Macrophage Colony Stimulating Factor Regulation by Nuclear Factor Kappa B: A Relevant Pathway in Human Immunodeficiency Virus Type 1 Infected Macrophages

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Macrophage colony stimulating factor (M-CSF) is a cytokine that promotes monocyte differentiation and survival. When overexpressed, M-CSF contributes to pathology in a wide variety of diseases, including osteoporosis, obesity, certain human cancers, and in human immunodeficiency virus type 1 (HIV-1) infection, particularly with respect to monocyte/macrophage infection and the development of HIV-1 associated central nervous system disorders. In this study, our aim was to expand the current knowledge of M-CSF regulation, focusing on nuclear factor kappa B (NF- κ B), a transcription factor playing a prominent role during inflammation and HIV-1 infection. Our results suggest that tumor necrosis factor alpha (TNF- α) promotes M-CSF secretion in primary macrophages and activates the $-1310/+48$ bp M-CSF promoter in Mono-Mac 1 cells. Inhibitors of the NF- κ B pathway diminish this response. We identified four putative NF- κ B and four CCAAT-enhancer-binding protein beta binding sites within the M-CSF promoter. Our findings, using promoter constructs mutated at individual NF- κ B sites within the M-CSF promoter region, suggest that these sites are redundant with respect to NF- κ B regulation. TNF- α treatment promoted NF- κ B p65 binding to the M-CSF promoter in phorbol 12-myristate 13-acetate (PMA) treated U937 cells chronically infected with HIV-1 (U1 cells), but not in PMA treated uninfected U937 cells, suggesting that the presence of HIV-1 increases the NF- κ B response. In conclusion, our findings demonstrate that NF- κ B induces M-CSF expression on a promoter level via multiple functional NF- κ B binding sites and that this pathway is likely relevant in HIV-1 infection of macrophages.

Introduction

MACROPHAGE COLONY STIMULATING FACTOR (M-CSF) is a hematopoietic growth factor that synergizes with other factors to regulate the survival, proliferation, differentiation, and migration of cells of the myeloid lineage (for review see, Pixley and Stanley, 2004; Haine *et al.*, 2006). Such activity is initiated when M-CSF binds to its cognate receptor, cFMS, leading to activation of class IA PI3K resulting in cell survival in monocyte-derived macrophages cells via signaling through Akt and Erk effectors (Kelley *et al.*, 1999; Bhatt *et al.*, 2002). Indeed, these processes lead to the survival and trafficking of monocytes to various compartments of the body, where macrophage emigrants carry out their normal functions. These same processes, however, may also contribute to disease. For example M-CSF plays a prominent role in osteoporosis, promoting osteoclast development and, therefore, bone reabsorption (for review see, Teitelbaum and Ross, 2003). Interestingly, the absence of M-CSF or M-CSF receptor, cFMS, leads to the opposite condition, namely, osteopetrosis in mouse models (Marks and Lane, 1976;

Wiktor-Jedrzejczak *et al.*, 1990; Yoshida *et al.*, 1990; Cecchini *et al.*, 1994; Yeung and Stanley, 2003).

M-CSF appears to play a central role in human immunodeficiency virus type 1 (HIV-1) progression and pathogenesis with HIV-1 and M-CSF forming a positive feedback loop, each promoting the expression of the other (Gruber *et al.*, 1995; Kutza *et al.*, 2000). Monocyte-derived macrophage cells pretreated with M-CSF are more susceptible to HIV-1 infection than control cells (Kalter *et al.*, 1991). Both CD4 and CCR5, receptors necessary for M-tropic HIV-1 infection into macrophages, are increased in response to M-CSF treatment of these cells (Bergamini *et al.*, 1994; Oravec *et al.*, 1997). The role of M-CSF in monocyte survival and differentiation further underscores the relevance of this cytokine to HIV-1 pathogenesis, as the macrophages appear to represent a significant reservoir of HIV infection. HIV-1-infected macrophages also express high levels of macrophage inflammatory protein-1 α in an M-CSF-dependent manner, which recruits uninfected macrophages to sites of infection (Kutza *et al.*, 2000). M-CSF may also play a critical role in HIV-1-associated central nervous system (CNS) disorders by

