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Dominant Negative MCP-1 Blocks Human Osteoclast Differentiation

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ABSTRACT

Human osteoclasts were differentiated using receptor activator of NFκB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) from colony forming unit-granulocyte macrophage (CFU-GM) precursors of the myeloid lineage grown from umbilical cord blood. Gene expression profiling using quantitative polymerase chain reaction (Q-PCR) showed more than 1,000-fold induction of chemokine MCP-1 within 24 h of RANKL treatment. MCP-1 mRNA content exceeds that of other assayed chemokines (CCL1, 3, 4, and 5) at all time points up to day 14 of treatment. MCP-1 induction preceded peak induction of calcium signaling activator calmodulin 1 (CALM1) and transcription factors JUN and FOS, which were at 3 days. Key osteoclast related transcription factors NFATc1 and NFATc2 showed peak induction at 7 days, while marker genes for osteoclast function cathepsin K and tartrate resistance acid phosphatase (TRAP) were maximally induced at 14 days, corresponding with mature osteoclast function. To test whether the early and substantial peak in MCP-1 expression is part of human osteoclast differentiation events, a dominant negative inhibitor of MCP-1 (7ND) was added simultaneously with RANKL and M-CSF, resulting in blockade of CALM1, JUN and NFATc2 induction and strong inhibition of human osteoclast differentiation. These data show that a cascade of gene expression leading to osteoclast differentiation depends on intact early MCP-1 induction and signaling in human osteoclasts. J. Cell. Biochem. 115: 303–312, 2014.

KEY WORDS: CFU-GM; MYELOID; HUMAN OSTEOCLAST; CHEMOKINE MCP-1 NFAT; INHIBITION; 7ND; JUN; CALMODULIN

Osteoclasts are large multinucleated cells that form by fusion from myeloid precursors within the monocyte/macrophage lineage [Scheven et al., 1986; Udagawa et al., 1990; Teitelbaum, 2000]. Popular osteoclast model systems include a number of species, with mouse and human being the most frequently used. Mouse models include the convenient immortal cell line RAW264.7 [Collin-Osdoby et al., 2003] or primary mouse mononuclear cells isolated from bone marrow (BMM) or spleen [Udagawa et al., 1990]. Human cell systems for studying osteoclast differentiation and function include peripheral blood mononuclear cells usually selected as CD14+ cells (PBMC) [Quinn et al., 1998], bone marrow aspirates [Matsuzaki et al., 1998; Quinn et al., 1998] or myeloid stem cells [Matayoshi et al., 1996]. Colony forming unit granulocyte/macrophage (CFU-GM) are a myeloid cell type derived from umbilical cord blood that can produce human osteoclasts at high frequency [Menaa et al., 2000; Hodge et al., 2004]. Osteoclasts are cultured in vitro from myeloid precursors by stimulation with receptor activator of NFκB ligand (RANKL), an osteoclast differentiation signal [Yasuda et al., 1998] and macrophage colony stimulating factor (M-CSF) a cytokine that is essential for human osteoclast precursor culture [Udagawa et al., 1999; Tsurukai et al., 2000; Boyce, 2013]. Cultured osteoclasts are frequently defined as multinuclear cells (MNC) with three or more nuclei that are positive for tartrate resistant acid phosphatase (TRAP+). Mature osteoclast function is characterized by notable marker genes including the protease cathepsin K (CTSK) and TRAP. Osteoclast differentiation is characterized by induction of transcription factors necessary for osteoclast differentiation including JUN, FOS, and NFATc1 [Takayanagi, 2007; Sitara and Aliprantis, 2010]. Although numerous roles for NFATc1 exist in other cell and organs systems, including activated T cells and indeed osteoblasts, NFATc1 is considered a master regulator of osteoclast differentiation since over-expression of

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NFATc1 in myeloid precursors appears sufficient for osteoclast differentiation.

Chemokines are small signaling molecules that mediate cross-talk between numerous cell types. We previously noted an important role for chemokine MCP-1 (CCL2) in human osteoclasts, defining a cell fate switch for osteoclast differentiation either to a dendritic-like cell or osteoclast [Kim et al., 2005]. In addition, MCP-1 treatment stimulated the formation of TRAP+ MNC and increased bone resorption activity in human osteoclasts, suggesting a role for chemokine signaling in osteoclast physiology [Kim et al., 2006a]. Similarly, other CCL chemokines and receptors are induced by RANKL in human osteoclasts [Granfar et al., 2005; Kim et al., 2006b]. Literature on gene expression analysis of CFU-GM derived human osteoclasts is limited. In this study, a range of genes are examined during 14 days of differentiation of CFU-GM into human osteoclasts using quantitative polymerase chain reaction (QPCR) analysis of transcript levels. Such genes include chemokines of the CCL family (1 through 5), calcium signaling mediator calmodulin, transcription factors involved in osteoclast differentiation and mature osteoclast functional markers. Furthermore, a dominant negative form of MCP1, known as 7ND [Zhang and Rollins, 1995; Ni et al., 2001] was used to show that chemokine signaling is essential for human osteoclast differentiation.

MATERIALS AND METHODS

CELL CULTURE METHODS

Colony forming unit, granulocyte macrophage (CFU-GM) are a particular sub-fraction of the myeloid lineage and were isolated by clonal selection in Methocult GF H4534 soft matrix medium (Stem-cell Technologies) from CD14 positive cord blood mononuclear cells purified over Ficoll-Paque using magnetic bead selection with anti CD14 antibody (Macsbeads, Mylteni) from healthy donors, as previously described [Nicholson et al., 2000; Hodge et al., 2004]. Basically, CFU-GM were cloned in methocult as colonies grown in medium supplemented with 30% fetal bovine serum (FBS), granulocyte colony stimulating factor (GM-CSF), interleukin (IL3), and stem cell factor (SCF). Colonies of CFU-GM were pooled for osteoclast assays as described [Hodge et al., 2004]. Osteoclast differentiation medium was Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% FBS, RANKL at 125 ng/ml and M-CSF at 25 ng/ml. Cells were seeded at 4×10^4 cells per well in a 96 well plates. CFU-GM derived osteoclast cultures form osteoclasts around day seven of culture [Hodge et al., 2004]. In most cases data are derived from three separate donor preparations of CFU-GM, differentiated into osteoclasts on different occasions and harvested as a time course, although some data are derived from up to six donor samples. Recombinant forms of human GM-CSF, M-CSF, and RANKL were obtained from Chemicon, Genetics Institute, and Peprotech, respectively.

