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NFAT regulates CSF-1 gene transcription triggered by L-selectin crosslinking

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ABSTRACT: L-selectin is a member of the selectin family that play an important role both in mediating the initial capture and subsequent rolling of leukocytes along the endothelial cells. Furthermore, L-selectin can function as a signal molecule. In our previous studies, we reported that L-selectin ligation could regulate CSF-1 (colony-stimulating factor-1) gene transcription, in which AP-1 acts as a crucial transcriptional factor. Here we investigated the function of the NFAT in the CSF-1 gene transcriptional events. We found that overexpression of WT NFAT induce CSF-1 gene transcription greatly in the activated Jurkat cells. Furthermore, we found that NFAT can be recruited to the nucleus after L-selectin ligation, and the nuclear NFAT interacts with the CSF-1 promoter region to regulate CSF-1 gene transcription in the L-selectin ligation activated Jurkat cells. These results indicate that nuclear NFAT can activate CSF-1 gene transcription by connecting with the CSF-1 promoter in the signaling events induced by L-selectin ligation.

Introduction

L-selectin, a member of selectin family and constitutively expressed on leukocytes, plays a fundamental role in the immune system by mediating lymphocyte homing and in the recruitment of leukocytes to inflammatory sites (Alon, 1997; Walcheck, 1996). Except for its adhesion role, engagement of L-selectin can result in cell activation which is related to the transcriptional factor activity and gene transcription. Clustering of L-selectin by antibody crosslinking leads to a rapid induction of actin assembly and CD18 co-localization with L-selectin in the plasma membrane (Simon *et al*., 1999). Crosslinking of L-selectin on lymphocytes or Jurkat cells with anti-L-selectin monoclonal antibody enhances some kinases phosphorylations (Kilian *et al*., 2004; Smolen *et al*., 2000; Waddell *et al*., 1995) and also initiates signaling cascades from L-selectin via tyrosine kinase $p56^{lck}$, Grb_2/Sos, Ras, MAPK, and Rac_2, resulting in O^{2-} generation and actin polymerization (Brenner *et al*., 1996, 1997a). Ligation of human L-selectin with monoclonal antibody mobilizes intracellular Ca^{2+} (Crockett-Torabi *et al*., 1995; Waddell *et al*., 1994), increases tumor necrosis factor α (TNF-α) and interleukin 8 (IL-8) mRNA expression (Crockett-Torabi *et al*., 1995). In our previous studies, we indicated that engagement of human L-selectin with its monoclonal antibody or sulfides induced CSF-1 gene transcription within 60 min, in which some kinases and AP-1 (activator protein 1) play important roles (Ba *et al*., 2005; Chen *et al*., 2006, 2008). It has been reported that NFAT (nuclear factor of activated T-cells), AP-1, NFκB (nuclear factor κb) transcription factors in the lymphocytes are activated following L-selectin ligation, and the c-fos gene transcription is regulated in a SRE dependent manner (Brenner *et al*., 1997b; Brenner *et al*., 2002; Crabtree and Clipstone, 1999; Rao *et al*., 1997; Turutin *et al*., 2003). These stud-

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ies indicated that engagement of L-selectin results in the activation of signaling pathways that were dependent on nuclear transcription factor activation.

NFAT is a family of transcription factors that regulate the differentiation and activation of a number of immune and related cell types, including T-cells and osteoclasts (Macian, 2005; Masuda *et al*., 1998). Inactive NFAT is maintained in the cytoplasm in a hyperphosphorylated state. Elevation of cytosolic free Ca^{2+} stimulates the phosphatase calcineurin, which in turn dephosphorylates multiple serine residues, exposing a nuclear localization signal that permits translocation of NFAT to the nucleus. Ferrari and coworkers examined the effect of ATP on NFAT activation in the N9 murine microglial cell line. They found that NFAT was activated within 1 min, reached a maximum after 15 min and diminished in the following 60 min (Ferrari *et al*., 1999). Brenner and co-workers indicated that NFAT was activated after L-selectin ligation with its mAb (Brenner *et al*., 2002). Activation of NFAT was also mediated by the P2X7 receptor (Ferrari *et al*., 1999).