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increasing monocyte/macrophage production and through alteration in monocyte/macrophage phenotype. M-CSF is known to induce CD16 expression on monocytes (Stohr *et al.*, 1998). Indeed, CD16⁺ monocytes are preferentially infected by HIV-1 *in vitro* (Ellery *et al.*, 2007) and have a more mature, macrophage-like phenotype (Pulliam *et al.*, 2004). These cells likely promote the establishment of a persistent reservoir of HIV-1 infection in the CNS. The expansion of this subset may be important in the pathogenesis of HIV-1 infection as CD163⁺/CD16⁺ monocyte expansion correlates significantly with viral load and inversely with CD4⁺ T cell count. Based on the co-expression of CD163, a marker associated with alternative macrophage activation, macrophages derived from such monocytes likely play a role in CNS disease and may contribute to the immune polarization and, consequently, the immunopathogenesis of AIDS (Fischer-Smith *et al.*, 2008). Indeed perivascular macrophages and microglia within the CNS exhibit this phenotype and constitute the major reservoir of HIV-1 infection in the brain (Fischer-Smith *et al.*, 2001).

HIV-1 seems unique among other viruses in its effect on M-CSF in monocyte-derived macrophages (Kutza *et al.*, 2000). However, the mechanism of HIV-1-induced M-CSF production in monocyte-derived macrophages is not fully understood. HIV-1 infection in macrophages promotes M-CSF production by a mechanism involving gp120 (Swingler *et al.*, 2007). This effect may be indirect, via gp120's role in tumor necrosis factor (TNF)- α induction (Lee *et al.*, 2005), which could impact the M-CSF promoter indirectly. Interestingly, TNF- α has been reported to promote the expression of neurotoxic agents, including glutamate and L-cysteine, in cooperation with HIV-1 tat and gp120, respectively (Yeh *et al.*, 2000; Koller *et al.*, 2001). HIV-1 infection is also associated with higher plasma levels of interferon- γ , TNF- α , TNF- β , interleukin (IL)-1, IL-3, transforming growth factor- β , IL-10, and IL-4, which may induce M-CSF production (for reviews see, Alonso *et al.*, 1997; Haine *et al.*, 2006; Orsilles *et al.*, 2006). Further, TNF- α , IL-6, granulocyte-macrophage CSF, and IL-1 β are reported to be elevated in the CNS during HIV-1 infection (for review see, Fischer-Smith and Rappaport, 2005). HIV-1-induced cytokines, whether expressed directly in macrophages or accumulating in extracellular fluids, may modulate M-CSF gene expression *in vivo* in the setting of HIV-1 infection.

In this report, we analyzed newly generated M-CSF promoter constructs spanning upstream regions not investigated previously evaluating indicator gene expression, gene ChIP, as well as western blot analysis to determine the role of nuclear factor kappa B (NF- κ B) as well as CCAAT-enhancer-binding protein beta (C/EBP β) factors, in controlling M-CSF gene expression in myeloid cell lines.

Materials and Methods

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood or buffy coats acquired from healthy seronegative donors by density-gradient centrifugation (Histopaque-1017; Sigma-Aldrich). PBMCs were incubated at a concentration of 4×10^6 /mL in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (FBS), 10% human AB serum, 2 mM L-glutamine and penicillin (50 U/mL)/

streptomycin (50 μ g/mL), and M-CSF (2 ng/mL) (R&D systems) overnight at 37°C. Nonadherent cells were removed and allowed to adhere for another 24 h. Adherent cells were cultured in RPMI 1640 supplemented with 10% human AB serum, 2 mM L-glutamine, penicillin (50 U/mL)/streptomycin (50 μ g/mL), and M-CSF (2 ng/mL) for an additional 4–6 days. Mono-Mac 1 cells were cultivated in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (50 U/mL)/streptomycin (50 μ g/mL), $1 \times$ nonessential amino acids (Invitrogen), and 1 mM sodium pyruvate (Invitrogen). U1 and U937 cells were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, and penicillin (50 U/mL)/streptomycin (50 μ g/mL). The cells were differentiated using 5 nM phorbol 12-myristate 13-acetate (PMA) for 24 h, then 48 more hours with additional media. U1 cells are latently infected with HIV-1 due to a mutation in the tat gene (Emiliani *et al.*, 1998). PMA differentiation activates virus production in these cells (Kim *et al.*, 1996) (data not shown). The compounds used for treatment of cells were as follows: recombinant human TNF- α (R&D systems), dexamethasone (Dex) (Sigma-Aldrich), RU486 (Sigma-Aldrich), and SC-514 (Merck KGaA).

