inflammation. Furthermore, CSF-1 triggers cytoskeletal changes and facilitates monocyte recruitment to sites of infection (Allen et al. 1997). High levels of the cytokine have been associated with enhanced viral replication in macrophages (Kalter et al. 1991). CSF-1 also promotes collagen-induced arthritis (Campbell et al. 2000), tumour growth and metastasis (Lin et al. 2001; Aharinejad et al. 2002). Understanding the role of CSF-1 will be key to understand macrophage biology in health and disease.

Effects of CSF-1 are mediated through a type III tyrosine kinase receptor, the CSF-1 receptor which is encoded by the proto-oncogene *cFms*. Binding of CSF-1 induces CSF-1 receptor dimerization and leads to the autophosphorylation of cytoplasmic tyrosine residues (Yeung and Stanley 2003; Bourette and Rohrschneider 2000). Phosphorylated tyrosin residues associate with a large number of proteins containing Src Homology-2 (SH2) domains and consequently recruit and/or phosphorylate many cellular substrates activating numerous signalling pathways (Lioubin et al. 1994). For instance, the adapter protein GRB-2, activates mitogen-activated protein (MAP) kinases, induces the expression of immediate-early transcription factors (c-Jun, c-Fos, AP-1, c-Myc), thus mediating proliferation and activation (Roussel et al. 1991; Langer et al. 1992; Buscher et al. 1993). Members of signal transducer and activator of transcription, STAT1 and STAT3, have also been implicated in CSF-1 signal transduction. They are phosphorylated by the Janus kinase Tyk2, and thought to transmit CSF-1 signals into the nucleus (Novak et al. 1995). The lipid kinase signalling molecule, phosphatidylinositol-3 kinase (PI3K), also binds to phosphorylated tyrosine residues and promotes survival and migration of macrophages (Kelley et al. 1999). CSF-1 receptor dimerization also triggers activation of the Raf/MEK/ERK pathway mediating cellular proliferation and activation (Valledor et al. 1999; Cheng et al. 1999).

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Although numerous studies have investigated CSF-1 signalling in mouse macrophages, the transcriptional changes in response to CSF-1 and the nature of transcription factors that mediate the effects of the cytokine are not well defined. Here, we performed global expression analyses to identify genes that are either repressed by or dependent on CSF-1 in mature bone marrow-derived macrophages (BMM). Interestingly, the removal of CSF-1 induced expression of a number of neutrophil-specific genes, suggesting an active role for CSF-1 in repressing these lineage-specific genes. The absence of common sequence motifs in promoters of CSF-1-repressed genes indicates the involvement of distal enhancers or post-transcriptional regulatory mechanisms. On the other hand, a large number of genes were downregulated after CSF-1 removal that were primarily associated with proliferation and depended on ERK signalling. *In silico* analysis of promoter sequences of CSF-1-dependent genes showed a highly significant enrichment of three sequence motifs, NF-Y, CHR, and E2F, with a strong positional preference on target promoters at −60, −30 and 0 bp upstream of the transcription start site, respectively. Thus, our analysis defines the common structure of promoters regulating CSF-1-dependent genes.

Materials and methods

Chemicals

All chemical reagents were purchased from Sigma-Aldrich (Berlin, Germany) unless otherwise noted. Protease inhibitors were obtained from Roche Applied Science unless otherwise noted. LY294002, SB203580, JNK Inhibitor II, and GW2580 were obtained from Calbiochem (CA, USA). U0126 was purchased from Promega (Mannheim, Germany). Oligonucleotides were synthesized by Metabion (Martinsried, Germany) or Operon Biotechnology GmbH (Cologne, Germany). Oligonucleotide primers were designed using PerlPrimer and controlled using UCSC *in silico* PCR and BLAT (http://genome.ucsc.edu/). Primer sequences are given in Supplemental Table 1**.** Antisera used for chromatin immunoprecipitation were purchased from Santa Cruz or Abcam.

Mice

C57BL/6J (8–12 weeks) mice were supplied by Charles River Laboratories (Sulzfeld, Germany).