In our previous work, we showed that CSF-1 gene transcription was regulated by c-Abl kinase and syk family kinases after L-selectin and PSGL-1 (P-selectin glycoprotein ligand-1) crosslinking with their monoclonal antibodies (Ba *et al*., 2005; Chen *et al*., 2006). Nuclear AP-1 transcriptional factors recruits to the nucleus and connects with the promoter of CSF-1 gene to regulate its transcription triggered by L-selectin ligation (Chen *et al*., 2008). To further understand the molecular mechanism involved in L-selectin induced CSF-1 gene transcription, we analyzed the function of the NFAT transcriptional factor in the signaling transduction induced by L-selectin crosslinking. The results we obtained show that NFAT can be recruited to the nucleus and connected with the promoter of human CSF-1 gene to regulate its transcription following L-selectin ligation.

Materials and Methods

Cell culture, reagents and antibodies

The human leukemic Jurkat T cell line was grown in RPMI 1640 (Gibco) containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. DREG56 (the anti-L-selectin monoclonal antibody, mouse IgG_1, sc-18851), PY99 (the anti-phosphotyrosine monoclonal antibody, mouse IgG_{2b}, sc-7020), and K18 (anti-NFAT antibody, rabbit IgG1) were purchased from Santa Cruz Biotechnology, Inc. DAPI and PMA were purchased from Sigma. ECL Plus western blotting detection reagents (RPN2132) were purchased from Amersham Biosciences.

Luciferase reporter assay

The luciferase reporter vector with a CSF-1 promoter, pREP4-CSF-1-luc, and the pREP7-Renilla-luc vector, were kindly provided by Dr. Keji Zhao (NIH, Maryland). The expression vectors encoding WT NFAT were the gift of Dr. Anne Goldfeld (CBR Institute for Biomedical Research, Harvard Medical School, Boston). Jurkat cells were transient transfected by electroporation. Briefly, 10^7 cells/ml in 10% fetal calf serum in RPMI1640 were electroporated at 350V, 950 μF, then the cells were rested for 10 min and transferred to culture plate. Twenty-four hours later, the transfected Jurkat cells were stimulated with the indicated antibodies for 6 h and then lysed in 25 μl of an appropriate reporter lysis buffer to measure both the Firefly and the Renilla luciferases (Promega). Luciferase activity was assayed in triplicate by luminometry using the Promega double luciferase assay system and expressed as fold increase relative to the basal activity seen in unstimulated cells.

Nuclear proteins extration

Jurkat cells were activated and lysed in lysis buffer A (10 mM HEPES–KOH, pH 7.4, 120 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml each of leupeptin and aprotinin), and then incubated at 4ºC for 10 min. After centrifugation, the cell pellet was resuspended in 200 μl of buffer A plus 1% Nonidet P-40, and incubated at 4ºC for 10 min with gentle shaking. Cell lysates were centrifuged at 13,000 *g* for 5 min. The pellet was resuspended in 150 μl of buffer B (20 mM HEPES–KOH, pH 7.4, 20% glycerol (v/v), 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml each of leupeptin and aprotinin), and then incubated at 4ºC for 20 min. The lysates were clarified by centrifuging at 13,000 *g* at 4ºC for 15 min. The supernatant was used as nuclear extract.

Immunoprecipitation and immunoblotting

Proteins isolated from the activated Jurkat cells nucleus extracts were resolved by SDS-PAGE. Proteins were transferred to the nitrocellulose membranes. After protein transfer, nitrocellulose membranes were

washed with TBST (20 mM Tris base, 500 mM NaCl, 0.05% Tween-20, pH 7.5) and immediately incubated with 3% BSA and then indicated with primary antibody at 37ºC for 1 h, and HRP-conjugated secondary antibody at 37ºC for another 1 h. Chemiluminescent detection was performed by using electrochemiluminescence plus western-blotting reagents.

Immunofluorescence Microscopy

To investigate the localization of NFAT in the resting and activated cells, after crosslinking with DREG56, Jurkat cells were fixed, permeabilized and then incubated with anti-NFAT polyclonal antibody (K18, 10 mg/ml) for 30 min, washed with PBS, and incubated with TRITC-conjugated goat anti-rabbit IgG (H+L) for another 30 min, then washed with PBS. The nuclei of the Jurkat cells were stained with DAPI. All these stained cells were observed under the fluorescence microscope.

