Evaluation of the lipopolysaccharide-induced transcription of the human TREM-1 gene in vitamin D3-matured THP-1 macrophage-like cells

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Abstract. Triggering receptor expressed on myeloid cells-1 (TREM-1) plays a role in inflammation by augmenting inflammatory responses through the production of pro-inflammatory cytokines. TREM-1 is expressed in mature macrophages, and is upregulated by stimulation with bacterial components, such as lipopolysaccharide (LPS). In the present study, the regulatory mechanisms responsible for the transcription of the human *TREM-1* gene were examined using a human monocytic cell line (THP-1 cells). Reverse transcription-polymerase chain reaction (RT-PCR) revealed that TREM-1 mRNA was constitutively expressed at a low level in resting cells, and that its expression was upregulated by treatment with vitamin D3 (VitD3), but not by LPS. Importantly, TREM-1 mRNA expression was further upregulated by stimulation of the VitD3-treated THP-1 cells with LPS. In addition, a luciferase reporter assay revealed that the serum response element (SRE) was involved in VitD3-induced promoter activity, whereas the activator protein-1 (AP-1) sites participated in the VitD3- and LPS-induced promoter activity. Of note, the CCAAT-enhancer-binding protein (C/EBP) site contributed not only to basal, but also to VitD3- and LPS-induced promoter activity. Transfection with transcription factor oligodeoxynucleotide (ODN) decoys indicated that transcription factors of the C/EBP and AP-1 families are likely involved in the basal, as well as in the VitD3- and LPS-induced TREM-1 transcription. Western blot analysis indicated that, of the members of the C/EBP family, C/EBPα was constitutively expressed in resting cells; its expression was enhanced by treatment with VitD3 and was further increased by treatment with VitD3 and LPS. Moreover, the expression of c-Fos and c-Jun (members of the AP-1 family) was augmented by treatment with both VitD3 and LPS. These observations indicate that members of the C/EBP family participate not only in basal, but also in the VitD3- and LPS-induced promoter activity of the human *TREM-1* gene, and that members of the AP-1 family are involved in the VitD3- and LPS-induced promoter activity.

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Introduction

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a novel receptor which participates in the amplification of inflammatory responses, and it is mainly expressed in neutrophils and monocytes/macrophages. Although the ligands of TREM-1 have not yet been identified, an agonistic monoclonal antibody elicits the interaction of TREM-1 with DNAX-activating protein 12 (DAP12), which is a transmembrane adaptor molecule, in order to activate the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways, thereby inducing the production of pro-inflammatory cytokines, such as interleukin (IL)-8, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α and IL-1β (1-3). By contrast, silencing TREM-1 expression has been shown to suppress the production of inflammatory cytokines, such as IL-1β, MCP-1, IL-10 and IL-2 by lipopolysaccharide (LPS)-stimulated macrophages (4). Furthermore, silencing TREM-1 expression and blocking TREM-1 responses have also been shown to suppress the production of cytokines (IL-1β, TNF-α and IL-6) and prolong the survival of mice or rats with bacterial sepsis (5-8). These observations suggest that TREM-1 modulates the production of cytokines, thereby amplifying the inflammatory response.

Based on the discovery that the mRNA expression of TREM-1 is higher in human mature $CD14^+$ monocytes compared with that in progenitor $CD34^+$ stem cells (9), it has been demonstrated that TREM-1 mRNA expression is increased by the maturation of monocyte progenitor cells into monocytes (9). Consistently, the mRNA expression of TREM-1 has been shown to increase following the treatment of

U937 cells (a human monocytic cell line) with 1,25-dihydroxycholecalciferol (vitamin D3, hereon referred to as VitD3), which promotes monocyte/macrophage maturation (9,10). Of note, the expression of TREM-1 has also been shown to be upregulated in monocytes/macrophages following stimulation with Gram-negative and Gram-positive bacterial components, such as LPS and lipoteichoic acid (LTA), respectively (5). Previous studies have noted that the transcription factor NF-κB (p65) and hypoxia-inducible factor (HIF)-1α are involved in the VitD3-induced upregulation of TREM-1 mRNA expression in phorbol myristate acetate (PMA)-matured U937 cells, based on the discovery that p65 and HIF-1α were increased by treatment with VitD3 and the upregulation of TREM-1 mRNA expression was inhibited by their inhibitors (10,11). By contrast, in a previous study of ours, we demonstrated, using a mouse TREM-1 promoter and a murine macrophage-cell line (RAW264.7), that the constitutive transcription of the *TREM-1* gene is regulated via the interaction of CCAAT-enhancer-binding protein (C/EBP)α and p50/p50 homodimers with the cAMP response element (CRE) and the NF-κB site, respectively, whereas the LPS-induced upregulation of the *TREM-1* gene is regulated via the interaction of c-Fos/c-Jun with the activator protein-1 (AP-1) site in the promoter (12). However, the *cis*-regulatory elements and transcription factors participating in the expression of the human TREM-1 gene during maturation or stimulation of monocytes/macrophages have not yet been elucidated.

Thus, in the present study, in order to elucidate the regulatory mechanisms responsible for the basal, and VitD3- and LPS-induced TREM-1 expression in human monocytes/macrophages, we evaluated TREM-1 promoter activity (using a luciferase reporter assay), the effects of transcription factor oligodeoxynucleotide (ODN) decoys on TREM-1 mRNA expression, as well as the expression of putative transcription factors (by western blot analysis) in resting, as well as in VitD3- and LPS-treated THP-1 cells, a human monocytic cell line.

Materials and methods

Reagents and antibodies. LPS [from *Escherichia coli* (*E. coli*) serotype O111:B4] was purchased from Sigma Chemical Co. (St. Louis, MO, USA); 1,25-dihydroxycholecalciferol (VitD3), rabbit anti-C/EBPα polyclonal antibody (sc-9314), rabbit anti-C/EBPβ polyclonal antibody (sc-150), rabbit anti-C/EBPζ polyclonal antibody (sc-130709), rabbit anti-serum response factor (SRF) polyclonal antibody (sc-335), rabbit anti-c-Jun polyclonal antibody (sc-1694) and rabbit anti-c-Fos polyclonal antibody (sc-253) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-TATA binding protein (TBP) monoclonal antibody (MA5-14739) was from Thermo Fisher Scientific (Rockford, IL, USA); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG/IgM were purchased from Chemicon International (Temecala, CA, USA); PE-labeled anti-human CD14 monoclonal antibody and PE-labeled anti-mouse IgG2b isotype control were from Beckman Coulter (Brea, CA, USA); and PE-labeled anti-human toll-like receptor (TLR)4 monoclonal antibody and PE-labeled anti-mouse IgG2a K isotype control were obtained from eBioscience (San Diego, CA, USA).