Promoter constructs

The M-CSF promoter (–1310/+48 bp) was amplified from human genomic DNA by PCR with the primers 5' GTGGAGAATGAATGGATGGCAAATGAC 3' (forward) and 5' CCGAGAGGACC CAGGCAAACCTTT 3' (reverse). The PCR product was ligated into the pGL3-basic vector (Promega Corporation) to form plasmid pGL3-M-CSF (pMCSF) (Fig. 1). This sequence was analyzed for transcription factor binding sites using Mac Vector 6.0 software. We identified four NF- κ B binding sites, three on the antisense and one on the sense strand. The location of three on the antisense and scores were –447 to –438 (81.9), –387 to –378 (85.4), and –60 to 51 (85.4). The location on the sense strand and score was –359 to –367 (97.5). The M-CSF promoter also contains four putative C/EBP β binding domains. The locations and scores for the antisense strand were –1269 to –1257 (84.9) and –1135 to –1121 (84.9) positions. The locations and scores for the sense were –815 to –804 (84.9) and –718 to –704 (85.5) locations. We used site-directed PCR mutagenesis to create four mutants for individual NF- κ B sites (pMCSF NF- κ B M1-4). Further, we created mutants mutated for all four C/EBP β , NF- κ B, or all eight putative sites (pMCSF C/EBP D4, pMCSF NF- κ B D4, and pMCSF D8, respectively). The primers are listed in Tables 1 and 2. This was done in a two-step PCR using the internal mutagenic primer and an external primer located in the pGL3 vector at 37°C annealing temperature for 25 cycles using Accuprime pfx (Invitrogen). The pGL3 vector primers used were as follows: pGL3F, 5' GCTCTCCATCAAAAACAAAACG 3' pGL3R, 5' GCGGTTCCATCTTCCAGCG 3'. The products of these reactions were then run for two cycles at 55°C annealing temperature. Then, external pGL3 primers were added and the reaction was run for an additional 25 cycles. The PCR products were digested using *EcoRV* and *NcoI* and ligated into a similarly digested, CIP-treated pGL3 vector. These products were then transformed into Top-Ten *E. coli* competent cells using electroporation and screened on the basis of restriction pattern. The combined mutants were produced in the exact same manner, except that multiple

FIG. 1. The *M-CSF* promoter contains four C/EBP and NF-κB sites. The *M-CSF* promoter (–1310/+48 bp) was amplified from human genomic DNA and ligated into the pGL3-basic vector (Promega Corporation) to form plasmid pGL3-*M-CSF*. Mutations in the putative C/EBPβ and NF-κB binding sites in the *M-CSF* promoter were generated by amplification of the whole plasmid pGL3-*M-CSF* using site-directed mutated primers. *M-CSF*, macrophage colony stimulating factor; NF-κB, nuclear factor kappa B; C/EBPβ, CCAAT-enhancer-binding protein beta.

PCRs were necessary to form the full-length product. All finished constructs were confirmed by DNA sequencing.

Transfection and luciferase assay

The pMCSF promoter constructs were transfected into Mono-Mac 1 cells using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Briefly, we used a 12 μL enhancer and 15 μL effectene using a total of 0.5 μg of DNA/.435 × 10⁶ cells (350 μL of 1.25 × 10⁶ cell/mL). The ratio of luciferase to renilla was 1:1. Dual Luciferase[®] Reporter Assay System (Promega Corporation) was used to detect both the production of pMCSF-driven firefly luciferase and *Renilla* from a control vector (pRL-TK) (Promega Corporation). The luminescence was detected on an EnVision 2104 multilabel reader (Perkin Elmer) at 10 s postluciferin addition. Luciferase activity was normalized using the *Renilla* control values and then expressed as fold-change relative to untreated cells for every individual promoter construct. All data represents an average of at least three separate triplicates.

Western blot and ELISA

Samples were run on SDS-poly acrylamide gels and electro-transferred to hybond-P membrane (Amersham, GE) using Tris/Glycine buffer. Antibodies used were NF-κB p65

