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TLR4 expression in mouse embryonic stem cells and in stem cell-derived vascular cells is regulated by epigenetic modifications ☆

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Abstract

Embryonic stem (ES) cells and ES cell-derived differentiated cells can be used in tissue regeneration approaches. However, inflammation may pose a major hurdle. To define the inflammatory response of ES and ES cell-derived vascular cells, we exposed these cells to LPS. With the exception of MIF no significant cytokine mRNA levels were observed either at baseline or after stimulation. Further experiments revealed that these cells do not express TLR4. Analysis of the DNA methylation status of the TLR4 upstream region showed increased methylation. Moreover, in vitro methylation suppressed TLR4 promoter activity in reporter gene assays. ChIP assays showed that in this region histones H3 and H4 are hypoacetylated in ES cells. Interestingly, 5-aza-dC or TSA partially relieves this gene repression. Finally, the increased levels of TLR4 observed in ES cells after treatment with 5-aza-dC or TSA confer responsiveness to LPS, as induction of IL-6 and TNFα mRNA was detected in endotoxin stimulated ES cells.

Keywords: Endotoxin challenge; DNA methylation; Histone hypoacetylation

Embryonic stem (ES) cells are totipotent cells derived from the inner cell mass of blastocysts. Their ability of unlimited self-renewal and differentiation to a variety of somatic cell types makes these cells an attractive target for new therapeutic approaches that involve tissue regeneration. In tissue engineering, the vasculogenic potential of human ES-derived endothelial cells has been explored. Differentiated ES cells grown on a biodegradable polymer scaffolds has been shown to form vascular networks within the engineered-tissue like structures [1–3]. ES cells have also been used in attempts to improve cardiac function. Cardiac committed-ES cells when transplanted into infarcted myocardium differentiate into cardiomyocytes and improve left ventricular function [4]. Transfusion of progenitor cells may improve organ function by various ways, e.g., improving neovascularization, inhibiting apoptosis, enhancing endogenous repair mechanisms or differentiating to specific cell types. Although it is still not known whether repopulation of the injured tissue is required or whether transient secretion of paracrine mediators by the transplanted cells can rescue organ function, stem cells represent a promising potential in cardiovascular, chronic and degenerative diseases [5,6].

Inflammation plays a central role in most of the pathological conditions where cell transplantation could be applied [7,8]. Complex interactions occur between inflammatory cells and the damaged tissue and expression of adhesion molecules, cytokines, matrix metalloproteinases and growth factors is extremely elevated. The use of stem cell-derived cells or progenitor cells that do not elicit an inflammatory response and thus do not further augment the damage in the injured area could have beneficial effects on the recovery of organ function. The study of the potential inflammatory response of the transplanted cells could provide valuable insights into the efficiency of such

☆ *Abbreviations:* LPS, lipopolysaccharide; SMC, smooth muscle cells; 5-aza-dC, 5-aza-2-deoxycytidine; TSA, trichostatin A; SAM, *S*-adenosylmethionine; ChIP, chromatin immunoprecipitation; AcH3, acetylated histone 3; AcH4, acetylated histone 4.

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therapeutic interventions. However, it is unknown whether ES and ES-derived cells have the ability to respond to inflammatory stimuli.

The innate immune system responds to infection through a panel of pattern recognition receptors (PRR) which recognize conserved sequences, such as bacterial lipoproteins and endotoxin (LPS) [9]. One of the main families of PRR is the family of Toll-like receptors (TLR). To date, 11 mammalian Toll homologues have been identified and designated, TLR1-11. Following ligation, TLRs signal through adapter molecules to activate the NF-κB pathway. This results in an immune response characterized by the production of cytokines, antimicrobial products and the regulation of costimulatory molecules. Interestingly, little is known about TLR expression and their functions in stem cells.

In the present study, we used the endotoxin model to investigate the inflammatory response of mouse ES, ES-derived endothelial cells (esEC) and ES-derived smooth muscle cells (esSMC). Our experiments showed that these cells do not upregulate cytokine expression and are unresponsive to LPS stimulation due to diminished levels of TLR4, the transmembrane receptor that recognizes LPS [10]. This repression of gene expression is mediated by epigenetic events that include methylation of the TLR4 promoter region and hypoacetylation of histones H3 and H4. These modifications have a profound effect on the promoter activity. Inhibition of DNA methylation or histone deacetylase activity can partially restore TLR4 expression in ES cells and confer responsiveness to endotoxin.

Methods

Cell culture. SMCs were isolated by enzymatic digestion of mouse aortas as described elsewhere [11] and were cultured in DMEM supplemented with 15% FCS, 2 mM L-glutamine and 100 mg/l gentamicin. Mouse ES cells (D3) were obtained from ATCC (Manassas, VA, USA) and grown on gelatin-coated flasks. Cell passages 3–15 were used for experiments. To maintain the ES cells in an undifferentiated state leukaemia inhibitory factor (LIF, 1000 U/mL) was added to the culture medium (DMEM, ATCC) supplemented with 10% FCS, 2 mM L-glutamine, 100 mg/l gentamicin and 10^{-4} M 2-mercaptoethanol (2-ME).

To obtain esEC cells, ES were plated on collagen IV slides and cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/l gentamicin and 5×10^{-5} M 2-ME for 4 days. The cells were subsequently subjected to shear stress at 12 dyn/cm^2 for 24 h. Expression of EC markers was detected by RT-PCR and confirmed by FACS.

To obtain esSMC, ES cells were cultured on type IV mouse collagen coated flasks in α-minimal essential medium (αMEM; Gibco), supplemented with 10% FCS, 2 mM L-glutamine, 100 mg/l gentamicin and 5×10^{-5} M 2-ME for 3 to 4 days. Sca-1$^+$ cells were sorted from the cell culture by magnetic labelling cell sorting (MACS) with anti-Sca-1 microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany). Sca-1$^+$ cells were resuspended and cultured in fresh ES cell growth medium. For SMC differentiation, Sca-1$^+$ cells were plated on collagen IV-coated dishes or flasks, and cultured in αMEM supplemented with 10% FCS and 5×10^{-5} M 2-ME and 10 ng/ml PDGF-BB (Sigma). esSMC passage 5 was used for all experiments. Expression of SMC markers was detected by RT-PCR and confirmed by FACS.