Cell culture. THP-1, a human acute monocytic leukemia cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co.) containing 10% fetal calf serum (FCS; endotoxin level <10 EU/ml; Cell Culture Technologies, Herndon, VA, USA), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) at 37˚C in an incubator with 5% CO_2.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The THP-1 ($1x10^6$) cells were cultured in RPMI-1640 supplemented with 10% FBS overnight in 35-mm dishes. Thereafter, the cells were incubated for 24 h in the absence or presence of VitD3 (100 nM), which acted as a differentiation-inducing agent, and then further stimulated with LPS (*E. coli* O111:B4, 100 ng/ml) for 24 h. Total RNA was purified using an RNeasy Plus Mini kit and QIAshredder (Qiagen, Valencia, CA, USA), and RT-PCR was performed using a ReverTra plus RT-PCR kit (Toyobo Co., Ltd., Osaka, Japan) in a thermal cycler (mastercycler gradient; Eppendorf, Hamburg, Germany) with the following set of oligonucleotide primers: TREM-1 forward, 5'-ATGAGGAAGACCAGGCTC-3' and reverse, 5'-CTAGGGTACAAATGACC-3'; and GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. In brief, cDNA was synthesized by reverse transcription of the total RNA (500 ng) using ReverTra Ace reverse transcriptase and oligo(dT)20. PCR amplification was performed with KOD -Plus- ver.2 polymerase in a thermal cycler. The PCR profile consisted of pre-denaturation [94˚C for 3 min, 24 cycles (for TREM-1) or 18 cycles (for GAPDH) at 96˚C for 10 sec, 58˚C for 30 sec and 68˚C for 45 sec] and a final extension of 7 min. PCR products were resolved by 1% agarose gel electrophoresis and stained with ethidium bromide. In our preliminary experiments, we attempted to semi-quantitatively detect mRNA expression by using different numbers of PCR cycles. The results indicated that the amounts of RT-PCR products increased, depending on the number of cycles. Thus, we decided to measure the mRNA levels by RT-PCR with the number of cycles indicated above. The detected bands were quantified using Multi Gauge (version 3.0; Fujifilm, Tokyo, Japan). The mRNA expression of TREM-1 was normalized to that of GAPDH mRNA expression, and expressed as a ratio relative to resting cells incubated without VitD3 and LPS.

Flow cytometry. The THP-1 ($1x10^6$) cells were incubated for 48 h in the presence or absence of VitD3 (100 nM), and washed twice with cold phosphate-buffered saline (PBS). The cells ($1x10^6$/200 μl) were incubated with PE-labeled anti-human CD14 monoclonal antibody (1 μg), PE-labeled anti-mouse IgG2b isotype control (1 μg), PE-labeled anti-human TLR4 monoclonal antibody (2 μg) or PE-labeled anti-mouse IgG2a K isotype control (2 μg) at 4˚C for 15 min. The cells were then washed with PBS containing 1% bovine serum albumin (BSA) and 0.1% NaN_3, and analyzed by flow cytometry (FACSCalibur™; BD Biosciences, San Jose, CA, USA). The expression of CD14 and TLR4 was determined by the mean fluorescence intensity (MFI), which was corrected for non-specific binding by subtracting the MFI values corresponding to the isotype-matched controls, and was expressed as a ratio relative to resting cells incubated without VitD3.

Plasmid construction. The transcription initiation site of human TREM-1 was estimated using the human chromosome 6 sequences and the mRNA database (NCBI reference sequence nos. AL391903 and AF287008). A 1.2-kbp fragment of the human TREM-1 promoter (-1200 to +64) was amplified from THP-1 genomic DNA using KOD -Plus- polymerase and a set of oligonucleotide primers as follows: -1200 sense, 5'-GGG ACGCGTCCTATACTTGAGTAGCAATC-3' and +64 antisense, 5'-GGGAGATCTCCTTCCTGTGCACCAGC-3' (underlined letters indicate the restriction sites, *Mlu*I and *Bgl*II, respectively). The PCR products were digested with *Mlu*I and *Bgl*II, and subcloned into a promoterless firefly luciferase-expression plasmid (pGL3-Basic; Promega, Madison, WI, USA) to generate a -1200 plasmid. The 0.6-, 0.4-, 0.2-, 0.1- and 0.05-kbp fragments of the human TREM-1 promoter (-600, -400, -200, -100 and -50 to +64) were amplified by PCR using a -1200 plasmid (as a template), KOD -Plus- polymerase and a set of oligonucleotide primers containing *Mlu*I and *Bgl*II restriction sites (indicated by underlined letters): -600 sense, 5'-GGGACGCGTCTGTTCTTGTTGGGTGGTG-3'; -400 sense, 5'-GGGACGCGTATGTTCTCACAAAAACCCTGAAG-3'; -200 sense, 5'-GGGACGCGTGTTGAAAGGTAATTGT CATTATTACC-3'; -100 sense, 5'-GGGACGCGTTCAGG AGTCAGAGCAACTGG-3'; -50 sense, 5'-GGGACGCGT CCAGGAATGGCCTCATATCC-3'; and +64 antisense, 5'-GGGAGATCTCCTTCCTGTGCACCAGC-3'. The PCR products were digested and subcloned into pGL3-Basic. The inserts were confirmed by sequencing with a BigDye® Terminator v3.1 Cycle sequencing kit and a 3730xl DNA Analyzer (both from Applied Biosystems, Foster City, CA, USA). The *cis*-regulatory elements were investigated using the TFSEARCH database (http://diyhpl.us/~bryan/irc/protocol-online/protocol-cache/TFSEARCH.html) in the 5' upstream region (-1200 to +64) of the human TREM-1 promoter.

The *cis*-acting motifs of AP-1-2 (-341 to -331), AP-1-3 (-97 to -90), AP-1-4 (-58 to -48), SRE (-353 to -342), CRE (-105 to -98), C/EBP-2 (-544 to -537), C/EBP-3 (-212 to -199), C/EBP-4 (-195 to -180), C/EBP-5 (-31 to -18), GATA-3 (-479 to -470) and GATA-4 (-376 to -368), which were deduced using the TFSEARCH database, were substituted by adenine nucleotides via PCR-based site-directed mutagenesis using the -1200 or -600 plasmid comprising of pGL3-Basic (as a template), KOD -Plus- polymerase and appropriate oligonucleotide sense and antisense primers listed in (Table I). Synthesized blunt-ended PCR products were purified with a MinElute Gel extraction kit (Qiagen), phosphorylated with polynucleotide kinase, and then self-ligated with T4 DNA ligase. Mutated *cis*-regulatory elements were confirmed by sequencing.

Transfection and luciferase assay. The THP-1 cells ($5x10^6$) were transfected with the -1200 plasmid, -600 plasmid or adenine mutant reporter plasmids (10 μg) with the *Renilla* luciferase control reporter vector phRL-TK (500 ng, as an internal control; Promega) by electroporation (220 mV, 960 mFD) using Gene Pulser™ (Bio-Rad, Hercules, CA, USA). The cells were then incubated without or with VitD3 (100 nM) for 48 h, or incubated with VitD3 for 24 h followed by further incubation with LPS for 24 h. Thereafter, the cells were washed twice with PBS and lysed in passive lysis buffer (Promega). Firefly and *Renilla* luciferase activity was then measured using a Dual-Luciferase® reporter assay system (Promega) and a microplate luminometer (SpectraMax® L; Molecular Devices, Sunnyvale, CA, USA). Promoter activity was normalized to *Renilla* luciferase activity and expressed as a ratio relative to the firefly luciferase activity of the -1200 plasmid-transfected cells incubated without VitD3 and LPS.