Electrophoretic Mobility Shift Assay (EMSA)

Synthetic oligonucleotides with the sequences corresponding to *cis*-acting elements of the M-CSF promoter were 5' ended labeled by biotin and the sequence was 5'-AGCGCGGAA**GGAAA**GGGTCGGT-3'. Equal amounts of nuclear extract from each of various cell treatments were incubated in 20 μl of binding reaction [40 mM KCl, 15 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonylfluoride, 5% glycerol and 1 μg of poly-(dIdC)] for 10 min. Then, twenty-five femtomoles biotin-labeled probe were added to the reaction mixture, and incubated further for an additional 15 min at room temperature. DNA/protein complexes were separated from unbound DNA probe by electrophoresis on native 5% polyacrylamide gels at 140 V in 45 mM Tris/borate (pH 8.0) containing 1 mM EDTA and then autoradiographed.

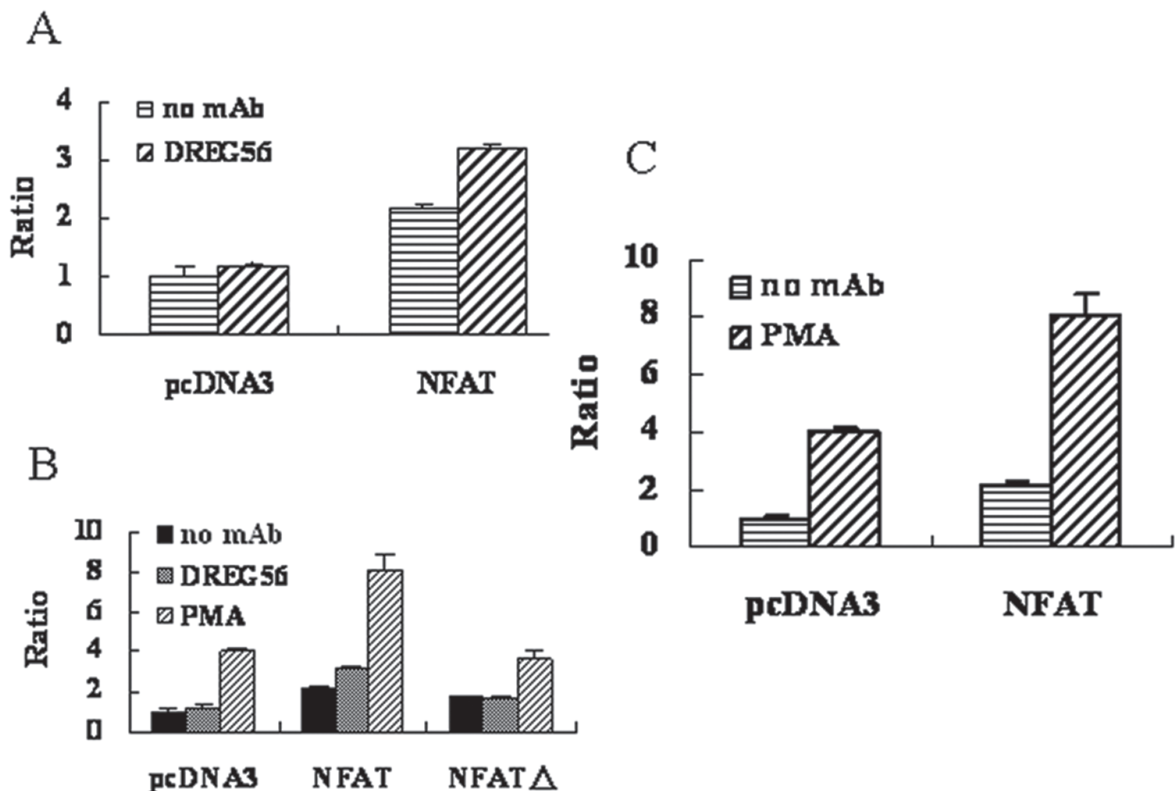

FIGURE 1. Nuclear NFAT regulates CSF-1 gene transcription in activated T cells. A. Jurkat cells were transfected with empty pcDNA3.1 or the same vector expressing WT NFAT in the presence of CSF-1-luc vector. The transfected cells were stimulated without or with antibody for 30 min, and then the cells were lysed and analyzed for luciferase activity. Results shown were the mean ±SD of three independent experiments performed in triplicate. B. Jurkat cells were transfected with empty pcDNA3.1 or the same vector expressing WT NFAT or NFAT NLS in the presence of CSF-1-luc vector. The transfected cells were stimulated DREG56 or PMA for 30 min, and then the cells were lysed and analyzed for luciferase activity. Results shown were the mean ±SD of three independent experiments performed in triplicate.