RNase protection assay (RPA). Total RNA was extracted using the Qiagen kit according to the manufacturer's instructions. To estimate the expression of cytokines, RNase protection assay was performed, using mCK2b and mCK5c multi-probe template sets (RiboQuant, Pharmingen, San Diego, CA) and [α-^{32}P]UTP (Amersham Biosciences) according to the manufacturer's recommendations. The "RNase-protected" fragments were purified and resolved on a 5% sequencing gel and autoradiographed. For quantification, signals for each sample of the blot were normalized to the housekeeping gene L32.

Reverse transcription-polymerase chain reaction. Total RNA was extracted using the Qiagen kit according to the manufacturer's instructions and any potential contaminating chromosomal DNA was digested using the DNA-free kit (Ambion). The procedure used for RT-PCR was similar to that described elsewhere [12]. In brief, 2 μg of RNA was converted to cDNA using Promega Reverse Transcription System (Promega, Madison, WI). cDNA products were amplified by PCR using gene-specific primers. The primers used were: TLR4 forward "GCT TTC ACC TCT GCC TTC AC," TLR4 reverse "AGG CGA TAC AAT TCC ACC TG," TNFα forward "AGC CCC CAG TCT GTA TCC TT," TNFα reverse "CTC CCT TTG CAG AAC TCA GG" IL-6 forward "CGA TGA TGC ACT TGC AGA AA," IL-6 reverse "GGA AAT TGG GGT AGG AAG GA," and GAPDH forward "CGG AGT CAA CGG ATT TGG TCG TAT" and GAPDH reverse "AGC CTT CTC CAT GGT GGT GAA GAC." PCR conditions were as follows: 94 °C for 3 min and then 40 cycles for TLR4, TNFα and IL-6 or 26 cycles for GAPDH at 94 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Real-time polymerase chain reaction experiments were performed with the Brilliant SYBR Green QPCR core reagent kit (Stratagene), using the Mx4000 (Stratagene) real-time thermocycler according to the company's instructions. Amplification was performed with 40 cycles and an annealing temperature of 58 °C. Copy numbers were calculated by the instrument's software from standard curves. The specificity of the amplification reaction was determined by a melting curve analysis. For quantification TLR4 and IL-6 and TNFα mRNA expression was normalized to the housekeeping gene GAPDH.

Western blot analysis. The procedure used was similar to that described previously [12]. Antibodies against TLR4 or β-actin were from Santa Cruz Biotechnology. Specific antibody–antigen complexes were detected by using the ECL Western Blot Detection Kit (Amersham Pharmacia Biotech, UK).

Plasmid construct. An expression vector harboring sequences of the mouse TLR4 promoter was created using genomic DNA from SMCs. A 0.7 kb fragment (−667/+29) from the TLR4 promoter was generated by PCR using TLR4 forward (−667) TCA GTC CTC GAG ACG AGC CTG CTC CTA TCT and TLR4 reverse (+29) TCA GTC AAG CTT GAA GTG AGA GTG CCA ACC primers. The PCR product was then digested with *Xho*I and *Hin*dIII (Promega), gel purified and cloned into the *Xho*I/*Hin*dIII site of the pGL3-basic vector to create plasmid pTLR4-Luc(−667). The construct was verified by sequencing.

Transient transfection. For transfection experiments, 5×10^4 cells per well were seeded in 12-well plates and left to adhere overnight. The cells were then transfected with the TLR4 expression and pCMV-β-galactosidase vectors using Fugene-6-Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. After 24 h, fresh medium was added to the transfected cells and the plates were returned to the incubator. In experiments where TSA was used, ES cells were exposed to 50 nM TSA for 24 h. The cells were then washed twice with ice-cold PBS and lysed in the Reporter Lysis Buffer (Promega, Madison, WI). The luciferase and the β-galactosidase activities were determined using luciferase and β-galactosidase enzyme assay systems, respectively (Promega, Madison, WI). The latter was used to calculate transfection efficiency in each experiment. At least three independent transfections were performed in triplicate.

In vitro DNA methylation. TLR4 promoter vectors were methylated with M.SssI methylase (New England Biolabs) in the presence of 160 μM SAM at 37 °C overnight. The completion of methylation was confirmed by resistance to *Bsa*AI digestion. Mock methylated controls were obtained by omitting the M.SssI methylase from the assays.

Region-specific methylation was performed by excision of the TLR4 promoter by *Xho*I and *Hin*dIII restriction digestion as described

previously [13]. The fragment was gel purified and in vitro methylated or mock methylated as described above. Methylated and mock methylated DNA was religated to the digested luciferase vector. Ligation reactions were purified and used directly for transfection experiments.

Analysis of the methylation status using methylation sensitive restriction enzymes. Genomic DNA (1 μg) isolated from cultured cells according to standard procedures [14] was digested with *Bce*AI or *Bsa*AI (New England Biolabs) for 2 h in a total reaction of 50 μl. The partially digested DNA was denatured for 5 min at 95 °C and fresh restriction enzyme was added. The samples were incubated overnight at 37 °C and the digested DNA was used for PCR amplification. Digestion with *Pst*I was used as a positive control. The primers used to amplify the TLR4 promoter were: TLR4 CL1 forward "ACG AGC CTG CTC CTA TCT TT" and TLR4 CL1 reverse "GGA GCA ACA TCC TCA TTC CT." PCR conditions were as follows: 94 °C for 3 min and then 35 cycles at 94 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Bisulfite modification. DNA bisulfite modification and purification was performed using the EZ-DNA Methylation kit (Zymo Research, Orange, CA) according to manufacturer's recommendations. The modified DNA was amplified using primers for bisulfite sequencing PCR that were designed using the program "Meth Primer: designing primers for Methylation PCRs." The primers used were: TLR4 BIS L "GAT GAT TTT TTG GGA TGA AAG TTA G" and TLR4 BIS R "CCC AAT AAA TAC CCC TCT ACA AAT AC." PCR conditions were as follows: 94 °C for 3 min and then 35 cycles at 94 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min. PCR products were gel purified and cloned into pGEM-T vector (Promega, Madison, WI) according to the company's instructions and 20 colonies were purified as minipreps and screened for correct insertion. In each case, 10 independent clones were sequenced. Additionally, the methylation status was evaluated by digestions of the PCR products using *Acl*I, *Hpy*99I and *Sau*3AI (New England Biolabs). Digestion products were separated by a 3% agarose gel electrophoresis and visualized by ethidium bromide staining.