Western blot analysis. Nuclear extracts were prepared from resting THP-1 cells, THP-1 cells treated with VitD3 or LPS, or THP-1 cells treated with VitD3 and LPS, as previously described (12,13). Aliquots of nuclear extracts (10 μg) were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and resolved proteins were then electrotransferred to polyvinylidene difluoride membranes (Immobilon™-P; Millipore, Billerica, MA, USA) using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad). The membranes were blocked in 5% BlockAce (Dainippon Pharmaceutical, Osaka, Japan) in TBS-T (10 mM Tris-HCl pH 7.5, 100 mM NaCl 0.05% Tween-20). The blotted membranes were probed with anti-C/EBPα antibody (1:200), anti-C/EBPβ antibody (1:200), anti-C/EBPζ antibody (1:200), anti-SRF antibody (1:200), anti-c-Fos antibody (1:1,000), anti-c-Jun antibody (1:1,000) or anti-TBP antibody (1:1,000). The membranes were washed with TBS-T 3 times and further probed with HRP-conjugated goat anti-rabbit IgG (1:2,000) or goat anti-mouse IgG/IgM (1:2,000). Proteins were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and detected with an LAS-3000 Image Analyzer (Fujifilm). TBP was used as a loading control to indicate that equal amounts of proteins were analyzed in each sample.

Tranesfection of transcription factor ODN decoys. Single-stranded sense and antisense phosphorothioate-bonded ODN decoys were synthesized by Operon (Alameda, CA, USA). The single-stranded sense sequences of consensus and mutant ODNs were as follows (the underlined letters indicate phosphorothioate-bonded bases): C/EBP consensus ODN, 5'-TGCAGATTGCGCAATCTGCA-3'; C/EBP mutant ODN, 5'-TGCAGAGACTAGTCTCTGCA-3'; AP-1 consensus ODN, 5'-CGCTTGATGACTCAGCCGGAA-3'; AP-1 mutant ODN, 5'-CGCTTGATGACTTGGCCGGAA-3'; SRF consensus ODN, 5'-GGATGTCCATAT TAGGACATCT-3'; and SRF mutant ODN, 5'-GGATGTCCATATTATTACATCT-3'. The double-stranded ODN was prepared by annealing the sense ODN to its antisense ODN. This was achieved by heating equal amounts of each single-stranded ODN at 95˚C for 5 min in TE buffer [10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA)], and then slowly cooling the mixture to room temperature. THP-1 cells ($5x10^5$) in 12-well plates were transfected with 250 pmol of consensus or mutant ODN using X-tremeGENE HP (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. After 24 h, the transfected cells were incubated without or with VitD3 (100 nM) for 48 h or incubated with VitD3 for 24 h followed by further incubation with LPS for 24 h. The cells were then washed twice with cold PBS, and total RNA was purified for use in RT-PCR.

Statistical analysis. The data are expressed as the means ± SD. Statistical analysis was performed by one-way analysis of vari-

Table I. Oligonucleotide primers for PCR-based site directed mutagenesis.

Putative motifs	Primer sequences
	-330 -307
AP-1-2	Sense: 5'-AAAAAATCACTACACTAAACTGGATGTG-3'
	Antisense: 3'-GTCGGAGAGGGATACACCCTTTTT-5'
	-360 -342
	-89 -67
AP-1-3	Sense: 5'-AAAAAGCAACTGGTGATGAAACAGAAC-3'
	Antisense: 3'-CAGAGACATAATAACACAACTACAGTTTTT-5'
	-119 -98
	-47 -29
AP-1-4	Sense: 5'-AAAAAAGGAATGGCCTCATATCCTG-3'
	Antisense: 3'-CACTACTTTGTCTTGGGTTTGAGTTTTT-5'
	-81 -59
	-341 -322
SRE	Sense: 5'-AAAAAACCTGACTCTCTTCACTACAC-3'
	Antisense: 3'-CTATGCTAACAGCGTCGGAGTTTTTT-5'
	-373 -354
	-97 -79
CRE	Sense: 5'-AAAAGGAGTCAGAGCAACTGGTG-3'
	Antisense: 3'-ACACAATAATACAGAGACCAAGACTCTTTT-5'
	-131 -106
	-536 -519
C/EBP-2	Sense: 5'-AAAAGCTCCCGAGGCCATGTCTG-3'
	Antisense: 3'-GTTTAACAAAGACCCCAGGGATGTGTTTT-5'
	-569 -545
	-198 -173
C/EBP-3	Sense: 5'-AAAAAAAAAGGTAATTGTCATTATTACCAC-3'
	Antisense: 3'-GTAAGTTCATAGTAATTTTTTT-5'
	-227 -213
	-179 -158
C/EBP-4	Sense: 5'-AAAAAAAATTACCACAGAAAGGAAAACTGG-3'
	Antisense: 3'-GAGGGAATCTAAAACATTCCAACTTTTTTTT-5'
	-218 -196
	-17 +1
C/EBP-5	Sense: 5'-AAAAAAATCCGAAGCCTCTAGGTCA-3'
	Antisense: 3'-GGTCCTTACCGGAGTATGTTTTTTT-5'
	-49 -32
	-469 -451
GATA-3	Sense: 5'-AAAAAAGGAGGTGCACCCCAGGTC-3'
	Antisense: 3'-GTTTACGTCCCACACCGGGAGGATTTTT-5'
	-503 -480
	-367 -351
GATA-4	Sense: 5'-AAAAAATTGTCGCAGCCTCTCC-3'
	Antisense: 3'-CAAGAGTGTTTTTGGGACTTCTTTTT-5'
	-397 -377

Underlined letters indicate the nucleotides for the adenine substitution of putative motif sequences in Fig. 3; AP-1-2 (-341 to -331), AP-1-3 (-97 to -90) and AP-1-4 (-58 to -48); SRE (-353 to -342); cAMP response element (CRE; -105 to -98); (CCAAT-enhancer-binding protein (C/EBP)-2 (-544 to -537), C/EBP-3 (-212 to -199), C/EBP-4 (-195 to -180) and C/EBP-5 (-31 to -18); GATA-3 (-479 to -470) and GATA-4 (-376 to -368).

ance (ANOVA), followed by Bonferroni's multiple comparison test or the unpaired Student's t-test (GraphPad Prism; GraphPad Software, Inc., San Diego, CA, USA). A P-value <0.05 was considered to indicate a statistically significant difference.

Results

VitD3 and LPS induce human TREM-1 mRNA expression in THP-1 cells. First, we examined the effects of treatment with VitD3 or LPS on the mRNA expression of human TREM-1 in monocytes/macrophages using THP-1 monocytic cells by RT-PCR. We found that TREM-1 mRNA was constitutively expressed at a low level in resting THP-1 cells that had not been treated with VitD3 and LPS, and was upregulated by treatment with VitD3, but not by LPS (P<0.001; Resting vs. VitD3; Fig. 1). Importantly, TREM-1 mRNA expression was further upregulated by the stimulation of the VitD3-treated THP-1 cells with LPS (P<0.01; VitD3 vs. VitD3 + LPS). In addition, we examined the effect of VitD3 treatment on the expression of CD14 as a differentiation marker of macrophages (Fig. 2A). VitD3, as a macrophage differentiation agent, significantly increased the expression of CD14 (approximately 90-fold, P<0.001; Resting vs. VitD3); however, the expression of TLR4 was not markedly altered by treatment with VitD3 (Fig. 2B). These observations indicate that VitD3 induces the mRNA expression of TREM-1, which is accompanied by the differentiation of THP-1 cells into macrophages, and LPS further upregulates the VitD3-induced expression of TREM-1.