HEK293T cells (ATCC, Maryland) were grown in DMEM augmented with 10% FBS. An expression construct of a dominant negative variant of MCP-1, known as 7ND, in the vector pcDNA was kindly donated by Ni et al. [2001]. Fugene (Roche) was used to transiently transfect the 7ND expression construct in HEK293T cells according to the manufacturer's instructions and fresh medium replaced after 4 h exposure to the Fugene-DNA containing medium. As a control for transfection and the presence of DNA in transfected cells, a control construct was a green fluorescent protein (GFP) expression vector in the parent vector pcDNA (Addgene). A further control was conditioned medium from un-transfected HEK293T cells. Transfected cell culture conditioned medium was harvested from the transfected cells after 6 days, centrifuged and filtered (0.44 μM). Osteoclast cultures were derived from PBMC using RANKL and M-CSF, stained and counted as previously described [Day et al., 2004]; conditioned medium was added to the culture at the rate of 100 μl/ml every 3 days with replacement. For studies of 7ND treatment of CFU-GM cells, 7ND peptide was purified from large amounts of transfected cell culture medium using FLAG affinity chromatography (Sigma–Aldrich), quantitated by Biorad protein stain, and added at 100 ng/ml to cultures simultaneously with RANKL and M-CSF. All experiments involving human samples were done after written informed consent was obtained in accordance with the Declaration of Helsinki approved by the Barwon Health Human Research Ethics Committee.

REAL-TIME PCR

Primers for gene specific transcript assays are listed in Table I. RNA was isolated using standard ultracentrifugation in caesium chloride

TABLE I. Primers used for Quantitative PCR Analysis

Gene	Forward 5′-3′	Reverse 5′-3′
18S rRNA	CTTAGAGGGACAAGTGGCG	ACGCTGAGCCAGTCAGTGTA
CALM1	GGCATTCCGAGTCTTTGACAA	CCGTCTCCATCAATATCTGCT
CCR2a	CATAGCTCTTGGCTGTAGGA	GTGAAGCCAGACGTGTGATT
CCR2b	AACAAACACGCCTTCCACTG	GTCAAAGTCTCTACCCACAG
CCR4	CTTATGGGGTCATCACCAGT	AGTAGGTATGGTTGCGCTCA
CCR5	ACCAAGCTATGCAGGTGACA	GAACAGCATTTGCAGAAGCG
MCP-1 (CCL2)	TCGCGAGCTATAGAAGAATCA	TGTTCAAGTCTTCGGAGTTTG
CCL3	CTATGGACTGGTTGTTGCCA	AGGGGAACTCTCAGAGCAAA
CCL4	TCCATGAGACACATCTCCTC	GCAACAGCAGAGAAACAGTG
CCL5	GAGCTTCTGAGGCGCTGCT	TCTAGAGGCATGCTGACTTC
CTSK	TGAGGCTTCTCTTGGTGTCCATAC	AAAGGGTGTCATTACTGCGGG
JUN	CGTTAGCATGAGTTGGCAC	GCATGAGGAACCGCATCGC
FOS	AGCCAAATGCCGCAACCGGA	GCAGGTTGGCAATCTCGGTC
NFATc1 (total)	GCATCACAGGGAAGACCGTGTC	GAAGTTCAATGTCGGAGTTTCTGAG
NFATc1 exon 1B	GACCCGCCATGACGGGGCTGGAGGA	CTCGTCGCGCTGGTTAAACTCGA
NFATc1 exon 1A	CAGAGCGAGACTCAGAGGCTCCGA	CTAGGACCTGCGCGGTGGCTCCGA
NFATc2	GATGGAAGCCACGGTGGAT	GCGGATATGCTTGTTCCGAT
TRAP	GACCACCTTGGCAATGTCTCTG	TGGCTGAGGAAGTCATCTGAGTTG

using a Beckman mini ultracentrifuge. cDNA was produced using the Improm-2 reverse transcriptase system (Promega) using both random (300 ng) and oligo-dT (100 ng) primers. Real-time PCR was carried out using the Bio-Rad iQ iCycler real-time PCR system using SYBR Green 1 supermix as previously described [Granfar et al., 2005; Kim et al., 2005; Kim et al., 2006a,b]. All Q-PCR assays were quantitative, highly linear in response to calibrated known samples and had similar high efficiencies, and were designed to amplify under the same conditions using BioRAD supermix as previously described [Granfar et al., 2005]. Regression analysis established the relevant equation for each standard. Data are represented as both absolute mRNA content (copies per million 18S molecules) and as fold change relative to either zero time or compared across treatments. Data are shown with error bars being one standard error, derived from replicate biological repeats of cell differentiation. Assays for NFATc1 total mRNA used primers spanning exons 5 and 6, which are common to all isoforms and variant mRNA of NFATc1 [Vihma et al., 2008]. Two assays were used that are specific for two alternative first exons: for exon 1A primers were in the 5′ leader sequence immediately upstream of the coding sequence beginning MPSTSFPVP and for exon 1B the 5′ primer was in the 5′ leader overlapping the initiation codon for the coding sequence beginning MTGLEDQEF and within the exon for the 3′ primer.

Although ΔCT values have a normal distribution, fold change relative to another treatment ($2^{(\Delta CT - \Delta CT)}$) has a log-normal distribution. Fold change in gene expression is expressed relative to the value at zero time, immediately prior to addition of RANKL and standard errors estimated by the standard method of propagation of errors for quotients. In the case of large gene expression changes, fold change is useful. Parametric statistical tests such as analysis of variance or *t*-tests were based on ΔCT data and quantitative data from standard curves. Gene expression changes in this study are so large relative to the standard errors that *P* values are not strictly necessary (all described phenomena are below $P = 0.0001$ unless otherwise mentioned) and are omitted for the majority of changes. Ribosomal 18S rRNA was used as the internal housekeeper gene in these studies. Prior examination of a number of different housekeepers gave high correlation ($R > 0.96$ for all comparisons) and similar results between all housekeepers. Equivalent housekeeper genes are: glyceraldehyde dehydrogenase (GAPDH), beta 2 microglubulin (B2M), 18S rRNA (18S), hypoxanthine-gauanine phosphoribosyl transferase (HPRT), porphobilinogen deaminase (PBDG). All data were pooled and no values were rejected.

RESULTS

GENE EXPRESSION IN CFU-GM DERIVED OSTEOCLASTS

Cultured CFU-GM provide osteoclast precursors that convert with high efficiency to mature osteoclasts under the action of RANKL and M-CSF. Replicate cultures from different donors were differentiated at different occasions and mRNA harvested at the indicated times after RANKL treatment. cDNA were analyzed using real time PCR and all data pooled for analysis. In order to verify that CFU-GM derived osteoclasts express standard markers of osteoclast function, a time course of expression of CTSK and TRAP is presented (Fig. 1A,B). In CFU-GM derived osteoclasts, these osteoclast marker genes related to osteoclast function were induced several thousand fold over time and still increasing in abundance at the last time point of 14 days (Fig. 1A,B). In this cell model, initial cell fusion to produce MNC occurs around day 7.