Chromatin immunoprecipitation (ChIP). The ChIP assays were performed as described previously [15] with minor modifications. In brief, cells were treated with 1% (v/v) formaldehyde at room temperature for 10 min and then quenched with glycine at room temperature. The medium was removed, and cells were harvested for sonication. The sheared samples were diluted into 1 ml immunoprecipitation buffer containing 25 mM Tris–HCl, pH 7.2, 0.1% NP-40, 150 mM NaCl, and 1 mM EDTA, and immunoprecipitation was conducted with rabbit anti-acetyl-histone H4, anti-acetyl-histone H3, together with single-strand salmon sperm DNA saturated with Protein G-Sepharose beads. Normal IgG was used as a control. Immunoprecipitates were pelleted by centrifugation. The immunoprecipitates were eluted from the beads using 100 μl elution buffer (50 mM $NaHCO_3$, 1% SDS). A total of 200 μl proteinase K solution was added to a total elution volume of 300 μl and incubated at 60 °C O/N. DNA was extracted, purified, and then used to amplify target sequences by PCR. The primers used to amplify the TLR4 promoter were Part 1: ChIP1 TLR4 forward "GAA CTG CAG AAG GCA CTC AA," ChIP1 TLR4 reverse "ATC AGT TGC CGT GTC TTG TG" and Part 2: ChIP2 TLR4 forward "CCA GCT TCC TCT TGC TGT TC" and ChIP2 TLR4 reverse "GGA AGT GAG AGT GCC AAC CT." Aliquots of chromatin were also analysed before immunoprecipitation and served as an input control.

Statistics. Statistical analyses were performed by one-way ANOVA, and $p < 0.05$ was considered statistically significant.

Results

ES cells, esEC and esSMC do not upregulate cytokine expression after endotoxin challenge

LPS treatment represents a strong proinflammatory stimulus and most cell types respond by inducing elevated levels of cytokines. In order to define the inflammatory response of embryonic stem cells (ES), endothelial (esEC) and smooth muscle cells (esSMC) derived from ES cells, we treated these cells with LPS and analysed the mRNA levels of various cytokines using RNase protection assay (RPA). Mature SMC were used as a positive control (Figs. 1A and B). Surprisingly, except MIF no significant cytokine mRNA levels were observed in the three cell lines even after 2 h treatment with an extremely high concentration of LPS (Figs. 1A–F). Very low levels of cytokines were detected after prolonged exposure of the film but no cytokine upregulation in response to LPS was identified. On the contrary, sca-1^+ adult stem cells isolated from the adventitia showed significant levels of cytokine mRNA at baseline and a marked increase in cytokine expression after endotoxin challenge (data not shown). Interestingly, stem cells in the absence of LIF expressed elevated levels of IP-10 that were not affected by the LPS treatment (Figs. 1D and F).

Embryonic stem cells, esEC and esSMC do not express TLR4

TLR4 functions as the signal transducing receptor for LPS, although recognition of LPS is complex and requires several accessory molecules [9,16]. Previous studies demonstrated that macrophages and B cells from TLR4-deficient mice did not respond to LPS. In order to determine whether ES cells are unresponsive to LPS due to low abundance of its receptor, TLR4 mRNA and protein levels were studied. As shown in Figs. 2A and B, expression of TLR4 mRNA and protein was diminished in ES cells. Similarly, TLR4 mRNA levels were undetectable in esEC and esSMC (Figs. 2C and D).

Previous studies have shown that the 700 bp fragment upstream of the transcription initiation site in the TLR4 gene possesses strong promoter activity [17]. We performed reporter gene assays using this fragment cloned into pGL3 basic vector and found comparable levels of luciferase activity in both ES and mature SMCs (Fig. 2E). These results indicate that although ES cells do not express TLR4 mRNA or protein they still maintain all the transcriptional machinery necessary to drive transcription from TLR4 promoter.

TLR4 promoter is methylated in ES, esEC and esSMC

Reversible methylation of cytosine is a major epigenetic modification in multicellular organisms. In mammals, cytosine methylation occurs almost exclusively at CpG dinucleotides. DNA methylation has previously been shown to mediate repression of transcription in vivo [18–21].

To explore the possibility that DNA methylation of the TLR4 promoter inhibits its expression in ES, esEC and esSMC, we did a sequence analysis of 5′ flanking region. Two regions enriched in CpG dinucleotides, Part 1 and Part 2 (Fig. 3A), were identified. In order to study