Sequence analysis of the 5' upstream flanking region of the human TREM-1 gene. In order to elucidate the mechanisms controlling the basal, VitD3- and LPS-induced human TREM-1 gene transcription, we analyzed the *cis*-regulatory elements in the TREM-1 promoter (from -1200 to +64) using the TFSEARCH database (version 1.3); the transcription initiation site (+1) was estimated using the sequence of human chromosome 6 and the mRNA database (NCBI reference sequencenos. AL391903 and AF287008). As shown in Fig. 3, the human TREM-1 promoter contained multiple potential binding motifs for the AP-1 family (AP-1-1, -2, -3 and -4), SRF, GATA (GATA-1, -2, -3 and -4), C/EBP (C/EBP-1, -2, -3, -4 and -5) and CRE, although the TATA-box sequence could not be detected in the promoter.

Luciferase assay of the human TREM-1 promoter containing adenine substitution mutants. In order to elucidate the potential *cis*-regulatory elements involved in the basal, VitD3- and LPS-induced transcription of human *TREM-1* gene, the motifs of AP-1-2 (-341 to -331), AP-1-3 (-97 to -90), AP-1-4 (-58 to -48), C/EBP-2 (-544 to -537), C/EBP-3 (-212 to -199), C/EBP-4 (-195 to -180), C/EBP-5 (-31 to -18), CRE (-105 to -98), GATA-3 (-479 to -470), GATA-4 (-376 to -368) and SRE (-353 to -342) in the promoter were substituted by adenine nucleotides (Fig. 4). The luciferase vectors containing these adenine-substituted promoter sequences were transfected into THP-1 cells and incubated without (Resting) or with 100 nM VitD3. As shown in Fig. 4, the plasmid containing the -1200 or -600 upstream region substantially enhanced the luciferase activity, which is consistent with the finding that the *TREM-1* gene is constitutively transcribed in resting THP-1 cells (Fig. 1). Importantly, the promoter activity of the -1200 plasmid was significantly enhanced by treatment with VitD3 (approximately 3.7-fold; Resting vs. VitD3, P<0.001). By contrast, VitD3-induced promoter activity was significantly decreased by the mutation of SRE (-1200 vs. SRE, P<0.05). In addition, promoter activity was

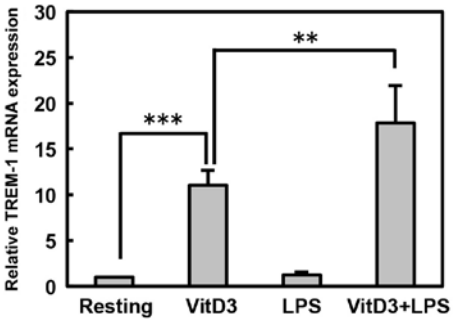

Figure 1. Induction of the mRNA expression of human TREM-1 by vitamin D3 (VitD3) and lipopolysaccharide (LPS) in THP-1 cells. THP-1 cells ($1x10^6$) were incubated without VitD3 (Resting) or with 100 nMVitD3 for 48 h (VitD3), incubated with VitD3 for 24 h, followed by further incubation with LPS for 24 h (VitD3 + LPS), or incubated with LPS alone for 24 h (LPS). TREM-1 mRNA expression was determined by RT-PCR and expressed as a ratio relative to the resting cells incubated without VitD3 and LPS. Data represent the means ± SD of 4 separate experiments. Values are compared between resting and VitD3-treated cells, and between VitD3-treated and VitD/LPS-treated cells. **P<0.01, ***P<0.001.

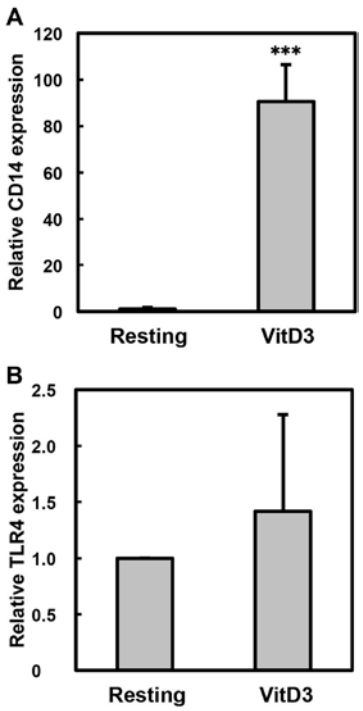

Figure 2. Upregulation of CD14 expression by vitamin D3 (VitD3) in THP-1 cells. THP-1 ($1x10^6$) cells were incubated without vitamin D3 (VitD3; Resting) or with VitD3 (100 nM) for 48 h (VitD3). Cells ($1x10^6$/200 μl) were incubated with PE-labeled anti-human CD14 monoclonal antibody (1 μg) or PE-labeled anti-mouse IgG2b isotype control (1 μg), and PE-labeled anti-human TLR4 monoclonal antibody (2 μg) or PE-labeled anti-mouse IgG2aκ isotype control (2 μg) at 4˚C for 15 min. The expression of (A) CD14 and (B) TLR4 was analyzed by flow cytometry. Data represent the means ± SD of 3-5 separate experiments, and are expressed as a ratio relative to resting cells incubated without VitD3. Values are compared between resting and VitD3-treated cells. ***P<0.001.

Figure 3. Sequence of the 5' upstream region of the human *TREM-1* gene. Transcription initiation site was estimated using the sequences of human chromosome 6 and mRNA database (NCBI reference sequence nos. AL391903 and AF287008). Analysis with a TFSEARCH program (version 1.3) revealed that the 5' upstream region of the human TREM-1 gene contained putative *cis*-regulatory motifs, such as AP-1 sites, SRE (-353 to -342), GATA-1 sites, CCAAT-enhancer-binding proteins (C/EBP) sites and CRE (-105 to -98); however, a TATA-box sequence cannot be detected in the promoter. AP-1 sites are termed AP-1-1 (-1114 to -1106), AP-1-2 (-341 to -331), AP-1-3 (-97 to -90) and AP-1-4 (-58 to -48); GATA-1 sites are termed GATA-1 (-1090 to -1081), GATA-2 (-889 to -879), GATA-3 (-479 to -470) and GATA-4 (-376 to -368); C/EBP sites are termed C/EBP-1 (-753 to -740), C/EBP-2 (-544 to -537), C/EBP-3 (-212 to -199), C/EBP-4 (-195 to -180) and C/EBP-5 (-31 to -18).

significantly reduced by the mutation of the AP-1-2 or AP-1-3 site (-1200 vs. AP1-2, -1200 vs. AP1-3, P<0.05). Moreover, other *cis*-regulatory elements in the TREM-1 promoter were analyzed using a -600 plasmid containing adenine-substituted promoter sequences. Similar to the results obtained with the -1200 plasmid, the promoter activity of the -600 plasmid was significantly enhanced by treatment with VitD3 (approximately 4.8-fold; Resting vs. VitD3, P<0.001; Fig. 4). Moreover, promoter activity was significantly decreased by the mutation of the AP-1-2 or AP-1-3 site (-600 vs. AP1-2, -600 vs. AP1-3, P<0.01). By contrast, promoter activity was not significantly affected by the mutations of C/EBP-2, -3 and -4 and GATA-3 and -4 sites. Notably, the mutation of the C/EBP-5 site markedly decreased not only the basal, but also the VitD3-induced promoter activity (-600 vs. C/EBP-5, P<0.001; Fig. 4). These findings suggest that the C/EBP-5 site is essential for the basal and VitD3-induced promoter activity, whereas the SRE, AP-1-2 and AP-1-3 sites are involved in the VitD3-induced promoter activity of the human *TREM-1* gene.