Results

Nuclear NFAT regulates CSF-1 gene transcription in activated T cells

As shown in Fig. 1A, the overexpression of WT NFAT could increase CSF-1 gene transcription, whereas transfection with equal amounts of control vector could not. To test which domain of NFAT is responsible for the CSF-1 gene transcription triggered by L-selectin, WT NFAT or NLS NFAT mutant and CSF-1-luc vector were co-transfected in the Jurkat cells. As shown in Fig. 1B, overexpression of the NLS mutant NFAT did not increase CSF-1 gene transcription significantly compared to the overexpression of WT NFAT in the DREG56 treated cells. We also evaluated the CSF-1 gene transcription activity in the PMA stimulated Jurkat cells. Treatment of Jurkat cells transfected with WT NFAT with PMA also resulted in the maximal activation of CSF-1 gene transcription, compared to the NLS mutant NFAT overexpression (Fig. 1B). These results suggested that nucleus NFAT played an important role in CSF-1 gene transcriptional events in the stimulated Jurkat cells.

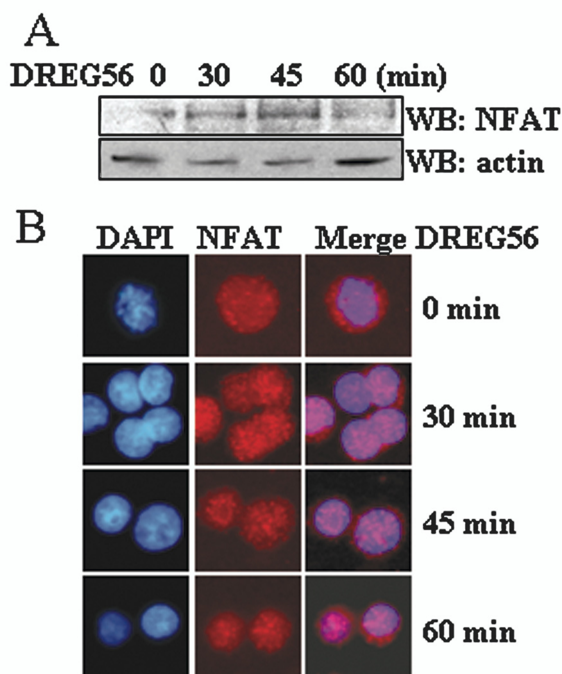

FIGURE 2. NFAT recruits to the nucleus following L-selectin ligation. A. Jurkat cells were stimulated with DREG56 for the indicated time, and then the cells were lysed for the extraction of nuclear proteins. The isolated nuclear proteins were separated by SDS-PAGE, and then the NFAT (top) was detected by western blotting. β-actin also was assessed by western blotting with its monoclonal antibody (the bottom land). B. Jurkat cells were incubated with DREG56, and then the cells were fixed, permeabilized and stained with anti-NFAT polyclonal antibody and TRITC-conjugated secondary antibody. The nuclei of the Jurkat and neutrophils were stained with DAPI. Bar=20 μm.