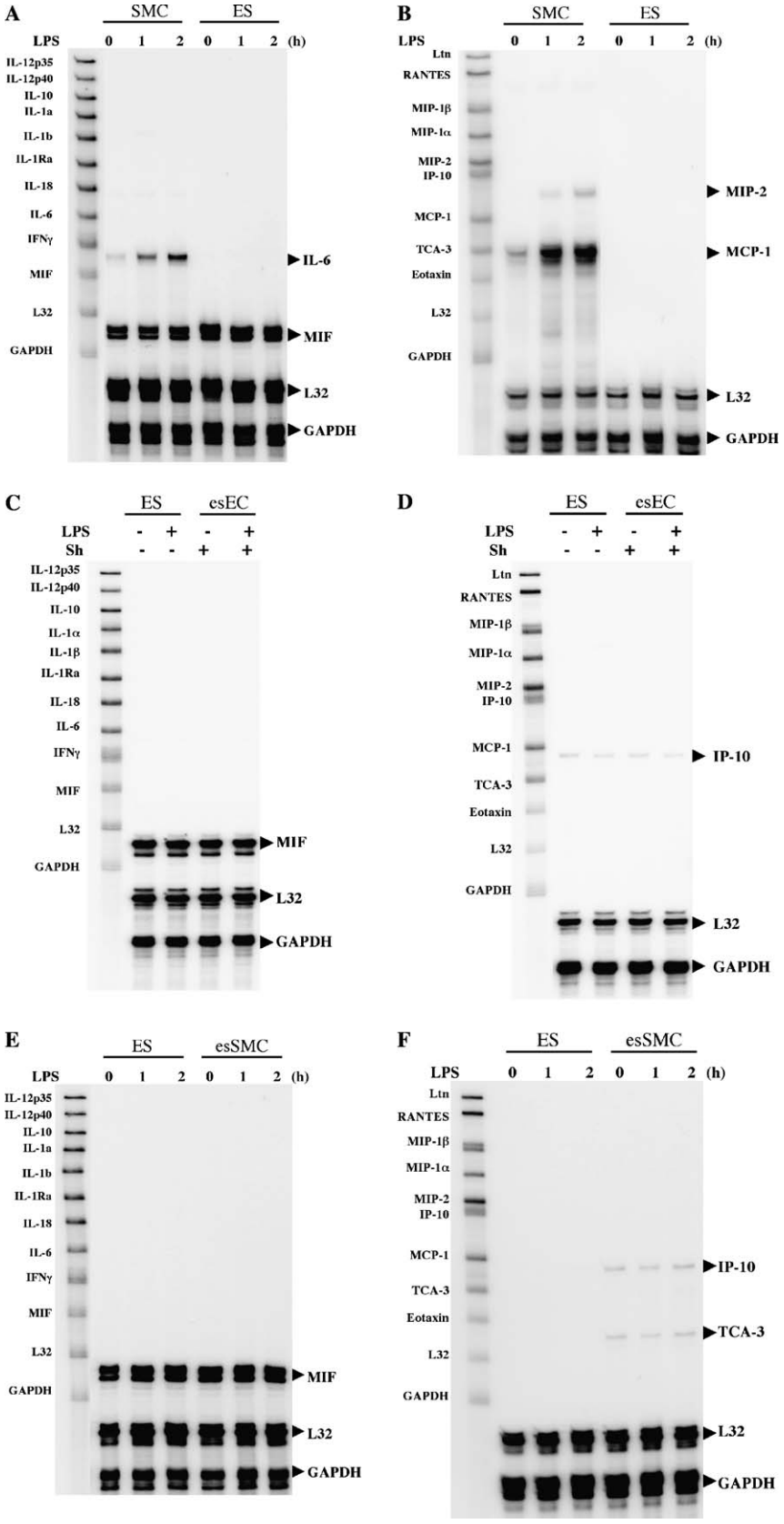


Fig. 1. ES, esEC and esSMC are unresponsive to LPS. (A–F) Cytokine expression after endotoxin challenge. Total RNA was isolated from cells after LPS treatment (10 μg/ml) for the indicated times. Samples were analysed by RPA. L32, a ribosomal protein mRNA was used as a loading control. Sh—shear stress.

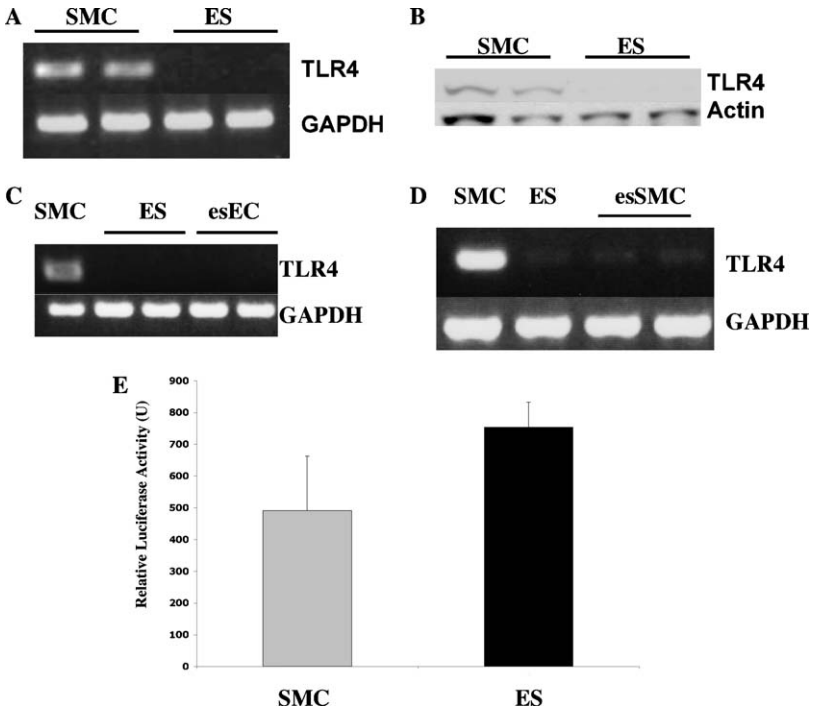


Fig. 2. ES, esEC and esSMC do not express TLR4. (A) TLR4 mRNA levels in ES cells as assessed by RT-PCR. (B) Western blot analysis of TLR4 protein expression in ES cells. Results were normalized to β-actin. (C,D) TLR4 mRNA levels in esEC and esSMCs as assessed by RT-PCR. Data from RT-PCR were normalized to GAPDH mRNA expression. Mature SMCs were used as a positive control. (E) SMC and ES cells were transfected with the mouse TLR4 promoter construct pTLR4-Luc(−667). Luciferase activity was normalized to the expression of co-transfected pCMV-β-galactosidase control plasmid. Values are means ± SD. Samples were run in triplicate. Data presented are the average of at least three independent experiments.

the methylation state, bisulfite modification of isolated genomic DNA from the three embryonic cell types and the mature SMCs and PCR amplification of Part 1 and Part 2 were performed. Despite repeated attempts, complete bisulfite modification of Part 1 could not be achieved and thus these results were not analysed further. However, in Part 1 two sites of methylation sensitive restriction enzymes, *Bce*AI and *Bsa*AI, were identified (Fig. 3B). To obtain information about the methylation state of these two CpGs we digested unmodified genomic DNA with the above restriction enzymes and performed a PCR using primers that would generate a product only if digestion is inhibited by methylation. Our experiments showed that the *Bsa*AI site is methylated in the ES and in ES-derived cells, while the *Bce*AI site is methylated in esEC and esSMCs and partially methylated in ES cells (Fig. 3C). Both sites were unmethylated in mature SMCs (Fig. 3C).