Next, we analyzed the *cis*-regulatory elements involved in the LPS-induced transcription of the *TREM-1* gene by transfecting the THP-1 cells with luciferase vectors containing adenine-substituted promoter sequences; the VitD3-treated cells were then stimulated with LPS. In accordance with the results obtained from the anlaysis of TREM-1 mRNA expression (Fig. 1), the promoter activity of the -1200 and -600 TREM-1 promoter sequences was enhanced by 1.5- to 1.9-fold, respectively, following the stimulation of the VitD3-treated cells with LPS (VitD3 vs. VitD3 + LPS, P<0.05) (Fig. 5). Of note, the LPS-induced promoter activity of the -1200 and -600 promoter sequences was significantly decreased by the mutation of the AP-1-2 or AP-1-3 site (-1200 vs. AP-1-2 or AP-1-3, P<0.05; -600 vs. AP-1-2, P<0.05; -600 vs. AP-1-3, P<0.01) (Fig. 5). Moreover, the mutation of the C/EBP-5 site markedly decreased the LPS-induced promoter activity (-600 vs. C/EBP-5, P<0.001), as well as the VitD3-induced promoter activity (Fig. 5).

Taken together, these findings indicate that the AP1-2 and AP1-3 sites participate in both the VitD3- and LPS-induced promoter activity of the human *TREM-1* gene, whereas the C/EBP-5 site is involved not only in the basal, but also in the VitD3- and LPS-induced promoter activity of the human

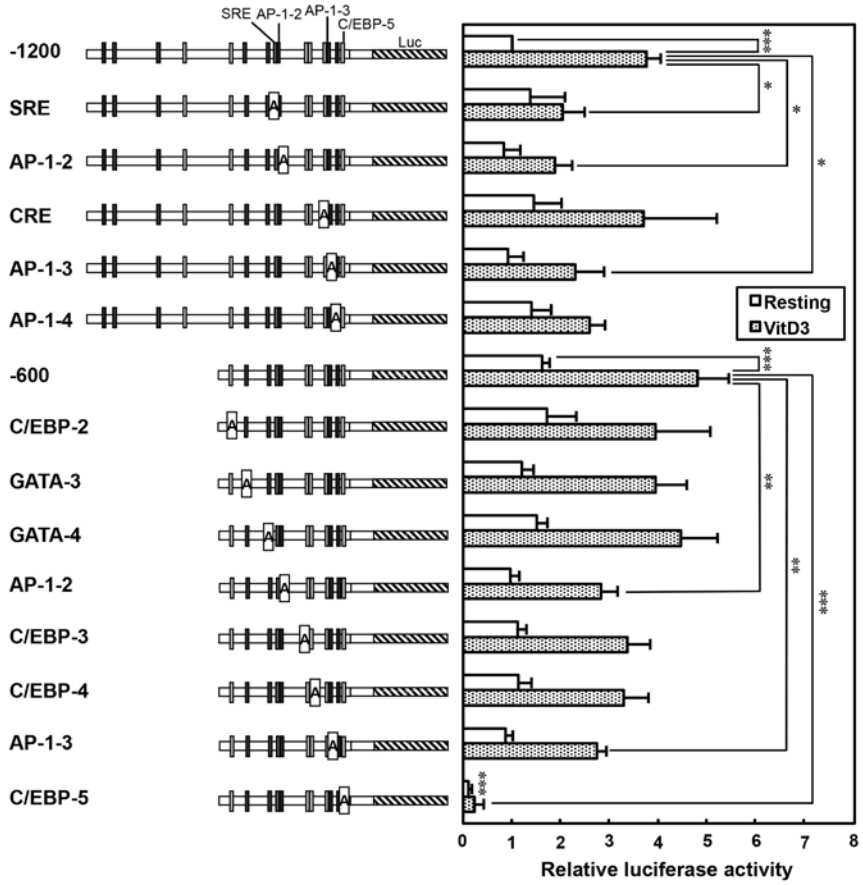

Figure 4. Basal and vitamin D3 (VitD3)-induced luciferase activity of the human TREM-1 promoter containing adenine-substituted constructs. Positions of putative *cis*-regulatory motifs (AP-1, SRE, CCAAT-enhancer-binding proteins (C/EBP), GATA and CRE) are indicated on the TREM-1 promoter flanking luciferase gene (Luc). THP-1 cells ($5x10^6$) were transfected with -1200 plasmid, -600 plasmid or adenine mutant-reporter plasmids (10 μg) with phRL-TK (500 ng, an internal control) by electroporation. Then, tThe cells were then incubated without VitD3 (Resting) or with VitD3 (100 nM) for 48 h (VitD3), and then firefly and *Renilla* luciferase activity was measured. Promoter activity was normalized to *Renilla* luciferase activity, and expressed as a ratio relative to the firefly luciferase activity of -1200 plasmid-transfected cells incubated without VitD3. Values are the means ± SD of at least 4 independent experiments. Values are compared between -1200 or -600 plasmids and adenine-substituted constructs in resting and VitD3-treated cells. *P<0.05, **P<0.01, ***P<0.001.

TREM-1 gene. These conclusions were supported by the experiments using the luciferase vectors containing 5' truncated promoter sequences, which indicated that the basal promoter activity of the -50 plasmid containing only C/EBP-5 was almost the same as that of the -1200 plasmid, and the VitD3- and LPS-induced promoter activity of the -400 plasmid containing AP-1-2, AP-1-3 and C/EBP-5 was equal to that of the -1200 plasmid (Fig. 6). Moreover, the VitD3- and LPS-induced promoter activity was slightly decreased by the deletion of the AP-1-2 site in the -200 plasmid, compared with that of the -400 plasmid; however, the VitD3- and LPS-induced promoter activity of the -100 plasmid containing the AP-1-3 site was almost the same as that of the -200 plasmid containing AP-1-3 (Fig. 6).