Cells and cell culture

To generate bone marrow-derived macrophages (BMM), bone marrow cells were flushed from femurs and tibias of 8–12 weeks old mice using cold PBS. Cells from several animals were pooled and plated on bacteriological 100 mm square plastic plates (Bibby Sterilin, Staffordshire, UK Ltd.) at 5×10^5 cells/ml in 20 ml endotoxin-free RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with glutamine, vitamins, pyruvate, nonessential amino acids, β-mercaptoethanol (all purchased from Invitrogen, Germany), 10% FCS (PAA Laboratories GmbH, Austria), and 200 ng/ml human rCSF1 (Cetus) per plate for 5 days. The complete medium was replaced on day 5, cells were harvested on day 6 and seeded with or without rCSF1 for 3, 24 and 48 h at a density of 10×10^6 cells/10 ml medium on 10 cm tissue culture dishes (Falcon). For inhibitor studies, BMM (on day 6), cultured with CSF-1, were incubated for 3, 24 or 48 h with W2580 (10 μM), LY294002 (100 μM), SB203580 (10 μM), JNK inhibitor II (40 μM), U0126 (10 μM) or vehicle (DMSO). In samples that were treated for 48 h, inhibitors were added on day 6 and additionally on day 7.

Mouse neutrophiles were isolated from peripheral blood taken by heart puncture with a 0.5 M EDTA coated syringe and 27-gauge needle. Erythrocytes were lysed as follows: 500 μl blood was mixed with 70 μl 0.05 M EDTA and 5 ml erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA pH 7.2- min and centrifuged (350*g*, 5 min). Cell pellets were washed twice with PBS, pooled, resuspended in PBS and counted. Neutrophiles were positively enriched from 10×10^6 total blood cells using MACS Anti-Ly-6G MicroBeads kit (Miltenyi Biotec, Germany) following the manufacturer's instruction except using 3 times the indicated volume of each reagent including the beads. The magnetic separation was performed using MS MACS columns and the suitable MACS separator (Miltenyi Biotec, Germany).

Flow cytometry analysis

BMM were harvested and resuspended in PBS containing 10% FCS and 10% supernatant from clone 2.4G2 containing anti-CD16/32 antibodies (kindly provided by Gerhard C. Hildebrandt, Department of Hematology, University Hospital Regensburg) to block FcRs. After blocking for 30 min on ice, cells were stained in blocking buffer with the specific antibodies for 45 min in the dark on ice. The following monoclonal antibodies were used: PE-labeled rat anti-mouse CD11b (Mac-1), CD115, CD204, F4/80 and isotype control (all purchased from AbD Serotec, UK); PE-labeled rat anti-mouse Ly-6G and isotype control (clone RB6-8C5) (purchased from BD Biosciences Pharmingen). After staining, cells were washed twice and incubated in PBS containing 10% FCS. FACS analysis was performed on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using CellQuest software (BD Biosciences).

Total RNA-preparation and quantitative real-time (RT) PCR

Total RNA was isolated using the RNeasy midi kit (Qiagen). RNA (1 μg) was reverse transcribed using Superscript II MMLV-RT (Promega). For elongation experiments, RNA (1 μg) was reverse transcribed using AffinityScript™ Multiple Temperature Reverse Transcriptase (Stratagene). Reverse transcribed products were diluted 1:4 in RNase free water and analyzed by quantitative RT-PCR on a LightCycler (Roche) or a Mastercycler Ep Realplex (Eppendorf) using the Quantitect SYBR Green PCR kit or the QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. Primers are given in Supplemental Table 1**.** Melting curves were analyzed to control for specificity of the PCR reactions. Expression data for all genes were normalized to the housekeeping gene *Hprt1*. The relative units were calculated from a standard curve plotting three different concentrations of log dilutions against the PCR cycle number (CP) at which the measured fluorescence intensity reached a fixed value. The amplification efficiency E was calculated from the slope of the standard curve by the formula: $E = 10^{-1/\text{slope}}$. For each sample, data of at least three independent analyses were averaged.

Microarray analysis and data handling

Two independent microarray experiments were performed using Affymetrix Mouse430A_2 arrays with RNA from BMM cultured with or without CSF-1 for 48 h (8 days in total). Hybridization, cRNA labelling, and data handling were performed by the Centre of Excellence for Fluorescent Bioanalysis GmbH (Regensburg, Germany). Data were imported into GeneSpring GX 7.3.1 (Agilent Technologies) for clustering and comparative analysis.