(Cell Signaling; C22B4, 1:1000), Lamin A (Abcam; ab26300, 1:1000), β-Actin (Sigma-Aldrich; A2228, 1:10,000), phospho-IκBα (Ser32; Cell signaling 2859; 1:1000), and total IκBα (Santa Cruz Inc.; sc-847, 1:1000). All antibodies were incubated overnight at 4°C in 5% BSA TTBS. The secondary antibodies mouse IgG-HPR (Santa Cruz Inc.; sc-2061, 1:10,000) or rabbit IgG-HPR (Santa Cruz Inc.; sc-2030, 1:10,000) were incubated for 1 h at room temperature in 5% milk TTBS. The signal was detected using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific; 34080). Nuclear cytoplasmic fractions were obtained using the PE-NER nuclear and cytoplasmic extraction kit (Thermo Scientific; 78833II). *M-CSF* ELISA was performed using the Quantikine Human *M-CSF* Immunoassay Ka kit (R&D systems; SMC00) according to the manufacturer's protocols. The western blots were quantified using representative blots by a protocol described below: www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted using the ChIP-IT[™] kit (Active Motif) using 2 μg of NF-κB p65 antibody (Santa Cruz; sc-372×). DNA concentrations were determined for all sheared chromatin samples and equivalent amounts were used between samples in experiments. ChIP assays using the differentiated U937 and U1

TABLE 1. CCAAT-ENHANCER-BINDING PROTEIN BETA MUTAGENIC PRIMERS

Name	Location	Sequence
M1F	–1257	5' GACTTATCAGAGCCAGCAGATATCAATGAGCCAAGTCCAATGGG 3'
M1R	EcoRV	5' CCCATTGGACTTGGCTCATTGATATCTGCTGGCTCTGATAAGTC 3'
M2F	–1121	5' GGAGTTTGTCTTCACCATGAAGCTTAAGGAGCATTACAGGCAGAGG 3'
M2R	HindIII	5' CCTCTGCCTGAATGCTCCTTAAGCTTCATGGTGAAGACAAACTCC 3'
M3F	–815	5' CACCTCAGTAAGTGCAATTTCAATCCCATCCAGGGAAATC 3'
M3R	EcoRI	5' GATTTCCTGGATGGGATTTCGAAATTGCATCTACTGAGGTG 3'
M4F	–718	5' GGGTACCAGCCAGCAAGCTTATCATCTAAGGGTCAGGTGCC 3'
M4R	HindIII	5' GGCACCTGACCCTTAGATGATAAGCTTGGCTGGTACCC 3'

Highlighted bases are mutated; bold are restriction sites.

TABLE 2. NUCLEAR FACTOR KAPPA B MUTAGENIC PRIMERS

Name	Location	Sequence
M1F	-438	5' GCGTCTTCAAAGG AGATCT CTCCCTTCCCAGTGCTTGTCCCTGC 3'
M1R	Bgl II	5' GGGAAAGGGAGAG ATCT CCCTTTGAAGACGCCCCCACCCC 3'
M2F	-378	5' GCTAAGATTGTTGGG AGATCT AGGCCTGGAGGAAAGTCCCTTGGG 3'
M2R	Bgl II	5' CCTCCAGGCCT AGATCT CCCCAAATCTTAGCAGAAAACGGACCG 3'
M3F	-367	5' GGCCTGGAG AGATCT CCCTTGGGACGATCATAGAGCGC 3'
M3R	Bgl II	5' CGTCCCAAGGG AGATCT CTCCAGGCCTGAAAAATCCCCAAATCTTAGC 3'
M4F	-51	5' GGCCAGGGT AGATCT CATAAACCACATGCCCCC 3'
M4R	Bgl II	5' GCATGTGGTTTAT AGATCT CACCCTGGCCAGGCGC 3'
M2/3F		5' GCTAAGATTGTTGGG AGATCT AGGCCTGGAG AGATCT CCCTTGGG 3'
M2/3R		5' CCAAGGG AGATCT CTCCAGGCCT AGATCT CCCCAAATCTTAGC 3'

Highlighted bases are mutated, bold are restriction sites.

cells were performed with equal amounts of starting chromatin (6 μ g total). TNF- α treatment, 10 ng/mL, was carried out for 1.5 h. PCR was performed using an annealing temperature of 55°C using primers recognizing the human *M-CSF* promoter: pMCSFchF, 5' TGCTCTCGGTCCGTTTCTG 3'; pMCSFchR, 5' CACTTCCAAGCCTTCAGCAAAC 3'.

