Histol Histopathol (2010) 25: 831-840

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Synthetic oligodeoxynucleotides induce MAP kinases activation in murine TIB-73 hepatocytes

Elena Martín-Orozco, Antonio Chicano, Antonio J. Ruiz-Alcaraz, Ana Lizana, María Martínez-Esparza, Trinidad Hernández-Caselles and Pilar García-Peñarrubia

Department of Biochemistry and Molecular Biology B and Immunology, School of Medicine, University of Murcia, Murcia, Spain

Summary. Aims: In this work we aimed to investigate the expression of TLR9 protein in the murine hepatocyte cell line TIB-73, compared to macrophage-like J774 cells, by Western blot analysis, and the role played by ERK 1/2 MAP kinase in the intracellular signals triggered by stimulation with CpG and non-CpG phosphodiester-ODN, and their more stable phosphorothioate-modified analogues. Results: TIB-73 hepatocytes express TLR9 protein. CpG and non-CpG ODN stimulation activated ERK 1/2 MAPK signal pathway in both hepatocytes and J774 murine macrophages. As expected, their phosphorothioate-CpG and non-CpG ODN analogues induced higher levels of ERK1/2 phosphorylation in TIB-73 cells, even higher than that induced in J774 cells under the same conditions. Phosphorylation of ERK 1/2 induced by synthetic ODN is dose-response dependent, being maximal at 100 μg/ml. Pretreatment of hepatocytes with an inhibitor of MEK-1 abrogated phosphorylation of ERK1/2 kinase. Conclusions: TIB-73 hepatocytes constitutively express TLR9 and respond to synthetic ODN stimulation through a high ERK1/2 phosphorylation independent of CpG motifs. Slight differences were found on ERK1/2 activation when using phosphorothioate *versus* phosphodiester oligonucleotides.

Key words: Hepatocytes, TLR9, ISS-ODN, ERK1/2

Offprint requests to: Pilar García-Peñarrubia, Department of Biochemistry and Molecular Biology B and Immunology, School of Medicine, University of Murcia, 30100 Murcia, Spain. e-mail: pigarcia@um.es

Introduction

The liver is a primary site of response to systemic infections. Uptake and clearance of pathogenic microorganisms and toxic products in the liver may be an important effector mechanism in host defences (Weiler-Normann and Rehermann, 2004).

Bacterial DNA act as an alert signal for eukaryotic cells through immunostimulatory unmethylated CpG sequences (Roman et al., 1997; Ishii and Akira, 2006). Current interest in the study of the response to immunostimulatory ODN arises from several distinct biological applications; namely, gene therapy, DNA vaccination, as vaccine adjuvants, antiallergens, and for the treatment of infectious diseases and cancer (Roman et al., 1997; Ishii and Akira, 2006). The active molecules are native phosphodiester DNA. Phosphodiester oligodeoxynucleotides (PD-ODN) containing CpG motifs mimic the direct effects of native bacterial DNA in vitro, including activation of macrophages, dendritic cells, and B lymphocytes (Roman et al., 1997; Akira and Takeda, 2004; Ishii and Akira, 2006). Most studies of CpG DNA action, as well as clinical applications of CpG DNA in immunotherapy and antisense therapy, now use phosphorothioate-stabilized ODN (PS-ODN), which has oxygen substituted for a non-bridging sulfur in the DNA backbone (Roman et al., 1997; Sester et al., 2000; Akira and Takeda, 2004; Ishii and Akira, 2006). PS-ODNs have an activity similar to that of bacterial DNA in macrophages *in vitro* and are potent immunomodulators *in vivo*. The superior potency of PS-ODN in these assays, and *in vivo* immunomodulation, has been attributed not only to their greater nuclease stability, but also to more efficient cellular uptake of PS-ODN (Sester et al., 2000).

These DNA sequences are recognized by an endosomal protein belonging to the Toll-like receptor