NFAT recruits to the nucleus following L-selectin ligation

The NFAT is highly phosphorylated in the resting cells and it can recruit to the nucleus after the cells are stimulated by BCR or TCR engagement. We also knew from Fig. 1 B that over-expression of NLS NFAT mutant could not greatly increase the CSF-1 gene transcription. It seems that the nuclear NFAT protein plays an important role in the CSF-1 gene transcription. As shown in Fig. 2A, the content of the nuclear NFAT increased after L-selectin ligation for 30 min, compared to the resting Jurkat cells. The content of NFAT protein reached a maximum when the cells were stimulated for 30 min, and diminished in the following 60 min. We also tested the NFAT location in the resting and stimulated Jurkat cells by using the immunofluorescene assay. As shown in Fig. 2B, treatment of the Jurkat cells with anti-L-selectin antibody for 30 min resulted in the translocation of NFAT from the cytoplasm to the nucleus. The fluorescence of NFAT protein decreased in the samples activated with mAb of L-selectin for 60 min. The results are consistent with that obtained from the SDS-PAGE, and suggested that NFAT was activated after L-selectin ligation firstly, and then the activated NFAT recruited to the nucleus after L-selectin ligation for 30 min. After incubation with mAb of L-selectin for 60 min, the content of NFAT decreased again. The NFAT shuttled down between the cytoplasm and the nucleus might balance the CSF-1 gene transcription to limit the extent CSF-1 transcription.

NFAT regulates CSF-1 gene transcription by interacting with its promoter

As mentioned above, NFAT was concentrated in the nuclei after the cells were activated by L-selectin ligation for 30 min. It has been reported that NFAT can regulate mouse CSF-1 gene transcription by interacting with the CSF-1 promoter. We assume that NFAT could regulate CSF-1 gene transcription by interacting with the

CSF-1 gene promoter. To test this point, Jurkat cells were crosslinked and the DNA protein complexes were used for EMSA assay. The results showed that the CSF-1 gene promoter region could be connected with NFAT protein, compared with that of mouse IgG (Fig. 3). These results suggested that NFAT recruited to the region of CSF-1 gene promoter in vivo, implying that NFAT as a component of transcriptional complex to regulate CSF-1 gene transcription.

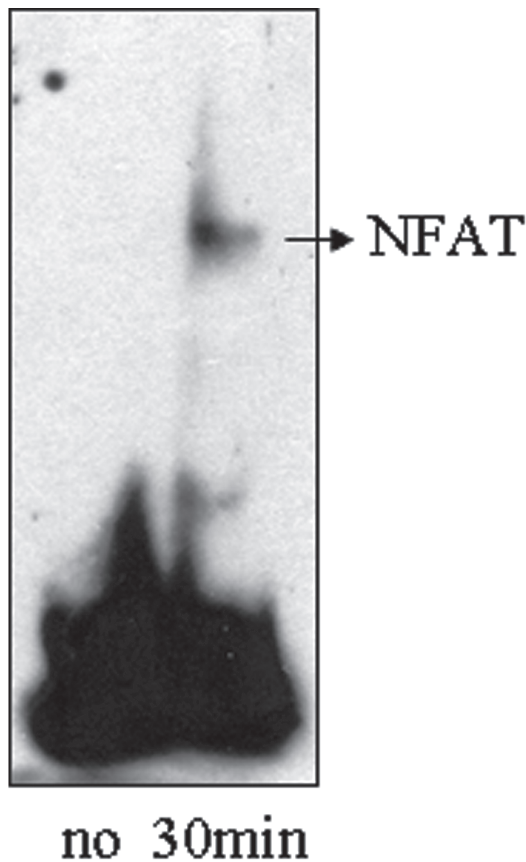

FIGURE 3. NFAT regulates CSF-1 gene transcription by interacting with its promoter. Jurkat cells were stimulated with DREG56 for the indicated time, and then the cells were lysed for the extraction of nuclear proteins. Nuclear extracts were incubated with biotin-labelled CSF oligonucleotide. The nuclear extracts without incubation with mAb were used as control (lane 1). CSF-1 oligonucleotide probes were incubated with 10 μg of nuclear extract/lane and resolved on 5% polyacrylamide gel.

Discussion

Selectins function as both adhesion molecules and transmembrane signaling receptors when leukocytes tether and roll on inflamed endothelium. Published data suggested that L-selectin crosslinking induced gene transcription which was related to many transcriptional factors (Brenner *et al.*, 1997b, 2002; Crabtree and Clipstone, 1999; Rao *et al.*, 1997; Turutin *et al.*, 2003). In this study, we tested CSF-1 gene transcription events in the L-selectin ligation activated Jurkat cells. Results showed that CSF-1 gene transcription induced by L-selectin ligation was regulated by NFAT transcriptional factor.