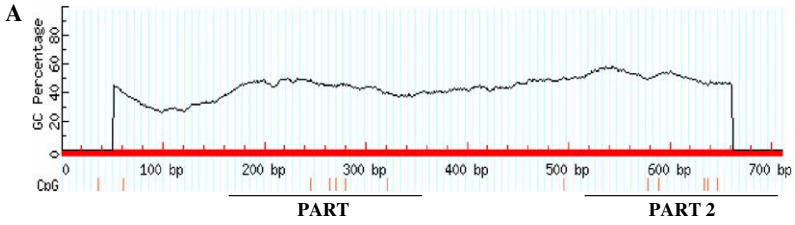
On the other hand, genomic DNA corresponding to Part 2 could be completely modified by Bisulfite. Sequencing of the PCR products from this part revealed that in mature SMC where TLR4 expression is detected, the promoter is completely unmethylated, whereas in ES, esEC and esSMC that do not express TLR4, significantly higher methylation levels occur (Fig. 3D).

Evaluation of the methylation status was also performed using restriction digestions of bisulfite PCR products. Bisulfite modification converts unmethylated cytosines to uracils and as a result bisulfite PCR fragments from the unmethylated TLR4 promoter are resistant to digestion with *Acl*I, *Hpy*99I and *Sau*3AI, while the above enzymes can digest PCR products from the methylated TLR4 promoter (Fig. 3E). As shown in Fig. 3F, contrary to the bisulfite PCR fragments from mature SMCs that are unmethylated and thus cannot be digested, PCR fragments from ES cell types can be partially or totally digested, depending on their degree of methylation.

In vitro methylation of the promoter region induces repression of the TLR4 promoter activity

To determine the contribution of the TLR4 promoter methylation to the downregulation of TLR4 gene expression, in vitro methylation of promoter construct pTLR4-Luc(−667) was performed using M.SssI methylase. Reporter gene assays using a methylated vector revealed a dramatic inhibition of promoter activity in both ES and mature SMC (Fig. 4A). Similar results were obtained with methylated or mock methylated TLR4 promoter in the absence of reporter methylation (Fig. 4B).

To further verify the role of methylation in TLR4 expression, ES cells were treated with 5-aza-deoxycytidine, a DNA methylation inhibitor. Significant increase in the TLR4 mRNA levels as estimated by RT-PCR analysis was identified after treatment of ES cells with various



B **PART 1**

(-539)

TTGAGAACTGAGAACTGCAGAAGGCACTCAATAGTGAACTCTGGCTTTTACACACACA

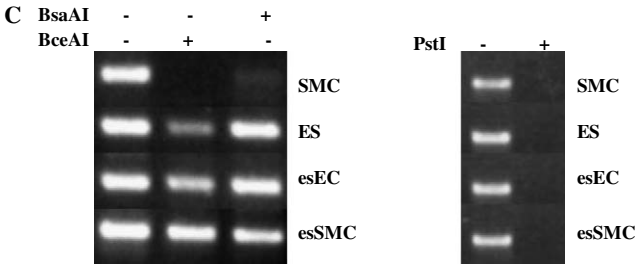
CACACACACACACACACACACACACACACACACACACACACACACACACACACACACACG

AAATATACATCCCCCCCGTGAACGAATGAACACGTACACACATAGGTAAAAGAAAGCA

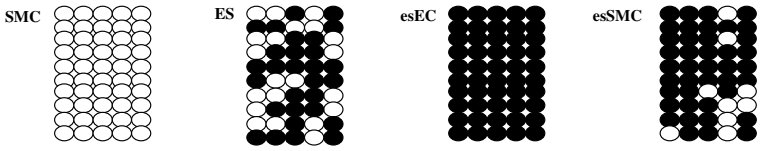
BsaAI

TCATGACACAAGACACGGCAACTGATGATATCTTCATCCTGGGTTT (-312)

BceAI



D **PART 2**



● Methylated ○ Unmethylated

E

(-131)

UNMODIFIED TGGGATGAAAGCCAGCTTCCTCTTGCTGTTCCTCCAGTCGGTCAGCAAACGCCTTCTTCC

MODIFIED TGGGATGAAAGTTAGTTTTTTTTTTGTTGTTTTTTTAGTCGGTTAGTAAACGTTTTTTTTT

AclI

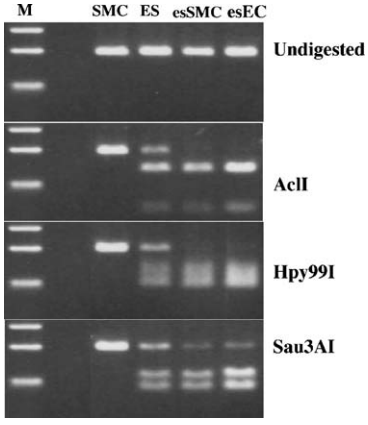
(-11)

UNMODIFIED TGTTCTAGTCTTCAGTCTTCTAACTTCCCTCCTGCGACGGGGCAGATCGATTCTAGAACA

MODIFIED TGTTTTAGTTTTTAGTTTTTTAATTTTTTTTTTGCGACGGGGTAGATCGATTTTAGAATA

Hpy99I **Sau3AI**

F



concentrations of the reagent (Fig. 4C). This upregulation was not affected by inhibition of protein synthesis (data not shown).

In ES cells TLR4 promoter methylation is associated with histone hypoacetylation

DNA methylation has been reported to affect chromatin structure, by altering the acetylation status of histones [22]. In order to see if modifications of the histone tails in the TLR4 promoter differ between ES cells and mature cells, we performed chromatin immunoprecipitation assays using antibodies against acetylated H3 and acetylated H4. As shown in Figs. 5A and B, ES cells exhibit lower levels of acetylation in both H3 and H4 compared to mature SMCs in both Part 1 and Part 2 of the TLR4 promoter.