GENE EXPRESSION CHANGES OF CCL CHEMOKINES

The mRNA content of CCL family chemokines (CCL 1–5) were assayed after RANKL treatment. MCP-1 (CCL2) was induced 1,180-fold at 24 h relative to zero time, reaching a mass of mRNA equivalent to that of

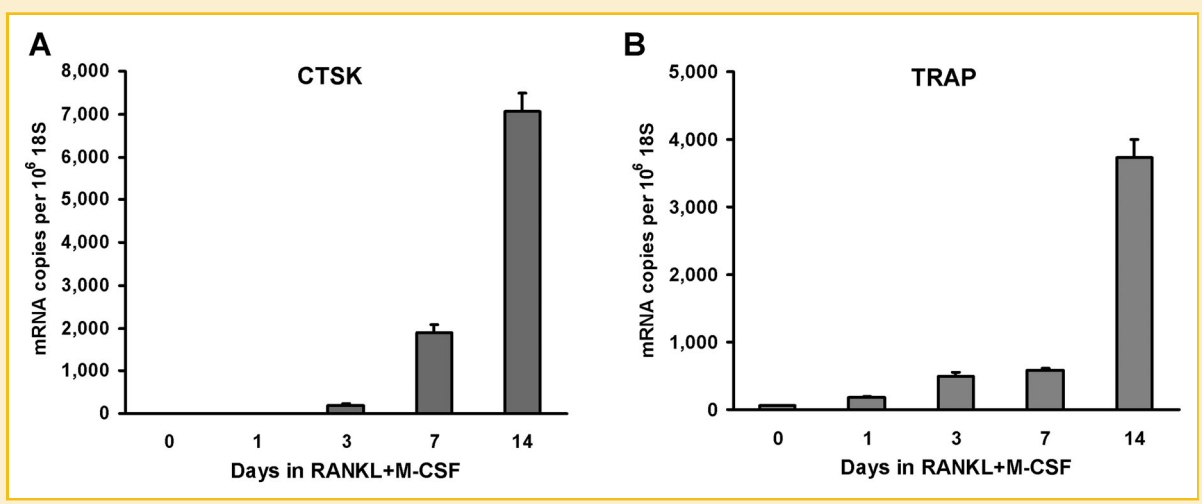

Fig. 1. Mature osteoclast gene markers in time course of CFU-GM precursors differentiated under the effect of RANKL and M-CSF. Cathepsin K (Graph A, CTSK) and tartrate resistant acid phosphatase (Graph B, TRAP) messenger RNA (mRNA) levels continually increase after treatment and are maximal at the last time point assayed (14 days). Graphs A and B show quantitative mRNA content in copies per million 18S molecules. Maximal induction in A and B represents 6,930-fold and 60-fold, respectively over zero-time levels. Error bars represent one standard error of the mean (SEM).

the 18S housekeeper control using calibrated quantitative real time PCR assays (Fig. 2A). After 3 days, induction was still high (416-fold) and the mass of mRNA considerable. These data indicate that MCP-1 transcripts are rapidly induced and reach an extremely high abundance in a short time. At all times the mass of MCP-1 mRNA exceeded by a great margin the mRNA content of all other assayed chemokines combined. Of assayed chemokines, CCL4 was next in rank of mRNA abundance (Fig. 2B) followed by CCL3 and CCL5 (Fig. 2C and D, respectively). CCL4 transcript was around 10 times more abundant than CCL3 and CCL5 combined. MCP-1 mRNA content exceeded all other assayed chemokines for both abundance at zero time and for fold change over zero time. Induction over zero time was about 40-fold for CCL4 (Fig. 2B) and about 9-fold for CCL3 (Fig. 2C) at day 1. When the mass of mRNA was calculated on an absolute basis relative to 18S, MCP-1 mRNA content at 24 h greatly exceeded the content of all other chemokine mRNAs combined. For MCP-1, the fold-change induction was large while the zero time content was also higher than all other assayed chemokines. From these data on levels of mRNA expression, MCP-1 induction would appear to be a significant early event in human osteoclast differentiation from CFU-GM cells. By 14 days, MCP-1 expression had decreased from the early high peak but was still about 25 times greater than at zero time and still exceeded the mass of other assayed chemokine mRNA by more than 1,000-fold.

GENE EXPRESSION CHANGES IN THE CALMODUIN–CALCINEURIN PATHWAY AND TRANSCRIPTION FACTORS

Gene expression patterns in osteoclasts derived from CFU-GM cells are interesting. Following in time after the early MCP-1 induction, calmodulin 1 (CALM1) showed strong induction. CALM1 transcript was induced more than 1,000-fold in a pulse, peaking at day 3 (Fig. 3A). JUN transcripts (Fig. 3B) were more abundant than FOS transcripts (Fig. 3C). When considered in terms of fold change relative to zero time values, JUN and FOS were induced to a similar extent (Fig. 3D). Both NFATc1 (Fig. 4A) and NFATc2 (Fig. 4B) were clearly induced after exposure of cells to RANKL and M-CSF. There was a much greater content of NFATc1 absolute mRNA copies compared to NFATc2 at all times. Induction of NFATc1 (9-fold, $P = 0.01$) and NFATc2 (2.5-fold, $P = 0.02$) was detectable at day 1 post RANKL addition, increasing with time to peak for both factors at 7 days. In terms of fold change from zero time, NFATc2 (60-fold, $P = 0.001$) was more highly induced in comparison to NFATc1 (27-fold) at 7 days, although the fold change is influenced by the relative zero time value, which was higher for NFATc1 transcripts. Fold change of NFATc2