family, named TLR9 (Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006). All members of the TLR family encode extracellular regions containing repeating leucine-rich motifs and intracellular regions homologous to that of the IL-1 receptor family proteins (Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006). TLR proteins recognize bacterial products and activate transcription factors and protein kinases, such as NF-κB and mitogen activated protein kinases (MAPK) (Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006). We have previously described that murine hepatocyte cell lines, TIB-73 and AML-12 incubated in the presence of synthetic ODN, respond to it by increasing the pool of intracellular peroxides and inducing Mn-SOD mRNA. This resulted in inhibition of *Salmonella typhimurium* intracellular growth when infected cells were stimulated by synthetic ODN (Sanchez-Campillo et al., 2004). We also showed that hepatocyte cell lines and murine liver constitutively express TLR9 mRNA, which is downregulated by LPS and the mix of IFNγ, IL-1ß and LPS (Sanchez-Campillo et al., 2004). These results reinforced other studies attributing to hepatocytes a role as a microbial product-responsive cell (Volpes et al., 1992; Gregory and Wing 1993; Szalay et al., 1995; Tanikawa et al., 1998; Lajarín et al., 1999; Liu et al., 1998, 2000, 2002; Vodovotz et al., 2001; Tian et al., 2007), and showed additional evidence of the capabilities of immunostimulatory-ODN (ISS-ODN) sequences as potent inducers of the innate immune response through the activation of a broad range of cell types (Roman et al., 1997; Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006). Herein, we have extended our previous work by studying the implication of extracellular signal-regulated kinase, ERK 1/2 kinase in the intracellular signals triggered in the TIB-73 mouse hepatocyte cell line, by CpG and non-CpG ODN stimulation, leading to the increase of intracellular pool of peroxides and the inhibition of intracellular growth of *Salmonella typhimurium*. We have also studied the constitutive TLR9 protein expression in TIB-73 cells compared to the murine macrophage-like cell line, J774. Our results showed that murine hepatocytes constitutively express TLR9 and respond to ODN stimulation by an intense phosphorylation signal of ERK 1/2 MAPK pathway. Slight differences were found on ERK1/2 activation when using phosphorothioate *versus* phosphodiester oligonucleotides.

Materials and methods

Cells and reagents

The murine cell line TIB-73 (from BALB/c mouse, embryonic) has been described as a hepatocyte cell line (Sanchez-Campillo et al., 2004). In addition, TIB-73 showed hepatocyte morphology and produced aldolase B (hepatic) (0.16 U/mg of protein), α-fetoprotein (3.6 ng/mg of protein), ferritin (0.02 mg/mg of protein), γ-glutamyltransferase (GT) (0.11 U/mg of protein) as well as other less specific markers like glutamate-oxalacetate-transaminase (GOT) (1.5 U/mg of protein) and glutamate-pyruvate-transaminase (GPT) (0.25 U/mg of protein). J774 cells are murine (BALB/c) macrophage-like cells. Both cell lines were obtained from the American Type Cultures Collection (Rockville, MD). All cell lines used on our study have been tested for mycoplasma contamination (Mykoplasmennachweis mit DNS-Färbetest Biochrom, Germany).

Cells were cultured in disposable 24-well plates or flasks from Costar (Cambridge, MA). Cells were grown and maintained in Iscove's modified DMEM with Glutamax-I from GibcoBRL (Carlsbad, CA, USA), supplemented with 10% heat-inactivated (30 min, 56ºC) FCS and 100 units/ml penicillin, and 100 mg/ml streptomycin (Gibco).

IFNγ was purchased from Genzyme, (Cambridge, MA), and human recombinant IL-1ß was purchased from DuPont, Co., (Wilmington, DE). LPS (from *S. typhimurium*) was purchased from Sigma Chemical Co., (St. Louis, MO). PD98059, a specific inhibitor of ERK kinase (MEK1) was purchased from Sigma. The following antibodies were used in our study: anti-ERK1/2 pAb, anti-phosphoThr/Tyr ERK1/2 pAb (Cell Signaling Technologies, Danvers, MA, USA), anti-TLR9 mAb (Hycult biotechnology, Uden, The Netherlands), and HRP-conjugated anti-mouse and anti-rabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

We used the following synthetic ODNs: CpG 1018 (CpG-B ODN): 5'-TGACTGTGAACGTTCGAGAT GA-3' and non CpG 1040 (CpG-B control): 5'-TGACTGTGAAGGTTAGAGATGA-3'. These synthetic ODNs were obtained from Tri-Link Biotech (San Diego, CA, USA). Both ODNs were used as PD and PS backbone linkages.

Immunofluorescence staining and confocal microscopy

TIB-73 hepatocytes and J774 cells were plated and cultured on 3-aminopropyltriethoxysilane (TESPA) (Sigma)-coated round glass slides in 24 well dishes. For TLR9 staining, the cells were permeabilized previously by incubating them for 10 minutes with saponin 0.1% in PBS.