Moreover, acetylation of core histones is known to augment promoter activity by enhancing transcription factor access to the chromatin template and increasing accessibility of nucleosomal DNA [23]. Aiming to elaborate on the role of histone acetylation on TLR4 promoter activity we used a deacetylase inhibitor, trichostatin A (TSA). Reporter gene assays performed using ES cells exposed to TSA revealed that activity of the methylated TLR4 promoter could be partially restored by inhibiting deacetylase activity (Fig. 5C). Increased transcription of TLR4 gene was also observed at the mRNA level by RT-PCR using various concentrations of TSA (Fig. 5D). Interestingly, combined treatment of ES cells with the demethylating agent 5-aza-dC and TSA resulted in even higher levels of transcription (Fig. 5E). Additional quantitative real-time PCR analysis confirmed that the maximum TLR4 mRNA levels occur when DNA methylation and histone deacetylation are inhibited (Fig. 5F). However, it is noteworthy that the detected TLR4 mRNA in ES cells is still significantly lower than that observed in mature SMCs, indicating that besides DNA methylation and histone acetylation, additional mechanisms are involved in the regulation of TLR4 gene.

Restoration of responsiveness to LPS following inhibition of DNA methylation or inhibition of deacetylase activity in ES cells

To test whether upregulation of TLR4 expression in ES cells was sufficient to confer LPS responsiveness, we assessed TNFα and IL-6 mRNA levels after endotoxin treatment using quantitative real-time PCR. More than 2-fold higher levels of cytokine mRNA were detected in stimulated cells that have been previously treated with the DNA demethylation reagent or the inhibitor of deacetylase activity (Figs. 6A and B).

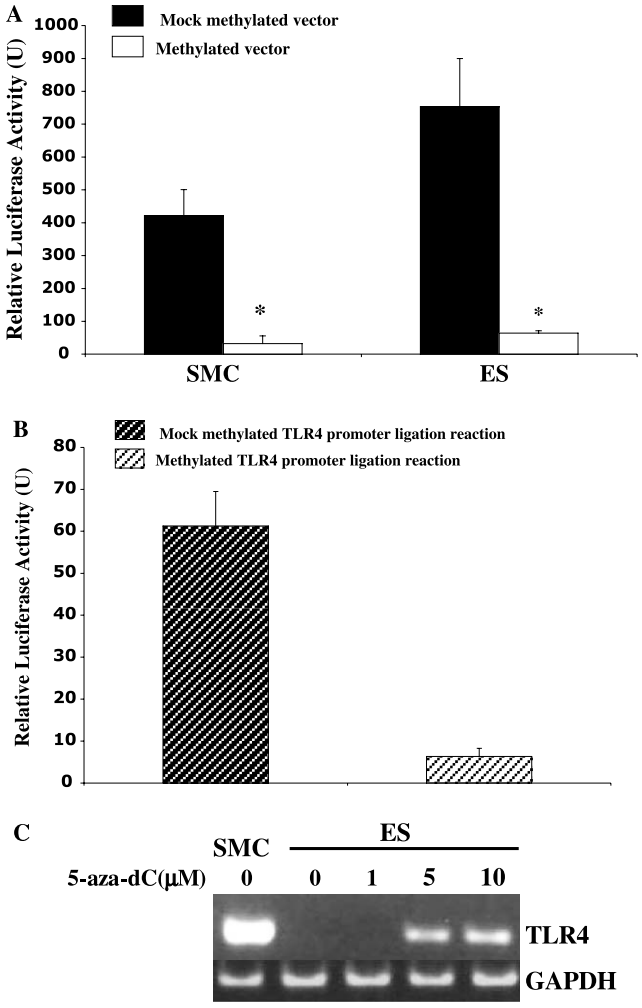


Fig. 4. In vitro methylation affects TLR4 promoter activity. (A) TLR4 promoter cloned into a luciferase vector was in vitro methylated using M.SssI methylase and transfected to ES and SMCs. (B) TLR4 promoter was either methylated or mock methylated and ligated into a luciferase vector. Ligation reactions were purified and used for transfection. Luciferase activity was normalized to the expression of co-transfected pCMV-β-galactosidase control plasmid. Values are means ± SD. Samples were run in triplicate. Data presented are the sum of at least three independent experiments. *Significant difference from unmethylated vector, $p < 0.05$. (C) TLR4 mRNA levels were assessed by RT-PCR in ES cells after treatment with various concentrations of the methylation inhibitor 5-aza-dC. Data were normalized to GAPDH mRNA expression.

Fig. 3. TLR4 promoter is methylated in ES cells, esEC and esSMC. (A) A schematic diagram of the TLR4 upstream region. CpG dinucleotides are depicted as black bars below the promoter. (B) Methylation sensitive restriction enzyme sites on Part 1. (C) Analysis of the methylation state using *Bce*AI and *Bsa*AI restriction enzymes. Undigested genomic DNA served as a positive control. Digestion with *Pst*I was used as a positive digestion control. (D) DNA methylation profile of the individual CpG elements at Part 2 in TLR4 5′ flanking region in SMCs, ES, esEC and esSMC, as identified by bisulfite sequence analysis of 10 separate clones. Unmethylated CpGs are represented with open circles and methylated CpGs with closed circles. (E) Sequence of bisulfite modified Part 2 assuming all CpGs are methylated. Restriction enzymes sites of *Acl*I, *Hpy*99I and *Sau*3AI are only present on the methylated TLR4 promoter after bisulfite modification (F) PCR products from bisulfite modified genomic DNA were digested with the above restriction enzymes. Only PCR fragments from unmethylated DNA are resistant to digestion.