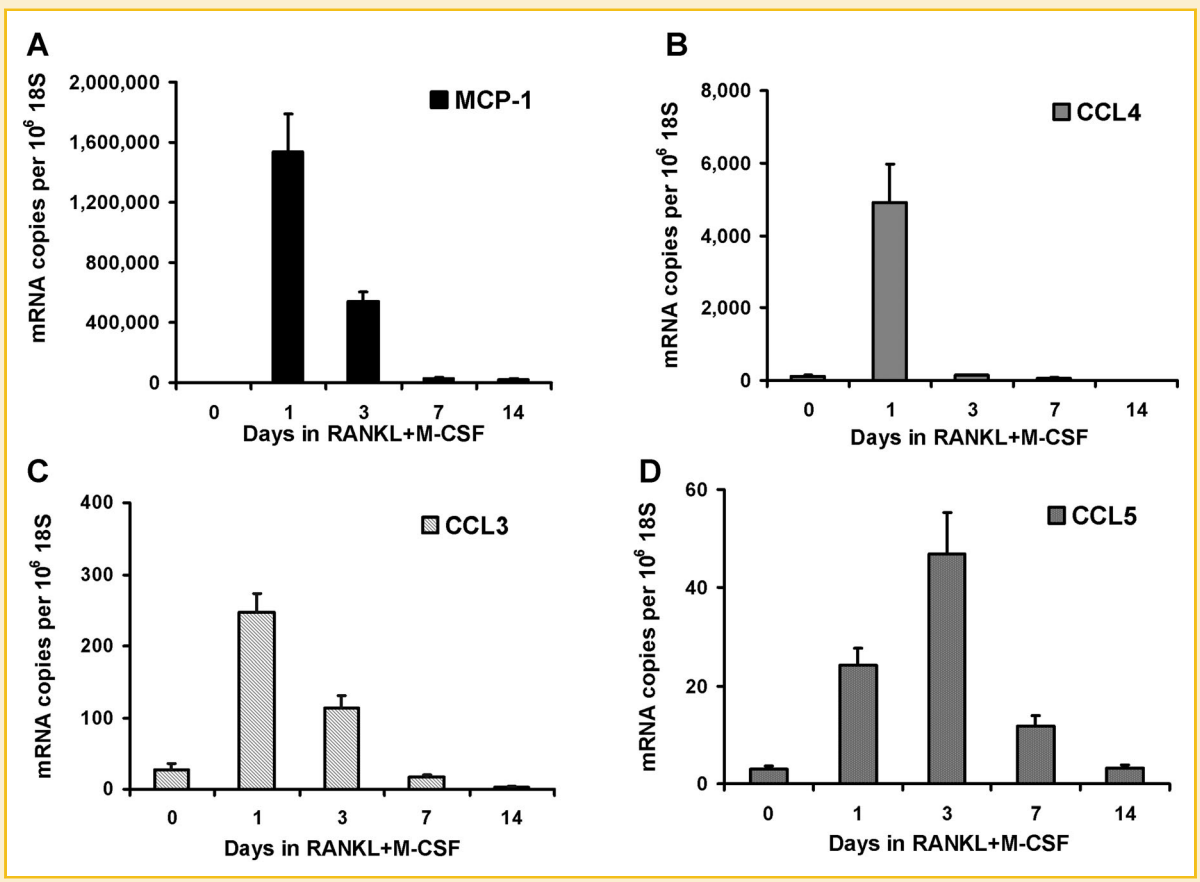

Fig. 2. Induction of CCL chemokines after treatment with RANKL and M-CSF in order of mRNA abundance. A: Strong induction of MCP-1 (CCL2) occurred with 1 day, decreased at day 3 and was still increased over zero-time values at day 14. Induction as fold change over baseline is 1,180-fold at day 1 and 416-fold at day 3. B: Of assayed chemokines, CCL4 (MIP1β) is reasonably induced at day 1 (40-fold over zero time) and decreases rapidly. C: Induction of CCL3 (MIP1α) is significant but much weaker in content than that of MCP-1 or CCL4. D: CCL5 (also known as RANTES) has a peak induction delayed relative to MCP-1, CCL3, and CCL4. In all graphs the scale is mRNA copies per million 18S molecules.

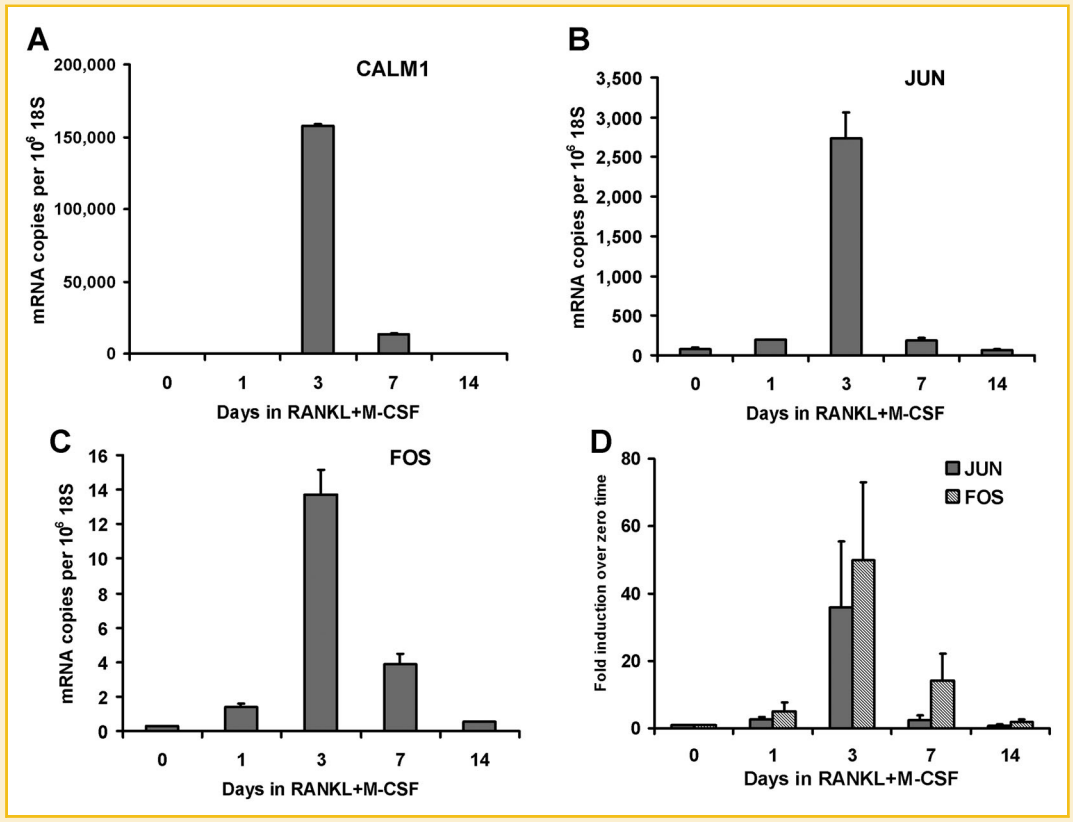

Fig. 3. Induction of agents for calcium signaling and transcription factors. A: Calmodulin 1 (CALM1), showed a strong induction at day 3 (1,000-fold compared to zero time) with substantial mRNA content. B: Transcription factor JUN followed a similar peak induction at day 3, although the mRNA content was much lower than that of CALM1 as expected for a nuclear factor. C: Transcription factor FOS had a similar profile to JUN but a much lower actual mRNA content. D: A comparison of JUN and FOS when data are expressed as fold change over zero time indicates very similar kinetics of induction. In graphs A–C the scale is mRNA copies per million 18S molecules and in D the scale is fold change over zero time values.

followed a slightly different profile with more relative induction (47-fold) at 3 days compared to that of NFATc1 (12-fold). Despite this slight difference in profile, the much greater content of NFATc1 transcript at all times was the dominant feature of this comparison.