Effects of C/EBP and AP-1 ODN decoys on TREM-1 mRNA expression. To further determine the involvement of C/EBP, AP-1 and SRF in the basal, as well as in the VitD3- and LPS-induced transcription of the human *TREM-1* gene, we examined the effects of C/EBP, AP-1 and SRF ODN decoys on the mRNA expression of TREM-1 by transfecting ODN decoys into the cells, followed by incubation with or without VitD3 and LPS. As shown in Fig. 7, the C/EBP consensus ODNs significantly suppressed not only the basal, but also the VitD3- and VitD3/LPS-induced TREM-1 mRNA expression, as compared with the mutant ODNs (resting cells, P<0.01; VitD3- and VitD3 + LPS-treated cells, P<0.05; Fig. 7A). In addition, AP-1 consensus ODNs substantially suppressed the VitD3- and VitD3/LPS-induced TREM-1 mRNA expression, although the effects were not statistically significant (Fig. 7B). By contrast, the SRF consensus ODNs did not affect the VitD3- and VitD3/LPS-induced TREM-1 mRNA expression (Fig. 7C).

Furthermore, we examined the expression levels of C/EBP, AP-1 and SRF in resting, VitD3-, LPS- and VitD3/LPS-treated THP-1 cells by western blot analysis. As shown in Fig. 8, of the members of the C/EBP family, C/EBPα was constitutively expressed in resting cells; its expression was enhanced by treatment with VitD3 and further increased by treatment with LPS (VitD3 +LPS). Moreover, c-Fos and c-Jun (members of the AP-1 family) were constitutively expressed in resting cells; their expression was enhanced by treatment with VitD3/LPS, but not by treatment with VitD3 alone. SRF was constitutively expressed in resting cells; however, its expression was not altered by treatment with VitD3, but was increased by treatment with VitD3 and LPS.

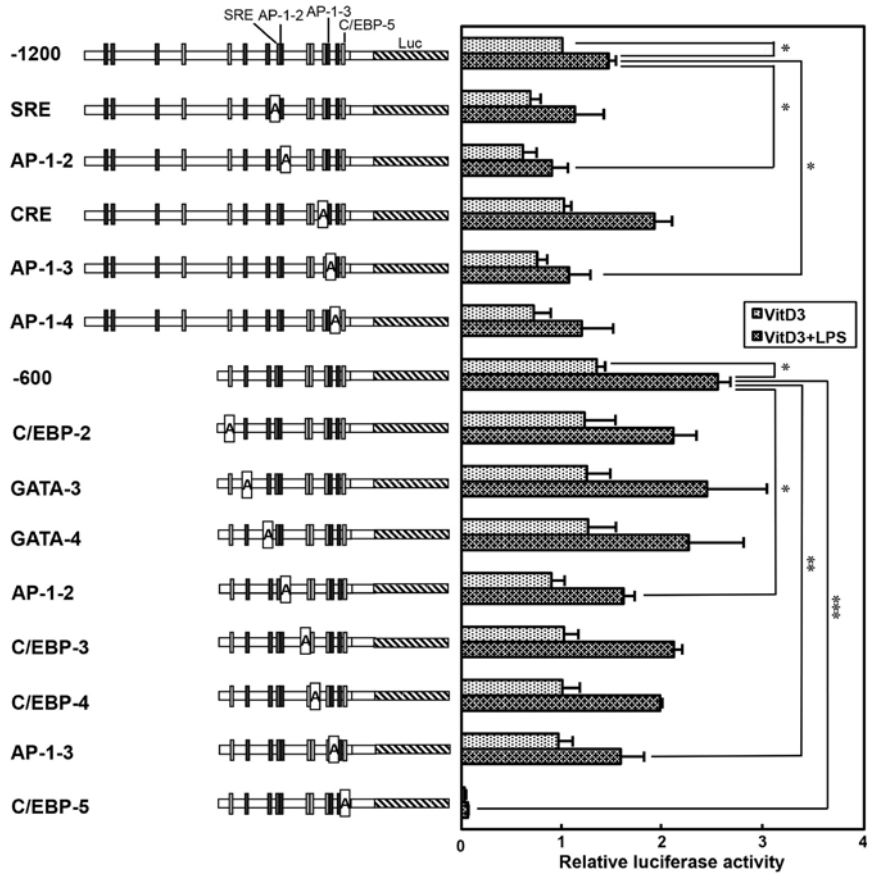

Figure 5. Lipopolysaccharide (LPS)-induced luciferase activity of the human TREM-1 promoter containing adenine-substitution constructs. Positions of putative *cis*-regulatory motifs [AP-1, SRE, CCAAT-enhancer-binding proteins (C/EBP), GATA and CRE] are indicated on the TREM-1 promoter flanking luciferase gene (Luc). THP-1 cells ($5x10^6$) were transfected with -1200 plasmid, -600 plasmid or adenine mutant reporter plasmids (10 μg) with phRL-TK (500 ng) by electroporation. The cells were then incubated with vitamin D3 (VitD3) alone for 48 h (VitD3), or incubated with VitD3 for 24 h, followed by further incubation with LPS for 24 h (VitD3 + LPS), and firefly and *Renilla* luciferase activity was then measured. Promoter activity was normalized to *Renilla* luciferase activity, and expressed as a ratio relative to the firefly luciferase activity of -1200 plasmid-transfected cells incubated with VitD3 alone. Values are the means ± SD of at least 4 independent experiments. Values are compared between -1200 or -600 plasmids and adenine-substituted constructs in VitD3-treated and VitD3/LPS-treated cells *P<0.05; **P<0.01; ***P<0.001.

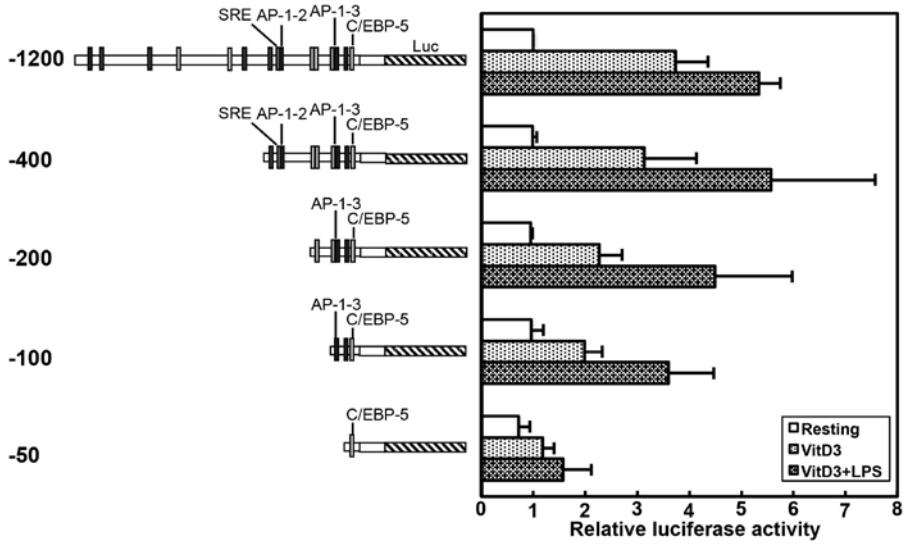

Figure 6. Basal, vitamin D3 (VitD3)- and lipopolysaccharide (LPS)-induced luciferase activity of the human TREM-1 promoter containing 5' deletion constructs. Positions of putative *cis*-regulatory motifs [SRE, AP-1 and CCAAT-enhancer-binding proteins (C/EBP)] are indicated on the TREM-1 promoter flanking luciferase gene (Luc). THP-1 cells ($5x10^6$) were transfected with a series of 5' deletions reporter constructs (-1200, -400, -200, -100 and -50 plasmids; 10 μg) and *Renilla* luciferase-expression plasmid phRL-TK (500 ng) by electroporation. Thereafter, the cells were incubated without VitD3 (resting) or with VitD3 (VitD3 for 48 h (VitD3), or were incubated with VitD3 for 24 h, followed by further incubation with LPS for 24 h (VitD3 + LPS). Firefly and *Renilla* luciferase activity was then measured. Promoter activities were normalized to *Renilla* luciferase activity, and expressed as a ratio relative to the firefly luciferase activity of -1200 plasmid-transfected cells incubated without VitD3 and LPS. Values are the means ± SD of at least 4 independent experiments.