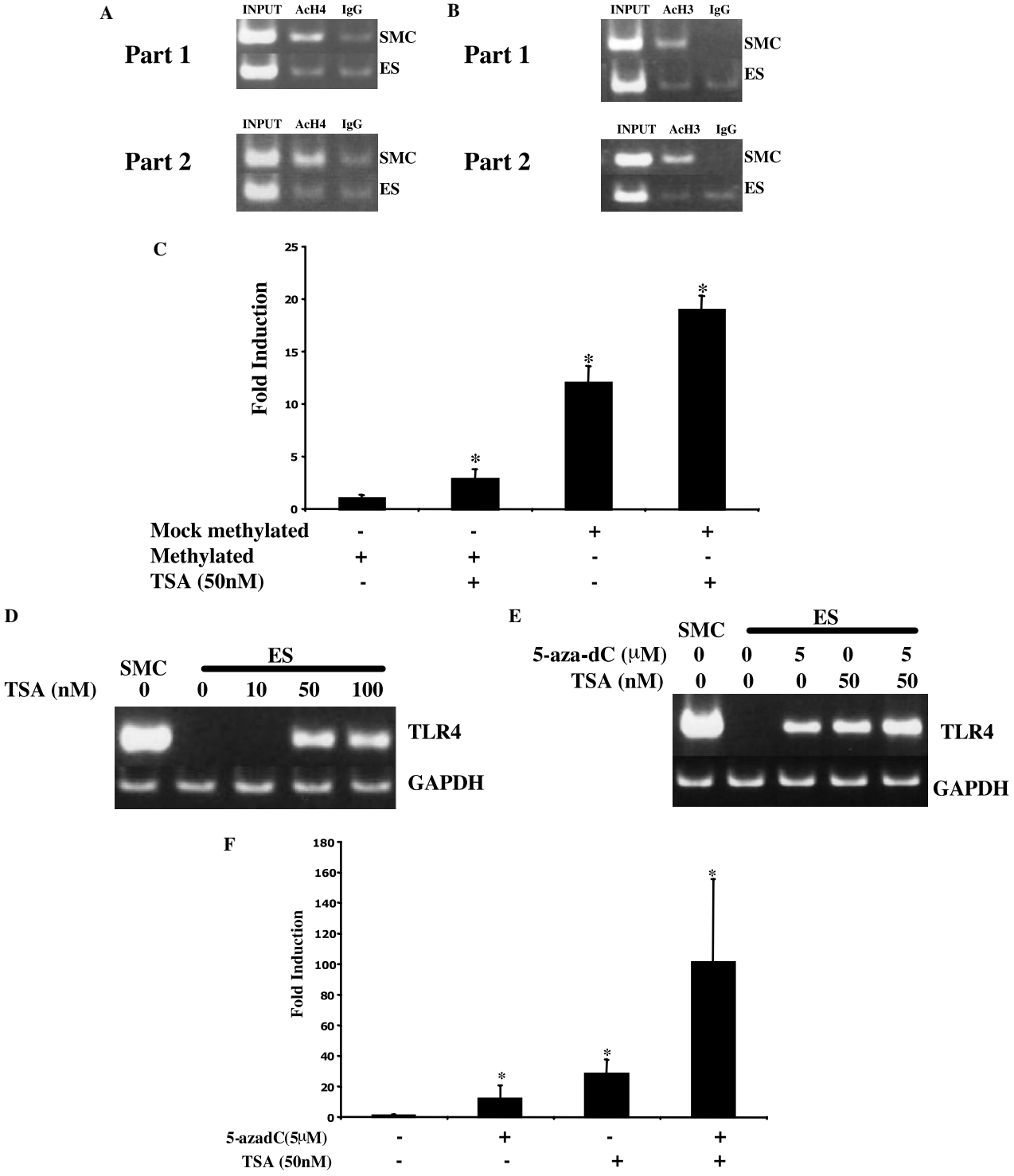


Fig. 5. Acetylation of H3 and H4 is involved in TLR4 promoter activity. Chromatin immunoprecipitation assays were performed using antibodies against (A) AcH4 and (B) AcH3. Normal rabbit IgG was used as a negative control. Aliquots of chromatin before immunoprecipitation served as an input control. (C) Inhibition of histone deacetylase activity can upregulate TLR4 promoter activity. ES cells were transfected with a reporter construct that was either in vitro methylated with M.SssI or unmethylated. After 24 h of treatment with 50 nM TSA, the luciferase activity was assessed. Results were normalized to the expression of β-galactosidase. Values are means ± SD. Samples were run in triplicate. Data presented are the sum of at least three independent experiments. *Significant difference from methylated vector, $p < 0.05$. (D) mRNA levels of TLR4 in ES cells treated with various concentrations of TSA for 24 h as assessed by RT-PCR. (E) mRNA levels of TLR4 after combined treatment of ES cells with TSA and 5-aza-dC. Data from RT-PCR were normalized to GAPDH mRNA expression. Mature SMCs were used as a positive control. (F) Quantitative real-time PCR of TLR4 mRNA levels in ES cells treated with various inhibitors. Data were normalized to GAPDH mRNA expression. Values are means ± SD. *Significant difference from untreated cells, $p < 0.05$.