Results

To begin investigating *M-CSF* gene regulation, we evaluated *M-CSF* gene expression in primary macrophages as well as in promoter-indicator plasmid-transfected cells. Macrophages produced *M-CSF* in response to TNF- α treatment as reported previously (Fig. 2A–C) (Oster *et al.*, 1987). Compared to media alone, treatment with I κ B α activator Dex (0.1 μ M) significantly reduced *M-CSF* expression in primary macrophages. A representative graph of one of three individual donor experiments conducted is shown (Fig. 2A). This effect was reversed with RU486 (1 μ M), an inhibitor of progesterone and glucocorticoid receptors, suggesting that this is specific to the glucocorticoid pathway. In the presence of TNF- α (10 ng/mL), the addition of Dex reduced expression of *M-CSF*, which was again reversed by RU486 (Fig. 2B). It is noteworthy that the levels of *M-CSF* in the TNF- α /Dex condition (Fig. 2B) were twice that of the Dex alone (Fig. 2A) ($p \leq 0.001$). Another inhibitor of the NF- κ B pathway, sc-514, which reduces TNF- α -mediated activation of IKK β , also decreased *M-CSF* gene expression in response to TNF- α treatment (Fig. 2B). Dex exhibited a dose-dependent inhibitory effect on *M-CSF* expression in macrophages (Fig. 2C). Interestingly, TNF- α stimulated *M-CSF* production at low concentrations of Dex, but not at concentrations above 0.01 μ M (Fig. 2C). Finally, these treatments produced the expected changes in I κ B α phosphorylation and degradation: phosphorylation was increased with treatments that induced NF- κ B phosphorylation and degradation, whereas inhibitory treatments had opposite effects. One representative experiment from three individual western blots using separate donors is shown (Fig. 2D, E). TNF- α (lane 2: T) treatment (10 min) also induced phosphorylation of I κ B α as compared to media treatment (M) (Fig. 2D, E). Further, co-treatment of these cells with Dex (lane 4: T + D) resulted in decreased I κ B α phosphorylation. The addition of RU486 to Dex reversed this effect (lane 5: D + R). These results taken together suggest that exposure of macrophages to TNF- α , Dex, and sc-514 alters *M-CSF* production in a manner that is consistent with

the known effect of these agents on NF- κ B signaling in cell culture conditions.

Mono-Mac 1 cells responded to TNF- α and Dex treatment in a manner similar to primary macrophages. Culture of Mono-Mac 1 cells with TNF- α for 3 days resulted in marked increase in *M-CSF* gene expression as determined by ELISA (Fig. 3A). Consistent with such a response, these treatments also resulted in expected inhibition (Dex) or activation (TNF- α) of NF- κ B p65 nuclear trafficking in these cells (Fig. 3B, C), whereas RU486 treatment had no such effect. As the Mono-Mac 1 cells produced *M-CSF* in response to TNF- α and NF- κ B in a similar manner to macrophages, we evaluated the role of TNF- α on *M-CSF* promoter activation in Mono-Mac 1 cells using the *M-CSF* luciferase construct (pMCSF). We observed that 24 h of TNF- α (10 ng/mL) treatment induced promoter activity of the wild-type construct and also activated the promoter of all four individual mutants for NF- κ B (pMCSF NF- κ B M1-4) (Fig. 4A). However, while the pMCSF C/EBP D4 mutants responded to TNF- α in a manner similar to wild-type (WT) pMCSF, the mutant lacking functional NF- κ B sites, pMCSF NF- κ B D4, failed to respond to such stimulation (Fig. 4B). In summary, Figure 4A and B suggests that there is a functional redundancy in the NF- κ B sites, at least under the conditions tested. Interestingly, the pMCSF D8 did respond to TNF- α , suggesting that sites other than those we have identified are TNF- α responsive and that the C/EBP β sites may play some inhibitory role in these cells. These results paralleled our studies using Dex. While the pMCSF C/EBP D4 mutants were inhibited by Dex in a manner similar to wild-type pMCSF promoter activity, the mutants lacking all functional NF- κ B sites, pMCSF NF- κ B D4 and pMCSF NF- κ B D8, failed to respond to such stimulation (Fig. 4C). Interestingly, the pMCSF D8 mutant exhibited reduced inhibition in response to Dex. As shown in Figure 4B, these results might suggest that the C/EBP β sites can exhibit inhibitory function, evident under these conditions. This finding, however, is observed only in the absence of NF- κ B binding sites.