Extracellular stimulation regulates gene expression via intracellular signaling cascades that control the activity of transcription factors. NFAT is a family of five transcription factors that regulate a variety of immune processes: apoptosis, anergy, T-cell development, and ageing of immune system (Macian, 2005; Masuda *et al.*, 1998). All NFAT proteins contain a highly conserved Rel-homology region that confers common DNA-binding specificity and NFAT homolog region (NHR) domain, containing intracellular localization signaling sequences (Rao, 1997). The NFAT proteins are cytosolic components of an inducible transcription factor complex and are constitutively serine phosphorylated. Stimulation of lymphocytes by the T cell receptor, the B cell receptor or Fc receptors leads to a rise in intracellular calcium and a rapid calcium-dependent activation of the phosphatase calcineurin that dephosphorylates NFAT (Clipstone and Crabtree, 1992). Dephosphorylation of NFAT stimulates a nuclear localization signal which results in the translocation of NFAT to the nucleus.

In this study, our data showed that L-selectin ligation with its mAb increases CSF-1 gene transcription (Fig. 1A). CSF-1, long known as a regulator of macrophage growth and differentiation, has immunomodulatory roles which make it a potential marker of tumor, inflammation, or both (Stanley, 1985; Stanley *et al.*, 1997). CSF-1 can modulate inflammatory response by stimulating the production of several cytokines and growth factors (Warren and Ralph, 1986).

Over-expression of the WT NFAT vector can increase the expression of the CSF-1 gene, otherwise, the over-expression of the NLS mutant NFAT vector could not increase the CSF-1 gene transcription (Fig. 1). This result suggested that the nucleus NFAT played an important role in the CSF-1 gene transcription. The immunofluorescence assay showed that NFAT could be stimulated and localized to the nucleus after the

cells were activated by L-selectin ligation for 30 min (Fig. 2).

CSF-1 is a member of a complex network of cytokines that regulate monocytic cell development and activity. It is produced in nearly all organs and control of CSF-1 gene expression in monocytes and fibroblasts is mediated by common and cell type-specific trans-acting factors (Konicek *et al*., 1998). Inspection of the CSF-1 promoter sequence with the use of the current list of transcription factor binding sites revealed the presence of several putative cis-regulatory elements that may participate in regulating CSF-1 gene transcription (Ladner, 1987; Yamada, 1991; Harrington, 1991). The motifs located between the transcription start site and nucleotide -565 nt could be recognized by AP-1, NFκB, the early growth response protein (EGR) and NF-IL6 (sometimes designated CEBP-b, CCAAT-enhancer binding protein) (Harrington *et al*., 1991). The interaction between these regulatory transcription elements would be expected to contribute to CSF-1 gene expression. A region containing the AP-1 binding site was required for the basal expression of murine CSF-1 gene (Konicek *et al*., 1998). Our results demonstrated that over-expression of WT NFAT otherwise the NLS NFAT mutant could increase CSF-1 gene transcription (Fig. 1B), which suggested that nuclear NFAT was able to regulate CSF-1 gene transcription in the L-selectin ligation activated cells. Importantly, a consensus derived from the alignment of NFAT-binding sites of several promoters resulted in a similar consensus sequence 5'-GGAAA- 3' (Macian *et al*., 2001). Our EMSA results suggest that the nucleus NFAT could be connected with the promoter of CSF-1.

NFAT proteins can enhance gene transcription by binding of AP-1 transcription factors to NFAT-AP-1-binding sites. There it cooperatively binds to DNA with other transcription factors like GATA4 (Macian *et al*., 2000). The components of the AP-1 transcription factor (Fos and Jun polypeptides) then mediate the induction of transcription by recruiting co-activators such as CBP (CREB binding protein), p300 and JAB1 (Jun activation domain binding protein), through their transcriptional activation domains (Harrington *et al*., 1991). In our previous studies, we demonstrated that the transcriptional complex including c-Abl and AP-1 could regulate CSF-1 gene transcription induced by L-selectin ligation. In this study, we found that the NFAT transcriptional factor was transferred to the nucleus and connected with the -564 nt to the CSF-1 promoter, which is near the AP-1 interaction site. So we suppose that NFAT enhance CSF-1 gene transcription by binding of AP-1 transcriptional factor. But more evidences are needed. All these results we obtained together showed that L-selectin ligation induced CSF-1 gene transcription was regulated by NFAT transcriptional factor.

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