Q-PCR of alternative first exons of NFATc1 gave much greater abundance for the exon 1B (Fig. 4C) after treatment with RANKL and M-CSF. Exon 1B transcripts (Fig. 4C) matched closely the measure of total NFATc1 mRNA content (measured using primers spanning exons 5 and 6, Fig. 4A), suggesting that the majority of NFATc1 transcripts in human osteoclasts derived from CFU-GM contain exon 1B sequences. Transcripts derived from exon 1A were of low abundance, but were induced up to 1,000-fold over zero time at day 7 (Fig. 4D). Despite the high induction of transcripts containing exon 1A, the abundance of the exon 1A containing mRNA was low in comparison to that containing exon 1B. Taken together the data indicate that NFATc1 and NFATc2 are induced by RANKL and M-CSF simultaneously within the cell, but that the set points of the relative expression are different, such that NFATc1 is more abundant than NFATc2 and that the predominant NFATc1 isoforms contain the exon 1B sequence.

GENE EXPRESSION CHANGES IN CHEMOKINE RECEPTORS

CCR2, CCR4, and CCR5 transcript levels were assessed only at days 1 and 14. Two alternative isoforms of CCR2 exist [Charo et al., 1994]: the mRNA content of CCR2b was strongly induced at 1 day (560-fold) and more so (1,120-fold) at day 14 (Fig. 5A). CCR2a was significantly induced at both time points but the magnitude (about 200-fold) and the mRNA content, was much less than that of CCR2b (Fig. 5B). CCR4 and CCR5 mRNAs were induced 430- and 107-fold, respectively at day 14 relative to zero time (Fig. 5C,D) and the mRNAs for these two receptors were reasonably abundant relative to 18S.

EFFECT OF BLOCKADE OF MCP-1 WITH DOMINANT NEGATIVE 7ND

MCP-1 mRNA (Fig. 2A) and an abundant high affinity receptor (CCR2b, Fig. 5A) were highly induced within the first day after exposure of CFU-GM to RANKL and M-CSF. The provision of ligand and receptors provides for an autocrine feed-back cycle that was tested using a truncated dominant negative form of MCP-1, known as 7ND, that blocks MCP-1 signaling. Due to limited material, zero time data were not taken and a small range of target genes were examined. For studies of MCP-1 mRNA content, cells were harvested at day 1 and day 2 (Fig. 6A). In control cultures, MCP-1 was highly expressed

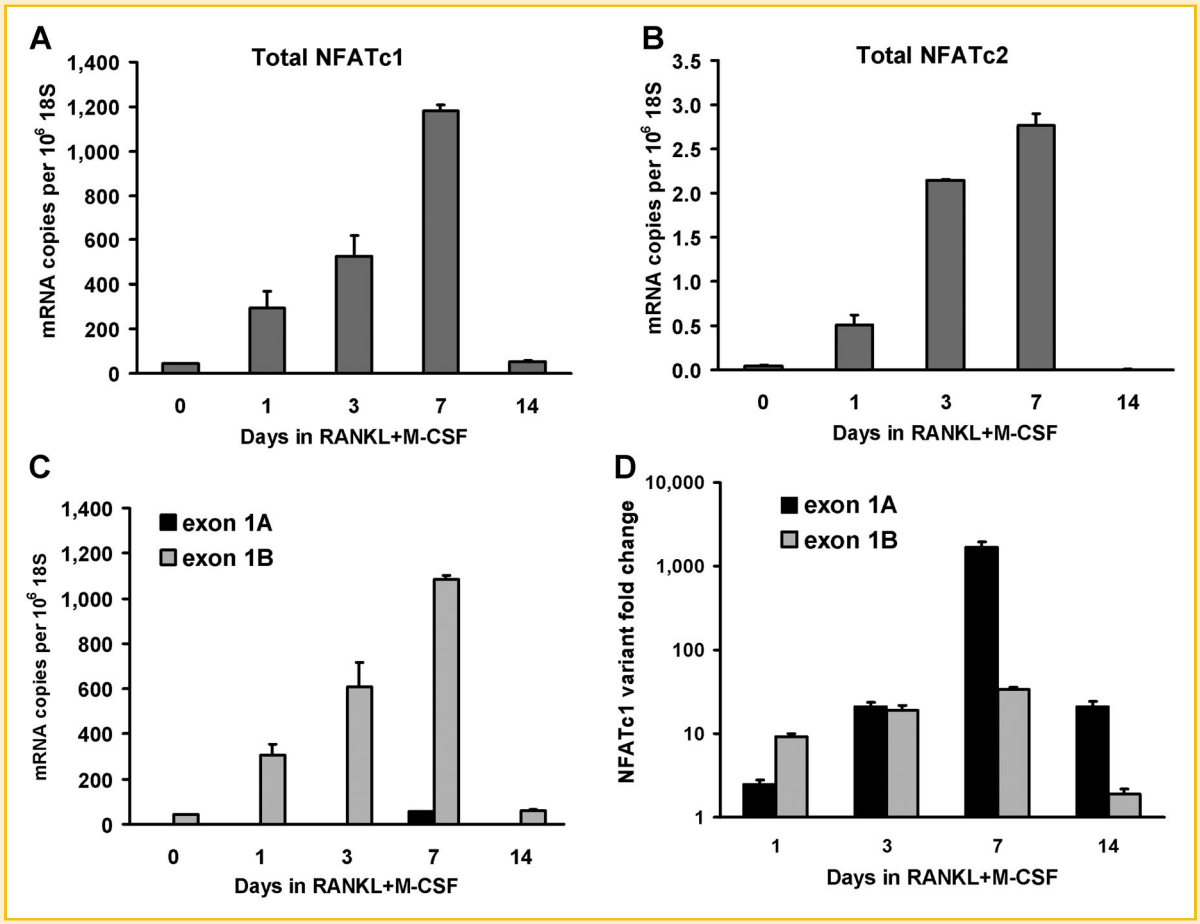

Fig. 4. Expression profiles of key transcription factors involved in osteoclast differentiation. A: Total NFATc1 mRNA content increased until a peak at day 7, although significantly changed at day 1 (ninefold over zero time). B: NFATc2 follows a similar profile of gene expression change to that shown by NFATc1, although the total mRNA content is lower. C: A comparison of the mRNA content of alternative NFATc1 exon 1A and exon 1B, shows a predominance of exon 1B in transcripts. D: NFATc1 alternative exon 1 usage expressed as a fold change over zero-time indicating that induction of both alternative exons is similar until day 7, when exon 1A transcripts are more than 1,000-fold induced relative to zero time. Despite the fold change, graph C shows that exon 1B variants are more abundant at all times tested. In graphs A–C the scale is mRNA copies per million 18S molecules and in D the scale is log-fold change over zero time values.