As TNF- α treatment results in uncoupling of NF- κ B p50/p65 heterodimer from I κ B α , we investigated if the p65 component of the complex, which is necessary for promoter activation (Baldwin, 1996), was bound to the *M-CSF* promoter in unstimulated and TNF- α treated cells. We used U1 and U937 cells to also assess the effect of HIV-1 on the responsiveness of *M-CSF* to NF- κ B in myeloid cells. Indeed, we were able to show that NF- κ B p65 binds to the *M-CSF* promoter in Mono-Mac 1, U937 and U1 cells via ChIP (Fig. 5A–

FIG. 2. M-CSF production in macrophages is NF- κ B responsive. **(A)** Macrophages were cultured for 3 days of with dexamethasone (Dex) (0.1 μ M), RU486 (1 μ M), or both treatments. M-CSF production shown, standardized by using protein content. **(B)** Three days macrophage culture in a TNF- α (10 ng/mL) context in combination with Dex (0.1 μ M), RU486 (1 μ M), and SC-514 (50 μ M) treatments. **(C)** Dose curve for Dex (0–0.001 μ M) on macrophage M-CSF production with and without TNF- α (10 ng/mL). **(D)** Western blot analysis of I κ B α phosphorylation and degradation shown in response to treatment of macrophages with Dex, TNF- α , or combined treatment for 10 min. [M: Media; T: TNF- α (10 ng/mL); D: Dex (0.1 μ M); R: RU486 (1 μ M)]. **(E)** Quantification of values from D standardized by levels of β -actin. (Student's *t*-test: **p* $\leq$ 0.05, ***p* $\leq$ 0.001, ns: nonsignificant). TNF, tumor necrosis factor.

C). The binding of p65 did not require TNF- α treatment in Mono-Mac 1 cells, in agreement with detectable levels of p65 in nuclear fractions of untreated cells (Fig. 3B). Interestingly, we observed binding of p65 to the M-CSF promoter in U937 cells but not in PMA treated U937 cells. In contrast, U1 cells treated with PMA, where HIV-1 virus was reactivated (data not shown), had far greater levels of p65 bound to the M-CSF promoter, as determined by ChIP assay using equivalent amounts of chromatin. Consistent with this observation, U1 cells that were differentiated for 24 h with PMA, and treated for 3 days with TNF- α , had significantly increased levels of

M-CSF compared to U937 cells (Fig. 5D). Overall, these findings suggest that the NF- κ B pathway contributes to M-CSF expression in a manner consistent with molecular mechanisms involving NF- κ B activation and interaction of p65 with *cis*-acting promoter elements.

Discussion

In this report, we demonstrated that M-CSF gene expression is activated by TNF- α in an NF- κ B-dependent manner in primary human macrophages. We used Mono-Mac 1 cells as

FIG. 3. M-CSF is NF- κ B responsive in Mono-Mac 1 cells. **(A)** Effect of 3 days of TNF- α (10 ng/mL) on levels of M-CSF excretion in Mono-Mac 1 cells. **(B)** Western blot analysis of NF- κ B p65 nuclear translocation after 1 h of treatment with media alone, Dex (0.1 μ M), RU486 (1 μ M), or TNF- α (10 ng/mL). Cytoplasmic (C) and Nuclear (N) fractions are shown. Lamin A is used as a positive control for nuclear extract. **(C)** Quantification of B with cytoplasmic fractions standardized for β -actin and the nuclear fractions standardized by Lamin A levels. The standardized nuclear and cytoplasmic values were then divided to determine the ratio of nuclear/cytoplasmic levels of NF- κ B p65 in each condition.

a model for M-CSF promoter activation by TNF- α , as these cells have similar M-CSF induction as compared to primary macrophages. By using promoter constructs mutated in individual and multiple putative C/EBP β and NF- κ B binding site(s), we demonstrated that multiple NF- κ B sites are involved in the activation of the promoter by TNF- α and in-

hibition by Dex. Mutation of the C/EBP β binding sites did not seem to affect the responsiveness of the M-CSF receptor to either TNF- α or Dex treatments, but may have had a small repressive effect in the absence of all four NF- κ B binding sites. We observed binding of NF- κ B p65 to the M-CSF promoter in Mono-Mac 1 cells as well as U937 and U1 cell lines. The induction of NF- κ B p65 binding to the promoter by TNF- α was far greater in U1 cells as compared to U937 cells, consistent with the higher expression levels M-CSF observed in U1 cells. The cell lines used and the experiments are summarized in Table 3.