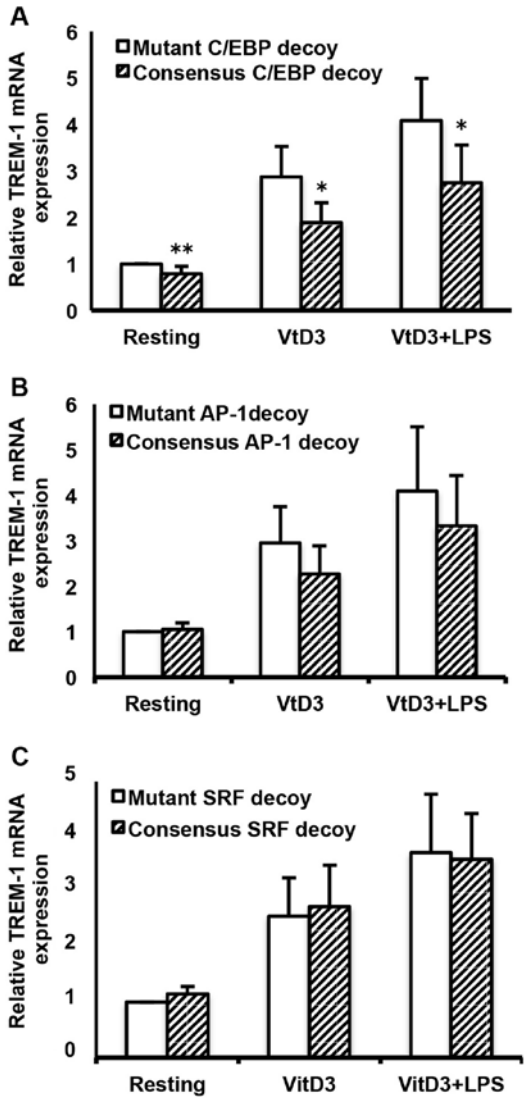

Figure 7. Effects of transfection of transcription factor oligodeoxynucleotide (ODN) decoys on the expression of TREM-1 mRNA. THP-1 cells ($5x10^5$) were transfected with 250 pml of consensus or mutant ODNs for (A) CCAAT-enhancer-binding protein (C/EBP), (B) AP-1 and (C) SRF using X-tremeGENE HP, according to the manufacturer's instructions. After 24 h, transfected cells were incubated without vitamin D3 (VitD3; Resting) or with VitD3 (VitD3) for 48 h (VitD3), or incubated with VitD3 for 24 h, followed by further incubation with LPS for 24 h (VitD3 + LPS). Thereafter, the cells were washed twice with cold PBS, and total RNA was purified for RT-PCR. Data are the means ± SD of 4 separate experiments. Values are compared between the consensus and mutant ODN-transfected cells. $^{*}P<0.05$, $^{**}P<0.01$.

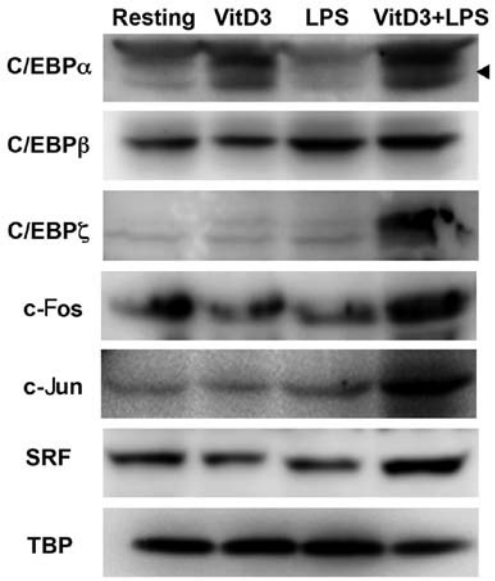

Figure 8. Western blot analysis of transcription factors in resting, vitamin D3 (VitD3)- and lipopolysaccharide (LPS)-treated THP-1 cells. THP-1 cells ($1x10^6$) were incubated without (Resting) or with VitD3 for 48 h (VitD3), incubated with VitD3 for 24 h, followed by further incubatedion with LPS for 24 h (VitD3 + LPS), or incubated with LPS alone for 24 h (LPS). Nuclear extracts (10 μg) were subjected to 10% SDS-PAGE, and then resolved proteins were electrotransferred onto PVDF membranes. The membranes were blocked and probed with anti-CCAAT-enhancer-binding proteins (C/EBP)α, anti-C/EBPβ, anti-C/EBPζ, anti-c-fos, anti-c-jun, anti-SRF and anti-TBP antibody. The membrane was further probed with HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG/IgM. Proteins were visualized with a chemiluminescent substrate. The arrowhead indicates C/EBPα. Images are representative of at least 3 separate experiments.

Discussion

In the present study, the regulatory mechanisms responsible for the transcription of the human *TREM-1* gene were examined using a human monocytic cell line (THP-1 cells). RT-PCR revealed that TREM-1 mRNA was constitutively expressed at a low level in resting cells, and was upregulated by treatment with VitD3, but not by LPS (Fig. 1). Importantly, TREM-1 mRNA expression was further upregulated by the stimulation of the VitD3-treated THP-1 cells with LPS (Fig. 1). In addition, a luciferase reporter assay revealed that the SRE site was involved in the VitD3-induced promoter activity (Fig. 4), whereas the AP-1 sites participated in the VitD3- and LPS-induced promoter activity (Fig. 5). Of note, the C/EBP site contributed not only to the basal, but also to the VitD3- and LPS-induced promoter activity (Figs. 4 and 5). Transfection with transcription factor ODN decoys indicated that the transcription factors of the C/EBP and AP-1 families were likely involved in the basal, VitD3- and LPS-induced TREM-1 transcription (Fig. 7); however, the role of SRF in TREM-1 transcription could not be clarified. Notably, western blot analysis indicated that, of the members of the C/EBP family, C/EBPα was constitutively expressed in resting cells; its expression was enhanced by VitD3 and was further increased by LPS. Moreover, the expression of c-Fos and c-Jun (members of the AP-1 family) was augmented by treatment with both VitD3 and LPS (Fig. 8). Taken together, these findings indicate that members of the C/EBP family participate not only in the basal, but also in the VitD3- and LPS-induced promoter activity of the human *TREM-1* gene, whereas members of the AP-1 family are involved in the VitD3- and LPS-induced promoter activity.