at day 1 (Fig. 6A) with a similar level as seen in previous experiments (see Fig. 2A). By day 2, a decrease in the peak of MCP-1 expression relative to the strong peak at day 1 was observed ($P = 0.002$). In contrast to control cultures, in 7ND treated cultures MCP-1 mRNA expression was abundant and did not decrease to the same extent at day 2. At day 1 there was no difference in MCP-1 mRNA content between control and treated cells ($P = 0.5$). At day 2, the effect of 7ND was to significantly sustain the level of expression of MCP-1 mRNA, being fivefold greater in 7ND treated cells compared to control ($P = 0.001$). The decrease over time in MCP-1 mRNA expression in control cultures suggests that once MCP-1 protein accumulates in the medium then there may be attenuation of expression due to autocrine signaling: such attenuation in MCP-1 mRNA content is blocked by 7ND treatment.

Other genes (NFATc2, CALM1, and JUN) were examined only at day 2 in 7ND treated and control cultures. The mRNA level of NFATc2, CALM1 and JUN at day 2 were closely equivalent to expectations from the prior time course data (see Figs. 3 and 4). The transcript levels of all three genes were substantially reduced in 7ND treated cultures (Fig. 6B,C). These data are consistent with chemokine signaling being necessary for the induction of NFATc2, CALM1, and JUN after exposure to RANKL and M-CSF. Such chemokine is either MCP-1 or some chemokine other than MCP-1 capable of being blocked by the dominant negative 7ND.

The effect of 7ND on differentiation of human osteoclasts, defined as TRAP + MNC, from PBMC was also tested in vitro. In this case 7ND was delivered as conditioned medium from HEK293T cells transiently transfected with 7ND expression vector and compared to control conditioned medium from identically transfected HEK293T cells using a GFP expression vector. 7ND protein was not measured in these media, but QPCR analysis of mRNA indicated around million-fold increase in 7ND mRNA from HEK293T cells transfected with 7ND construct relative to GFP transfected HEK293T cells. A further control consisted of PBMC treated with conditioned medium from

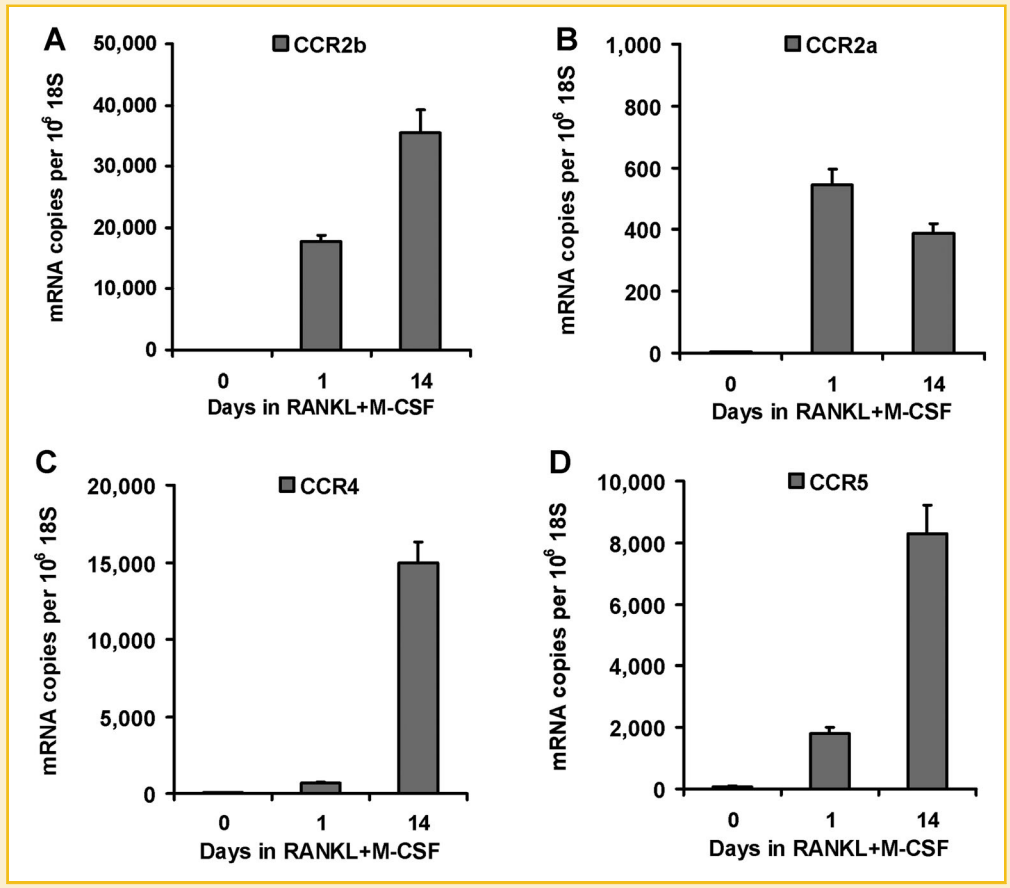

Fig. 5. Expression of chemokine receptors after treatment with RANKL and M-CSF. A: The prime MCP-1 receptor alternative splicing variant CCR2b is a highly abundant transcript and is induced by RANKL. B: CCR2a, a lower affinity MCP-1 receptor variant is of lower abundance than CCR2b. C: An alternative receptor for MCP-1, CCR4 is abundantly expressed and induced by RANKL. D: Receptor CCR5, a receptor for CCL5, is induced by RANKL and is reasonably abundant, despite the low expression of CCL5 in this system. In all graphs the scale is mRNA copies per million 18S molecules.

untransfected HEK293T cells. Conditioned medium was added with replacement every 3 days at the rate of 100 μl/ml culture and TRAP + MNC cells counted after 14 days. A striking inhibition of osteoclast formation was observed in cultures treated with 7ND (Fig. 6D) relative to controls.