Previous studies have shown that several regions of the M-CSF promoter are responsive to TNF- α stimulation, although smaller regions of the promoter were used relative to the studies presented here. Also, the molecular mechanisms have been incompletely understood in previous works. It was originally described that the region from -419 to -304 of the human M-CSF promoter was responsive to TNF- α stimulation (Yamada *et al.*, 1991). This region also produced a shift in response to TNF- α as seen by electromobility shift assay. Consistent with these findings, DNA footprinting of this construct showed that TNF- α stimulation protected the -379 to -366 region of the promoter. Another study identified an additional responsive element at -65 in same promoter (Sater, 1994). The report also documented the presence of a 50 kDa protein bound to this site. Finally, a third study found that a 93bp region located in -420 to -328 region had activation due to M-CSF (Yao *et al.*, 2000), in apparent agreement with findings mentioned above (Yamada *et al.*, 1991). The authors also reported that this region bound the NF- κ B p50 subunit, but not p52, p65, c-Rel, or Rel B subunits. Interestingly, it was noted by the authors that the -1239 to +15, -969 to +15, and -719 to +15 M-CSF promoter constructs failed to respond to TNF- α treatment, suggesting that some inhibitory elements may be present on the promoter. As a whole, these studies demonstrated that M-CSF is TNF- α responsive due to NF- κ B binding of the promoter; however, there has been a lack of consensus regarding the relevant *cis*-acting binding sites involved in this effect. Also, p50 binding alone cannot account for transcriptional regulation of this gene, as this subunit lacks a transactivational domain (Ghosh *et al.*, 1998). Therefore, additional clarity was needed regarding the elements important in the regulation of human M-CSF, particularly in the context of HIV-1 infection in myeloid cells. While it has previously been shown that M-CSF is NF- κ B responsive, the exact mechanistic nature of this relationship has not been fully elucidated. In this report, we showed that the M-CSF promoter has multiple, redundant NF- κ B binding sites and demonstrated that NF- κ B p65 binds to the M-CSF promoter in myeloid cells.

Results from studies using treatments activating or inhibiting NF- κ B support that this transcription factor pathway is involved in M-CSF regulation. In macrophages, TNF- α induced M-CSF expression, whereas inhibitors for NF- κ B inhibited the secretion of M-CSF (Fig. 2). The induction of M-CSF by TNF- α was lower in macrophages than in the Mono-Mac 1 cell line (Figs. 2C and 3A). This may be due to the differences in the level of differentiation in these cells. Indeed, the magnitude of the inhibitory effects observed with Dex and sc-514 suggests that the NF- κ B pathway is activated to a large extent in macrophages under standard culture

FIG. 4. The *M-CSF* promoter contains functional redundant NF- κ B binding sites. (A) *M-CSF* promoter response of single NF- κ B mutants (NF- κ B M1-M4) to 1 day of TNF- α treatment in Mono-Mac 1 cells as determined by luciferase activity. Firefly luciferase values were standardized for transfection by using *Renilla* luciferase values from the same sample. *M-CSF* promoter response of quadruple NF- κ B, C/EBP β , or both promoter element mutants (NF- κ B D4, C/EBP D4, and pMCSF D8, respectively) to 1 day of TNF- α (B) or Dex (C) treatment in Mono-Mac 1 cells. (Student's *t*-test: * $p \leq 0.05$, ** $p \leq 0.001$, ns: nonsignificant).

conditions. Further, the enhanced induction of *M-CSF* in U1 cells by PMA and TNF- α treatment relative to U937 cells, NF- κ B induction of *M-CSF* may be augmented by HIV-1 infection.

It is surprising that the C/EBP β binding sites appeared to have an inhibitory effect on *M-CSF* activity. This result was

only observed where all the NF- κ B sites were mutated and with TNF- α treatment (Fig. 4B) or Dex treatment (Fig. 4C). In previous studies, *M-CSF* promoter regions longer than the basic promoter with NF- κ B sites alone failed to respond to TNF- α stimulation (Yamada *et al.*, 1991; Sater, 1994; Yao *et al.*, 2000). Therefore, it is possible that C/EBP β sites in the *M-CSF* promoter, which are found in areas upstream of NF- κ B binding sites, promote some of these previously described inhibitory effects with respect to TNF- α induction. However, in our hands, we did not observe inhibition by these sites in the presence of wild-type NF- κ B sequences. Further, while previous results were obtained using a human promoter and human myeloid cell lines similar to ones used in this study, our results differ in that our findings clearly indicate that individual NF- κ B mutant constructs retain the ability respond to TNF- α treatment. Finally, these previous reports emphasize the role of NF- κ B p50, but not NF- κ B p65 in *M-CSF* promoter activation due to *M-CSF* (Sater, 1994; Yao *et al.*, 2000). Our results clearly show, however, that NF- κ B p65 does bind to the promoter via CHIP assay in both Mono-Mac 1 cells and U937 cells (Fig. 5A–C). One reason for this discrepancy may be due to the use of electromobility shift assay rather than the ChIP studies described here. If this is indeed true, the binding of p65 to the *M-CSF* promoter may occur indirectly in the context of myeloid cells.