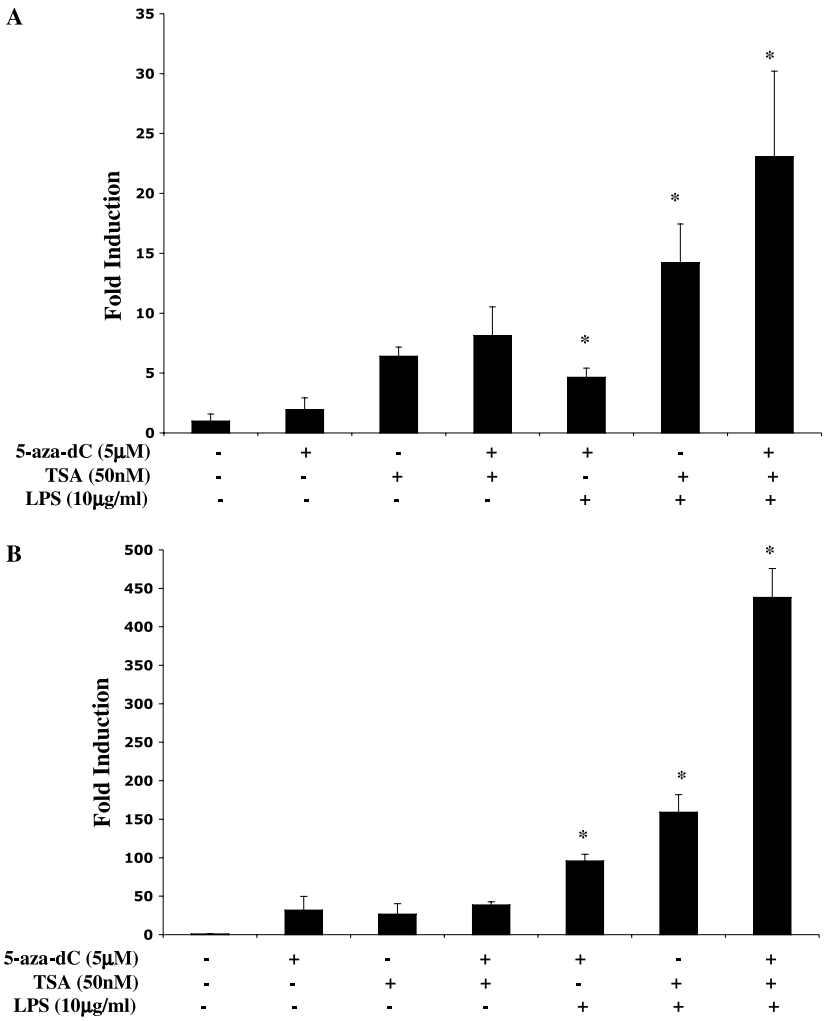


Fig. 6. Increased levels of TNFα and IL-6 after LPS stimulation of ES cells treated with 5-aza-dC or TSA. Quantitative real-time PCR of TNFα and IL-6 mRNA levels in ES cells incubated with the above inhibitors and then stimulated with LPS (10 μg/ml) for 1 h or left untreated. Data were normalized to GAPDH mRNA expression. Values are means ± SD. *Significant difference from the unstimulated sample incubated with the same inhibitor, $p < 0.05$.

Discussion

A critical component of host defences against microbes is the ability to recognize and respond to bacterial products, e.g., LPS. Recent studies have established the central role of TLRs in inflammatory responses and cardiovascular diseases [9]. Mutations that attenuate receptor signalling and diminish vascular inflammation influence the progress of various chronic diseases [24,25]. Among TLRs, TLR4 has been extensively studied and shown to play an important role in vascular disease development. Asp299Gly TLR4 polymorphism is associated with decreased risk of atherosclerosis and coronary artery disease and it also seems to be involved in the progression of chronic disease like Alzheimer and diabetic neuropathy. Additionally, increased levels of TLR4 correlate with allograft endothelial dysfunction in patients receiving heart transplant [26,27].

In the present report, we demonstrated for the first time that mouse ES cells and esEC and esSMC do not upregulate cytokine expression after endotoxin challenge due to diminished levels of TLR4. This repression of TLR4 seems to be a specific event rather than part of a global suppression of TLR expression, as mRNA from other members of the Toll-like receptor family was detected in ES cells (data not shown). Our results indicate that the immature TLR4 signalling in embryonic stem cells and stem cell-derived vascular cells could influence the inflammatory responses in vivo and might have an impact on the application of stem cell-based therapy. Previous studies have shown that transplantation of ES cells into the ischemic myocardium can elicit a vigorous inflammatory response [28], while injection of ES cells to infarcted myocardium does not lead to inflammatory cell infiltration [4]. Further experiments using in vivo animal models are required in order to clarify the potential of ES and ES-derived cells in such therapeutic interventions.

Suppression of TLR4 gene in mouse embryonic stem cells is mediated at least in part by epigenetic modifications. Increased DNA methylation was found in all three embryonic cell types. In contrast to the unmethylated TLR4 promoter observed in mature smooth muscle cells (SMC),

almost complete methylation of the CpG dinucleotides was detected in the stem cell-derived vascular cells. These methylation levels were even higher than those detected in the undifferentiated ES cells supporting the dynamic nature of the methylation status in early development that occurs by de novo methylation and active demethylation as indicated in previous studies [29,30].

Reporter gene assays using an in vitro methylated pTLR4-Luc(−667) vector revealed that this epigenetic modification sharply reduces TLR4 promoter activity. Additional evidence for the functional relationship between DNA methylation and transcription came from experiments using the DNA methylation inhibitor 5-aza-dC. ES cells treated with the inhibitor displayed elevated TLR4 mRNA levels.

DNA methylation is believed to repress transcriptional activity at least in part by promoting the formation of an inactive chromatin structure. Previous studies suggest that this process is mediated by methyl-specific binding proteins that recruit enzymes capable of altering histone modifications such as histone deacetylases [19,20,31]. Removal of the charge-neutralizing acetyl group from the histone lysine tails by histone deacetylases leads to compaction of the chromatin structure and results in repression of gene transcription.

Our data support the involvement of histone deacetylase activity in the suppression of the transcriptional activity of the TLR4 promoter. Chromatin immunoprecipitation assays show hypoacetylation of histones H3 and H4 in TLR4 upstream region in mouse stem cells. Moreover, inhibition of histone deacetylase activity using trichostatin A (TSA) partially relieves transcriptional repression as shown by reporter gene assays and significant levels of TLR4 mRNA are detected in ES cells treated with this reagent.

Combined inhibition of DNA methylation and histone deacetylase activity leads to a robust induction of TLR4 but the expression levels are still dramatically low compared to mature SMCs. These data suggest that even though recruitment of histone deacetylases is an important step, other mechanisms that augment DNA methylation-dependent repression of TLR4 gene are in place. Methyl-CpG-binding proteins that are recruited to DNA methylated regulatory regions can alter chromatin structure in various ways. Recent papers linking methyl-CpG-binding proteins with histone methylation [22,32] may provide a possible mechanism for the observed TLR4 gene repression. Further experiments will verify whether DNA methylation coincides with histone methylation on the TLR4 promoter in ES cells.

Interestingly, the TLR4 upregulation observed after treatment with 5-aza-dC or TSA is sufficient to confer LPS responsiveness in ES cells. Stimulated ES cells exhibit significantly elevated levels of TNFα and IL-6 mRNA, indicating that the induced TLR4 is functional. The detected cytokine expression, though, is relatively low compared to mature SMCs. However, TLR4 signalling involves accessory molecules such as MD2, a secreted protein that is required for cell surface expression of TLR4 [16]. MD2 is expressed in ES cells but its levels are affected by the treatment with the above reagents (data not shown), suggesting that cytokine induction as a response to LPS is a more complex process.

Overall, we have shown that mouse embryonic stem cells and in vitro derived vascular cells are unresponsive to LPS due to low levels of TLR4. Epigenetic modifications, DNA methylation and histone deacetylation, on the TLR4 promoter contribute to this downregulation and inhibition of these alterations results in enhanced levels of TLR4 that can confer responsiveness to endotoxin. Our findings provide basic information for understanding the mechanisms of the repressed inflammatory response by embryonic stem cells and their in vitro derived vascular cells, which could be an important issue in stem cell-based therapy.

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