DISCUSSION

Donor bloods from human umbilical cords provided CFU-GM as described previously [Hodge et al., 2004]. Such cells produce osteoclasts at high efficiency after exposure to RANKL and M-CSF, giving an opportunity to examine gene expression changes with defined precursors during human osteoclast differentiation. Although the amount of CFU-GM cells available for this study was limited, we were able to assay the expression of a number of genes important to human osteoclast biology. In this cell system osteoclasts become abundant at about 7 days and increase over the 14 day period of study. Increasing gene expression of CTSK and TRAP, marker genes associated with osteoclast function, is consistent with this time frame.

Perhaps a remarkable outcome of this study is the very early potent induction of the chemokine MCP-1 that, both in time frame (peaking at 1 day) and magnitude, exceeded all other measured chemokines. Nevertheless, MCP-1 gene expression was still substantial at later time points, being 25-fold over zero time at 14 days, a figure similar to our prior data on 21 day old human osteoclasts derived from adult blood mononuclear cells [Kim et al., 2006a,b]. Other chemokines such as CCL3 and CCL4 were also induced by treatment with RANKL and M-CSF, but the mass of mRNA in a quantitative sense was much less than that of MCP-1, which appears to be the dominant chemokine (of those assayed) in this human cell model system. We previously showed that MCP-1 is important in human osteoclast biology, determining a cell-fate switch in the presence of GM-CSF between osteoclasts and a dendritic like alternative cell type [Kim et al., 2005]. Exogenous MCP-1 increases osteoclast differentiation and bone resorption in mouse [Miyamoto et al., 2009; Goto et al., 2011; Sul et al., 2012], rat [Li et al., 2007; Asano et al., 2011], and human cell systems [Kim et al., 2005], consistent with a role in stimulating differentiation and function. Although we did not investigate the molecular mechanism of MCP-1 induction, NF-κb transcription

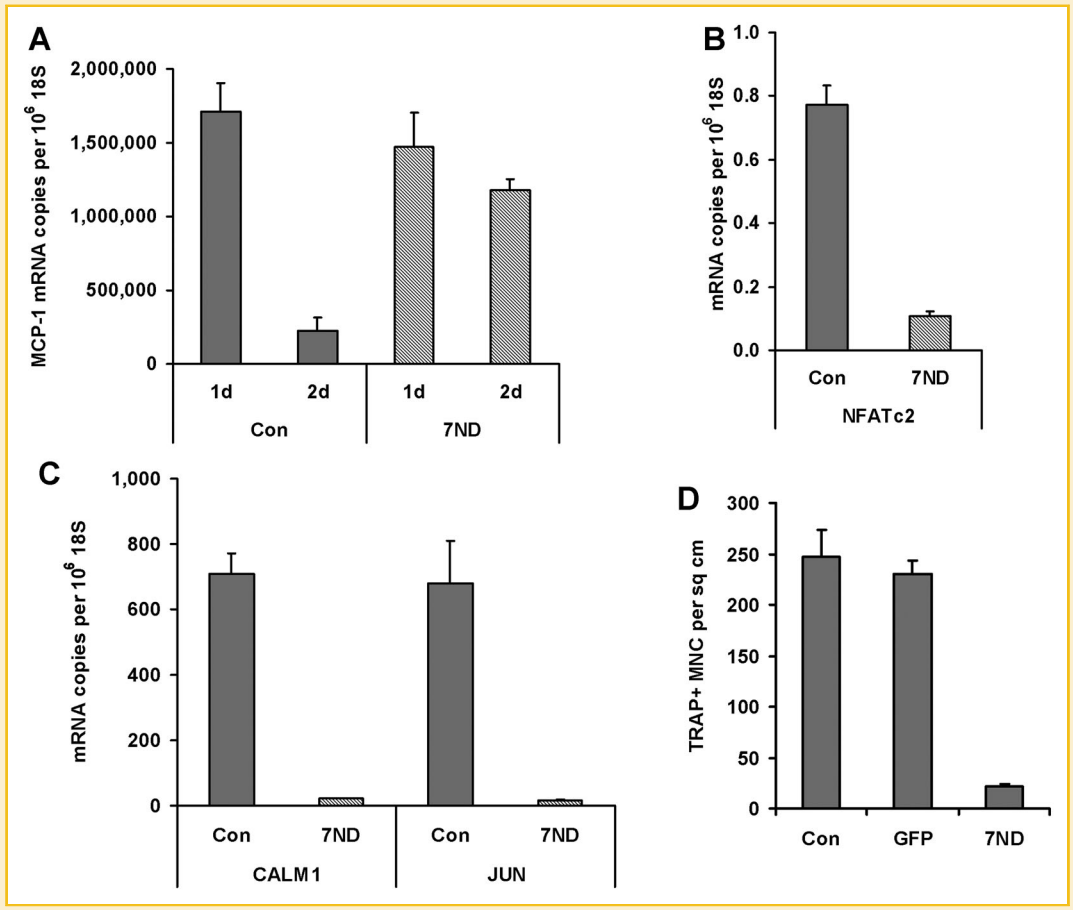

Fig. 6. The effect of MCP-1 blockade by dominant negative 7ND against gene expression in CFU-GM cells and osteoclast formation from PBMC cells. A: Gene expression of MCP-1 after RANKL + M-CSF treatment (columns marked Con, gray columns) compared to RANKL + M-CSF treatment in the presence of 7ND (columns marked 7ND, cross hatching). MCP-1 gene expression subsides on day 2 in control cells treated with RANKL + M-CSF whereas in the presence of 7ND, MCP-1 gene expression persists at a high level. B: NFATc2 mRNA levels are inhibited by 7ND treatment in the presence of RANKL + M-CSF, meaning that gene induction is prevented by 7ND. C: Similar to NFATc2, the RANKL-mediated gene induction of CALM1 and JUN are both suppressed by treatment with 7ND in the presence of RANKL + M-CSF. D: Formation of tartrate resistance acid phosphate positive MNC (TRAP + MNC) is strongly inhibited by 7ND treatment. All columns had treatment with RANKL + M-CSF: Con indicates addition of conditioned medium from HEK293T cells, GFP indicates addition of conditioned medium from HEK293T cells transfected with a green fluorescence protein expression vector (GFP) and 7ND indicates addition of conditioned medium from HEK293T cells transfected with 7ND expression construct. Experiments in graphs A–C used CFU-GM cells treated with purified 7ND (100 ng/ml) and graph D used PBMC cells treated with 7ND in conditioned medium as described.

factor is a strong inducer of MCP-1 in other cell systems [Deng et al., 2013], suggesting that RANKL mediated activation of NF-κb is a reasonable explanation for induction of MCP-1 via RANKL, which is a known activator of NF-κb.