CD14 and TLR4 function as receptors for LPS (14-17). The expression of CD14 was markedly upregulated by treatment of the THP-1 cells with VitD3. By contrast, TLR4 was constitutively expressed in the THP-1 cells, and its expression was not markedly altered by treatment with VitD3. Of note, the expression of TREM-1 was increased by treatment of the THP-1 cells with VitD3, and its expression was further upregulated by LPS. By contrast, TREM-1 expression was not increased

by the stimulation of resting THP-1 cells with LPS, which expressed a low level of CD14. These observations suggest that the LPS-induced upregulation of TREM-1 in VitD3-treated cells is largely dependent on the increased expression of CD14 following the maturation of THP-1 cells with VitD3.

The transcription factors NF-κB (p65) and HIF-1α have been shown to be involved in the VitD3-induced upregulation of TREM-1 mRNA expression in PMA-matured U937 cells, based on the findings that p65 and HIF-1α were increased by treatment with VitD3, and the upregulation of TREM-1 mRNA expression was inhibited by their inhibitors (Bay 11-7082, YC-1) (10,11). However, in the present study, the putative motifs of NF-κB and HIFs could be identified in the 5' upstream region (-1200 to +64) of the human TREM-1 promoter (Fig. 3). Thus, it remains to be elucidated whether NF-κB and HIF-1α are involved in the regulation of *TREM-1* gene expression in VitD3-treated THP-1 cells. Furthermore, it has been reported that the vitamin D receptor (VDR) participates in the upregulation of TREM-1 mRNA expression in VitD3-treated human airway epithelial cells, based on the findings that a vitamin D receptor response element (VDRE) was identified in the TREM-1 promoter using computer-based analysis (MatInspector; www.genomatix.de) and the protein level of VDR was increased by VitD3 (18). In this study, we localized a putative VDRE (-209 to -186) in the human TREM-1 promoter using the MatInspector algorism. However, the luciferase assay revealed that the mutations of the C/EBP-3 and C/EBP-4 sites overlapping VDRE (-209 to -186) did not significantly affect the VitD3-induced promoter activity of the *TREM-1* gene (Fig. 4). Thus, it is unlikely that putative VDRE was involved in the VitD3-induced TREM-1 promoter activity in monocytes/macrophage using U937 cells (10,11).

The luciferase assay indicated that the C/EBP-5 site plays a role in the basal, and in the VitD3- and LPS-induced TREM-1 promoter activity. In line with this, the transfection of ODN decoys revealed that transcription factors of the C/EBP family were involved in the basal, and in the VitD3- and LPS-induced TREM-1 mRNA expression. Furthermore, western blot analysis revealed that, of the members of the C/EBP family, C/EBPα was constitutively expressed in resting cells; its expression was enhanced by treatment with VitD3 and was further increased by treatment with VitD3 and LPS. These observations suggest that members of the C/EBP family, possibly C/EBPα, participate not only in the basal, but also in the VitD3- and LPS-induced promoter activity of the human *TREM-1* gene.

In addition, the luciferase assay indicated that the AP1-2 and AP1-3 sites are involved in both the VitD3- and the LPS-induced promoter activity of the human *TREM-1* gene. Consistently, the transfection of ODN decoys revealed that transcription factors of the AP-1 family contribute to the VitD3- and LPS-induced TREM-1 mRNA expression. Of note, the expression of c-Fos and c-Jun (members of the AP-1 family) was markedly increased by treatment with both VitD3 and LPS, suggesting that c-Fos and c-Jun may participate in the VitD3/LPS-induced *TREM-1* gene transcription. However, the levels of c-Fos and c-Jun were not affected by treatment with VitD3 alone, although VitD3 enhanced TREM-1 promoter activity. Importantly, it has been reported, using U937 and HL-60 cells, that treatment with VitD3 induces the activation of the c-Jun-N-terminal (JNK) kinase, and that phosphorylated c-Jun can bind with AP-1 sites to enhance the transcription of target genes (19). Thus, it is interesting to hypothesize that c-Jun, which is phosphorylated by JNK, may interact with AP-1 sites and increase the TREM-1 promoter activity without substantially altering the level of c-Jun level, although we did not confirm the phosphorylation of c-Jun in the present study.

The luciferase reporter assay indicated that SRE was involved in the VitD3-induced TREM-1 promoter activity. However, the VitD3-induced mRNA expression of TREM-1 was not affected by transfection with consensus SRF ODN decoys that bind with SRF. Thus, it may be speculated that transcription factors other than SRF may bind to the SRE in the TREM-1 promoter to regulate the VitD3-induced promoter activity, since the homology of the SRE (CCCTATGTGG) is at most 80% of the consensus SRE sequence CC(A/T)6GG, and the SRE is neighbored with the functional AP-1-2 site.

In a previous study, we revealed the transcriptional regulation of the mouse *TREM-1* gene using RAW264.7 macrophage-like cells (12). In another study, pairwise sequence alignment using the EMBOSS Stretcher Algorithm indicated only 47.4% homology between the 1.2-kbp human and mouse TREM-1 promoters (http://www.ebi.ac.uk/Tools/psa/emboss_stretcher/nucletide.html) (20). Our previous and present studies indicate that an NF-κB site (-743 to -730 in the mouse promoter) involved in the basal and LPS-induced transcription of the mouse *TREM-1* gene was not identified in the human TREM-1 promoter. By contrast, several AP-1 sites were identified in both mouse and human promoters: an AP-1 site (-907 to -898) was involved in the LPS-induced transcription of the mouse *TREM-1* gene, whereas AP-1-2 (-341 to -331) and AP-1-3 (-97 to -90) sites participated in the VitD3- and LPS-induced transcription of the human *TREM-1* gene. Furthermore, CRE was identified in both mouse and human promoters; however, it is not likely that CRE (-105 to -98) was involved in the transcription of the human *TREM-1* gene (Figs. 4 and 5), although CRE (-99 to -92) participated in the basal and LPS-induced transcription of the mouse *TREM-1* gene. Notably, the C/EBP-5 site (-31 to -18) was involved in the basal, and VitD3- and LPS-induced transcription of the human *TREM-1* gene; however, it is not likely that a C/EBP-like sequence (-38 to -25), which was identified in the mouse promoter, contributed to the transcription of the mouse *TREM-1* gene, since a -50 plasmid-retaining C/EBP sequence completely lost the basal and LPS-induced transcription of the mouse *TREM-1* gene. These observations suggest that the transcription of mouse and human *TREM-1* genes is differentially regulated by multiple *cis*-acting motifs and transcription factors, although members of the AP-1 family may play a role in the modulation of both genes.

In conclusion, the present study revealed the mechanisms responsible for the basal, and VitD3- and LPS-induced promoter activity of the human *TREM-1* gene, which encodes a novel inflammation-amplifying molecule. It is interesting to note that silencing or blocking TREM-1 represses cytokine production, thereby prolonging the survival of animal models with bacterial sepsis (5-8). Thus, the findings of the present study provide important insight into the modulation of TREM-1 expression, a therapeutic target in inflammation, which may assist in controlling inflammatory disorders, based on the regulatory mechanisms responsible for the transcription of the *TREM-1* gene in humans.

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