Next, cells were treated with the corresponding primary antibody (TLR9 at 10 μg/ml) or isotype-matched antibody control for 20 min at room temperature, washed three times, stained with Alexa Fluor 568 goat anti-mouse secondary antibody at 10 μg/ml for 20 min at room temperature, washed in cold medium and fixed with paraformaldehyde 2% for 10 min. The coverslips were then washed once and mounted with Dako Fluorescent Mounting Medium (Dako, Carpinteria, CA). Cells were imaged by laser-scanning confocal fluorescence microscopy (TrueConfocal Scanner Leica TCSSP2). Confocal

sections were obtained through an x100 (numerical aperture = 1.4) objective.

Cell culture and preparation of cellular extracts

To prepare cellular extracts, cells were plated at $5x10^4$ cells/ml (TIB-73) or at $2.5x10^4$ cells/ml (J774) in 24-well culture plates and incubated for 48 hrs at 37ºC with DMEM media supplemented with 10% FCS and penicillin/streptomycin. Then, cells were incubated for 2 additional hours with DMEM media w/o FCS, and after that the different stimuli were added to the cells and kept on culture for 30 minutes. For experiments involving signalling inhibitors, cells were pre-treated for 1 hr at 37ºC with a dose of 100 μM of PD098059 (MEK1 Inhibitor). Cell protein extracts were obtained by collecting total cells, washing them with phosphate buffer saline (PBS) and resuspending them in Cell Signaling lysis buffer (Cell Signaling Technologies) following the manufacturer instructions.

Assessment of ERK kinase activation by Western blotting

Once the cell extracts were obtained, equal amounts of proteins (5-10 μg/lane) were subjected to polyacrilamide gel electrophoresis (10%) and transferred to polyvinylidine difluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking (2% BSA-T-TBS), the membranes were incubated with each primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized using the ECL detection system (Amersham Biosciences, Buckinghamshire, UK). Positive bands were quantified on digitised images using the "Scion Image" Software by referring the intensity of phosphorylated ERK to the corresponding total ERK band.

Statistical analysis

Data are represented as the mean ± SE. Data were analyzed with Student's t-test. Statistical significance was defined as $p<0.05$ (*) or $p<0.01$ (**).

Results

Constitutive expression of TLR9 protein in murine TIB-73 hepatocytes and J774 macrophages determined by both confocal microscopy analysis and Western blot

We have previously shown that TIB-73 hepatocytes express TLR9 mRNA by RT-PCR (Sanchez-Campillo et al., 2004). To confirm here the expression of TLR9 protein in hepatocytes compared to myeloid cells, we examined by confocal microscopy TIB-73 murine hepatocytes and J774 macrophages after secondary immunofluorescence staining with mAb recognizing TLR9 protein. As it has been described that TLR9 is predominantly expressed in the endosomal compartment, staining of TIB-73 and J774 cells with mAb anti-TLR9 was performed after cellular permeabilization to allow labelling of intracellular TLR9, and then observed under confocal microscopy. Fig. 1A and 1B, show that TLR9 proteins are expressed in both TIB-73 hepatocytes and J774 cells. Additionally, total cell lysates from TIB-73 hepatocyte and J774 macrophage cell lines in the absence of stimulation (constitutive) were analysed by Western blotting for expression of TLR9 as described in materials and methods. Fig. 2 shows that TIB-73 hepatocytes and J774 macrophages constitutively express a similar level of TLR9.

Phosphorylation of ERK 1/2 (Thr 202/Tyr 204) in TIB-73 hepatocytes and J774 macrophages after treatment with CpG and non-CpG PD-ODN and PS-ODN

It has been described that the extracellular signal-regulated kinase (ERK) signaling pathway is activated in response to ISS-ODN in both macrophages and non myeloid cell types (reviewed by Akira and Takeda, 2004; Ishii and Akira, 2006). Thus, we planned to investigate whether the treatment with PD-ODN and their nuclease resistant analogues, PS-ODN, lead to ERK activation in TIB-73 hepatocytes compared to J774 cells. Total cell lysates from both cell lines incubated in the absence of stimulation (constitutive) and after 30 minutes in the presence of LPS, a mixture of IFNγ (50 U/ml), IL-1ß (1.25 U/ml) and LPS (2.5 μg/ml); PD-ODN, PD-mODN (non-CpG control), PS-ODN or PS-mODN (non-CpG control), all of them at 100 μg/ml, were subjected to Western blotting analysis of phosphorylated ERK1/2 (Thr 202/Tyr 204). As shown in Figure 3, phosphorylation of ERK1/2 (Thr 202/Tyr 204) relative to total ERK 1/2 in TIB-73 hepatocytes (A) and macrophage cell line J774 (B) varied under the experimental conditions assayed. Densitometric quantitative analysis revealed that incubation of TIB-73 hepatocytes with LPS alone increased between 1.5 and 2 fold the baseline level of ERK1/2 phosphorylation, subsequent addition of IFNγ and IL-1ß induced higher increases of ERK1/2 phosphorylation (more than 2.5 fold), which were higher than those induced in J774 cells (1.5 fold). In turn, both PD-ODN and PD-mODN also induced increases of ERK1/2 phosphorylation in both cell lines (2 and 1.8 fold with PD-ODN and 1.5 and 1.3 fold with PD-mODN, respectively). In this line of evidence and according to the reported superior potency of PS-ODN (Sester et al., 2000), 30 min incubation in the presence of PS-ODN and PS-mODN induced higher increases of ERK1/2 phosphorylation in both cell lines independently of CpG motifs (2.8 and 2.3 fold for TIB-73 cells and 1.5 and 1.7 fold for J774 cells, respectively). It is noteworthy that the increase of ERK1/2 phosphorylation induced by PS-ODN in TIB-73 cells was near 2 fold higher than that induced in J774 cells.