As HIV-1-infected macrophages and myeloid cells are well known to exhibit NF- κ B activation (Roulston *et al.*, 1995; Choe *et al.*, 2002), it is likely that at least some HIV-1-mediated induction of *M-CSF* is due to NF- κ B signaling. Consequently, latently HIV-1-infected U1 cells can be reactivated using PMA stimulation (Kim *et al.*, 1996), likely promoting NF- κ B activity and possibly accounting for the observed increase in p65 binding to the *M-CSF* promoter. This is consistent with our findings that there is indeed greater p65 binding in U1 cells as compared to U937 cells. Considering that TNF- α , and other NF- κ B inducing agents, are up-regulated during HIV-1 infection, the increased responsiveness of infected cells to such stimulation may further account for the high levels of *M-CSF* seen in the disease. Therefore, in addition to the already known activation of NF- κ B activity, including HIV-1 long terminal repeat activation (West *et al.*, 2001), NF- κ B may promote cell survival in HIV-1-infected macrophages via *M-CSF* induction. Indeed, such protective effects of *M-CSF* on the survival of HIV-1-infected macrophages with TRAIL treatment have been demonstrated previously (Swingler *et al.*, 2007). Therefore, the NF- κ B pathway seems to be an important mediator of infection and survival signals in myeloid cells, perhaps contributing to persistence of HIV-1 infection within this lineage.

Conclusion

The results presented here demonstrate that NF- κ B induces *M-CSF* expression at the promoter level via multiple functional NF- κ B binding sites. The C/EBP β sites found in the *M-CSF* promoter appear to have little function with regard to NF- κ B responsiveness or may be inhibitory based on our findings comparing pMCSF NF- κ B D4 and pMCSF D8 constructs (Fig. 4B, C). It is possible that the C/EBP β sites and corresponding binding factors regulated the activity of *M-CSF* in monocytes/macrophages under alternative conditions that have not yet been examined. Considering that

FIG. 5. NF- κ B p65 binds to the *M-CSF* promoter in myeloid cell lines. **(A)** ChIP analysis of NF- κ B p65 binding to the *M-CSF* promoter in undifferentiated and PMA (5 nM) differentiated U937 in response to 1.5 h of TNF- α treatment. Amplification shown at 45 cycles of PCR. **B.** NF- κ B ChIP for *M-CSF* promoter in chronically infected, PMA differentiated U1 cells in response to TNF- α **(B)**. Amplification shown at 38 cycles. **(C)** NF- κ B ChIP for *M-CSF* promoter in Mono-Mac 1. Amplification shown at 45 cycles. **(D)** Levels of *M-CSF* expression than U937 cells after 1 day of PMA treatment followed by 3 days of TNF- α treatment (10 ng/mL) **(D)**. (Student's *t*-test: **p* $\leq$ 0.05, ***p* $\leq$ 0.001, ns: nonsignificant). PMA, phorbol 12-myristate 13-acetate; ChIP, chromatin immunoprecipitation.

TABLE 3. DESCRIPTION OF CELL TYPES USED

Cell Line	Macrophages	Mono-Mac 1	U937	U1
Cell Type	PBMC derived	peripheral monoblastic leukemia	promonocytic cells	U937 clone with inducible HIV-1 (Tat mutant)
M-CSF production	+++	++	+	+
TNF- α reactivity	Yes	Yes	Yes	Yes
Summary of findings	M-CSF production in response to NF- κ B (Fig. 2).	M-CSF production in response to NF- κ B (Fig. 3); Transcriptional regulation of M-CSF by NF- κ B (Figs. 4 and 5)	NF- κ B p65 binding to M-CSF promoter (Fig. 5)	NF- κ B 65 binding to M-CSF promoter and the role of HIV-1 (Fig. 5)

M-CSF, macrophage colony stimulating factor; HIV-1, human immunodeficiency virus type 1; NF- κ B, nuclear factor kappa B ; TNF, tumor necrosis factor.

NF- κ B signaling is activated in HIV-1-infected cells and our findings that p65 binding to the *M-CSF* promoter is greater in U1 cells, it is likely that increased M-CSF expression during HIV-1 is driven, at least in part, by NF- κ B activation, possibly via autocrine and paracrine mechanisms involving HIV-induced TNF- α secretion. Other cytokines dysregulated during HIV-1 infection may also contribute to increased M-CSF production in macrophages in the setting of HIV Infection.

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Disclosure Statement

No competing financial interests exist.

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