Chemokine receptors are seven-transmembrane loop G-protein (G*i*) receptors that inhibit adenyl cyclase, reduce cAMP, and result in calcium influx after signaling. Receptors for MCP-1 are induced after RANKL treatment, providing the capacity for autocrine activity that directly activates calcium signaling. CCR2 is considered the prime receptor for MCP-1 although considerable promiscuity of ligand receptor interactions are known for CCL–CCR interactions [White et al., 2013]. CCR2b is reported to have higher affinity than CCR2a [White et al., 2013]. CCR2b was particularly abundant when measured as mRNA content using highly specific primers. The alternative, less active form (CCR2a) was only a minor component of CCR2 mRNA. CCR4 has a number of ligands, including MCP-1 [White et al., 2013]. The induction and relative abundance of CCR2b, CCR2a, and CCR4 in CFU-GM derived osteoclasts was similar to our prior observations in PBMC derived human osteoclasts [Kim et al., 2006b]. At peak induction, CCR4 content was about fivefold less than that of CCR2b, suggesting that the majority of MCP-1 signaling in an autocrine setting may pass through CCR2b in this model. It is notable that the dominant negative form of MCP-1 with 7 amino acids truncated (7ND) prevented RANKL mediated induction of NFATc2, CALM1, and the transcription factor JUN. 7ND treatment blocked the decrease in MCP-1 transcripts seen at day 2, an effect consistent with autocrine feedback. Indeed, 7ND treatment strongly inhibited human osteoclast differentiation using the PBMC cell system. These data suggest that early chemokine signaling via MCP-1 is necessary for induction of transcription factors (at least JUN and NFATc2), CALM1 induction and finally osteoclast formation. Although we did not measure other possible target genes (such as NFATc1), these observations are

sufficient to indicate the importance of chemokine signaling in human osteoclast differentiation and function.

In mouse studies NFATc2 is not reported as RANKL induced, but pre-existing NFATc2 is suggested as an early mediator of NFATc1 induction [Asagiri et al., 2005]. In contrast, both NFATc1 and NFATc2 were detectably induced within 24 h in CFU-GM derived human osteoclasts treated with RANKL and M-CSF. We report early and continued NFATc2 induction time points up to 7 days with a return to baseline at 14 days. In studies of mouse osteoclasts, Asagiri et al. [2005] confirmed the scheme of Chuvpilo et al. [2002], proposing that RANKL activates pre-existing NFκB and NFATc2, which then results in induction of the P1 promoter of NFATc1 producing the NFATc1/A isoform, which is shorter relative to the NFATc1/C isoform. In mouse, the NFATc1/A isoform is proposed as the dominant osteoclast form and is auto-inducible on the P1 promoter, leading to high levels of a shorter isoform NFATc1/A. The scenario from mouse, of early gene induction of NFATc1 by pre-existing non-induced NFATc2, does not fit exactly with our human data. First, we observe NFATc2 induction by RANKL and M-CSF. Second, pre-existing NFATc1 mRNA at zero time exceeded that of NFATc2, meaning pre-existing NFATc2 is not required for auto induction of NFATc1, as sufficient NFATc1 would already be present, if mRNA corresponds to protein level.

NFATc1 has multiple protein isoforms derived from alternative first exons, differential splicing, and alternative polyadenlyation sites as reviewed by Vihma et al. [2008]. Within close proximity on the DNA an inducible P1 promoter (that responds to NFAT) and a constitutive promoter (P2) were identified in studies of mouse T-lymphocytes [Chuvpilo et al., 2002]. Furthermore, alternative first exons (exon 1A and 1B) exist that are 4 kb pairs apart in the human genome [Vihma et al., 2008]. The relative ratios of different alternative first exons that we observe (where exon 1B predominates) suggest that the majority of NFATc1 isoforms in osteoclasts are the so called β isoforms, being NFATc1/βA, NFATc1/βB, and NFATc1/βC, according to the nomenclature for NFATc1 isoforms laid out by Chuvpilo et al. [2002]. All variants that contain the exon 1B will commence the amino acid sequence with MTGLEDQEFD rather than the alternative sequence MPSTSFPVP. Recent data from lymphocytes shows that the exon 1A encoded α peptide leads to a shorter half-life of NFATc1 protein [Hock et al., 2013]. The significance of the amino terminal sequence function of NFATc1 in osteoclasts remains to be determined. To our knowledge, a specific study of NFATc1 promoter activity and alternative isoforms has not been published for mouse or human osteoclasts. Our data are dependent on mRNA studies only: recent data suggests that NFATc1 mRNA levels are not strictly related to protein levels. For instance, the Serfling laboratory [Hock et al., 2013] report that, in B and T cells, high levels of mRNA transcripts for NFATc1/B and C exist with no detectable protein by Western blot. Despite this caveat, available Western blot data of human osteoclasts is limited but does show high molecular weight forms of NFATc1. Yarilina et al. [2011] used long-term exposure to tumour necrosis factor (TNF) to produce TRAP + MNC that were calcitonin receptor positive and degrade bone: Western blot showed high molecular weight isoforms of NFATc1 are induced by TNF at 3, 4, and 7 days and return to lower levels at 10 days. The data of Yarilina et al. [2011] is in broad agreement with high molecular weight NFATc1 isoforms existing in human osteoclasts [Day et al., 2005]. Further work is required to understand the differential role of pre-existing isoforms of NFATc1 in precursor cells, the mechanism of induction of NFATc2 as well as NFATc1 promoter function in human osteoclasts relative to other cells. Another similarity between our work and Yarilina et al. [2011] relates to JUN with early FOS induction (1 h) and later (72 h) sustained upregulation of JUN with undetectable FOS. Both JUN/JUN homodimers and JUN/FOS heterodimers are capable of interacting with NFAT proteins. Although both JUN and FOS are induced in our studies, we find a greater content of JUN mRNA compared to FOS, similar to previous data [Day et al., 2005].

In conclusion, CFU-GM are an excellent model for human osteoclast differentiation and gene expression studies. These studies show induction of calmodulin 1, JUN/FOS, and induction of NFATc2 and NFATc1 isoforms as points of interest. Most importantly, we observed a very strong induction of chemokine MCP-1 as the first potent gene induction event after treatment of CFU-GM cells with RANKL and M-CSF. The peak induction of this chemokine exceeded all other measured chemokines and came days before peak induction of NFATc1, a regulator of osteoclast specific gene expression. The provision of chemokine receptors, induced similarly, provides the setting for an autocrine loop that, in the case of chemokines, is known to involve calcium signaling. Blocking potential MCP-1 autocrine action through the dominant negative 7ND prevented induction of NFATc2, calmodulin 1 and JUN, and prevented human osteoclast differentiation suggesting that MCP-1 is a reasonable clinical target for blocking osteoclast differentiation.

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