Given the maximum efficiency of the PS backbone in TIB-73 activation, the rest of the assays were performed with these synthetic ODNs.

Dose-response phosphorylation of ERK 1/2 (Thr202/Tyr204) in TIB-73 hepatocytes to synthetic ODN treatment

Next, we aimed to determine whether ERK 1/2 activation induced by synthetic ODN in TIB-73 cells was dose-dependent. Thus, four different doses of PS-ODN (from 0.1 to 100 μg/ml) were assayed, and after 30 min incubation, total cell lysates were subjected to Western blot analysis for phosphorylated ERK1/2. As seen in Fig. 4, TIB-73 cells are able to detect and subsequently to respond to PS-ODN at doses as low as 0.1 μg/ml. An increase in ERK1/2 activation was obtained at a concentration of 1 μg/ml. Next, ERK1/2 activation increased with exposure to higher doses of PS-ODN up to the highest assayed concentration of 100 μg/ml.

Effect of pre-incubation with a MEK inhibitor in the phosphorylation of ERK 1/2 MAPK induced by treatment with synthetic CpG and non-CpG PS-ODN in TIB-73 hepatocytes.

The availability of specific inhibitors of signal pathways has allowed rapid progress in the knowledge of the role played by these signalling molecules in various biological processes. To further demonstrate the implications of MAPK signalling molecules in the hepatocyte response to PS-ODN, we treated TIB-73 and J774 cells for 1 h with a specific MEK1 inhibitor before

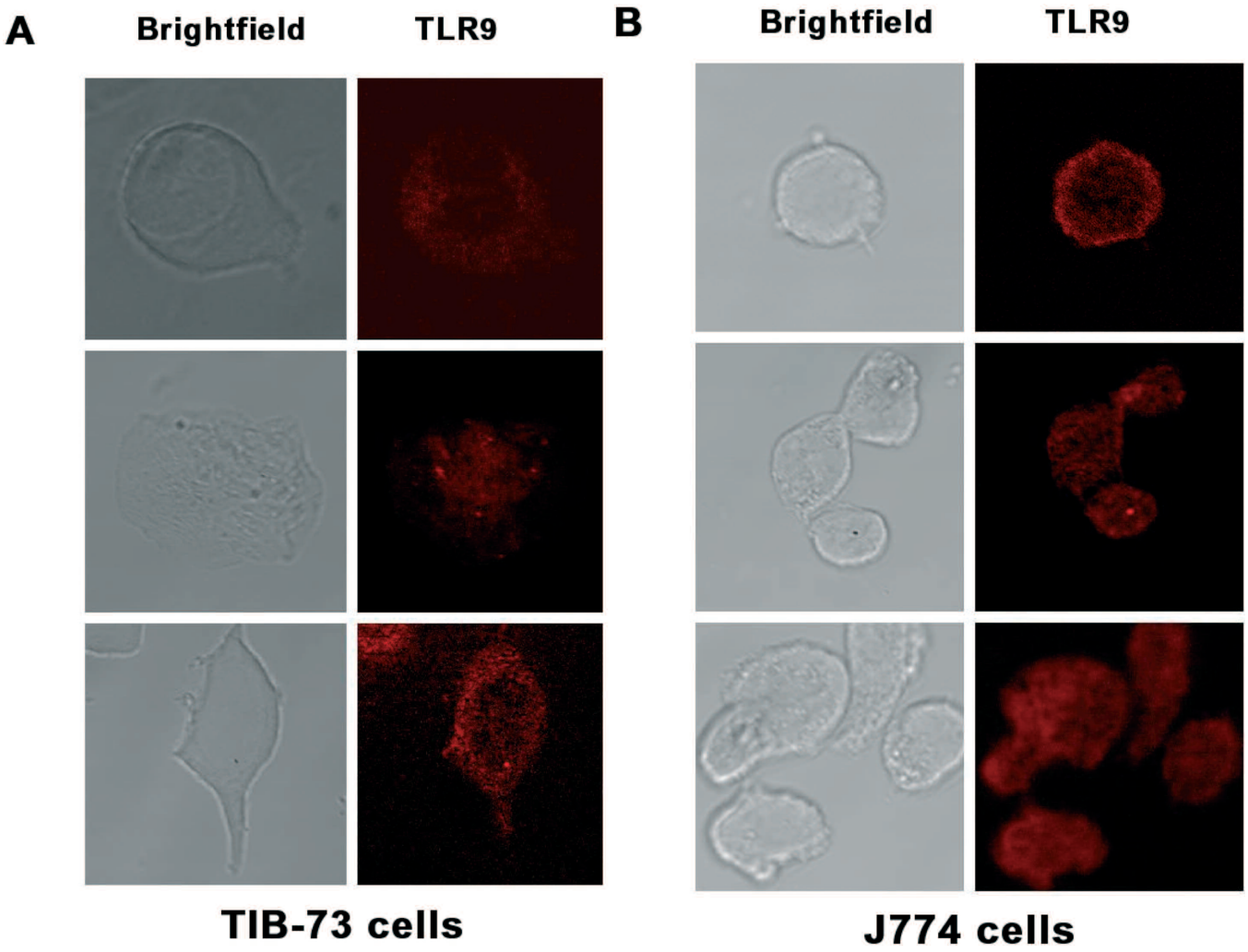


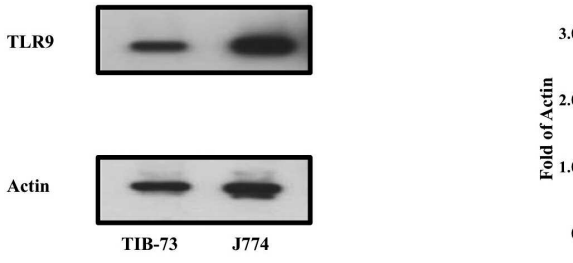
Fig. 1. Confocal microscopy analysis of TLR9 expression on murine TIB-73 hepatocytes and J774 macrophages. TIB73 hepatocytes **(A)** and J774 cells **(B)** were plated on round glass slides in 24 well dishes. Then cells were washed, permeabilized with 0.1% saponin in PBS, labelled with mAb anti TLR9, formalin-fixed and mounted for confocal microscopy analysis.

PS-ODN stimulation. As negative controls we used cells incubated with DMSO at the same concentration as it was present in the cultures of cells treated with the inhibitor.

Figures 5A and 5B show phosphorylation of ERK 1/2 (Thr 202/Tyr 204) in TIB-73 hepatocytes and J774 cells respectively, following 30 min treatment with PS-ODN or PS-mODN alone (50 μg/ml), or after 1 hour pre-incubation with the specific inhibitor of MEK1, PD98059 (100 μM). As expected, pretreatment with 50 μM (data not shown) and 100 μM of PD98059, significantly inhibited ERK 1/2 phosphorylation induced by both PS-ODN and PS-mODN in TIB-73 (Fig. 5A) and in J774 cells (Fig.5B).

Expression of TLR9 protein in murine TIB-73 hepatocytes and J774 macrophages under different stimulating conditions

Finally, expression of TLR9 protein in TIB-73 and J774 cells after 24 h incubation in the presence of LPS, a mixture of IFNγ (50 U/ml), IL-1ß (1.25 U/ml) and LPS



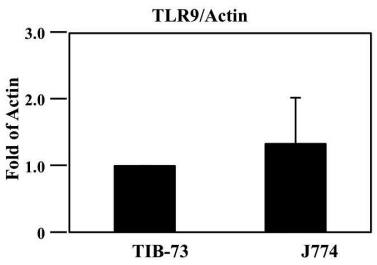


Fig. 2. Western blot analysis of TLR9 expression in TIB-73 hepatocytes and J774 macrophages. Total lysates of TIB-73 hepatocytes and macrophage cell line J774 on basal state were blotted with specific antibodies. Histograms represent quantification of TLR9 relative to basal expression levels of actin in TIB-73 hepatocytes and J774 macrophages as the mean ± standard error from three different experiments. *: P<0.05; **: P<0.01

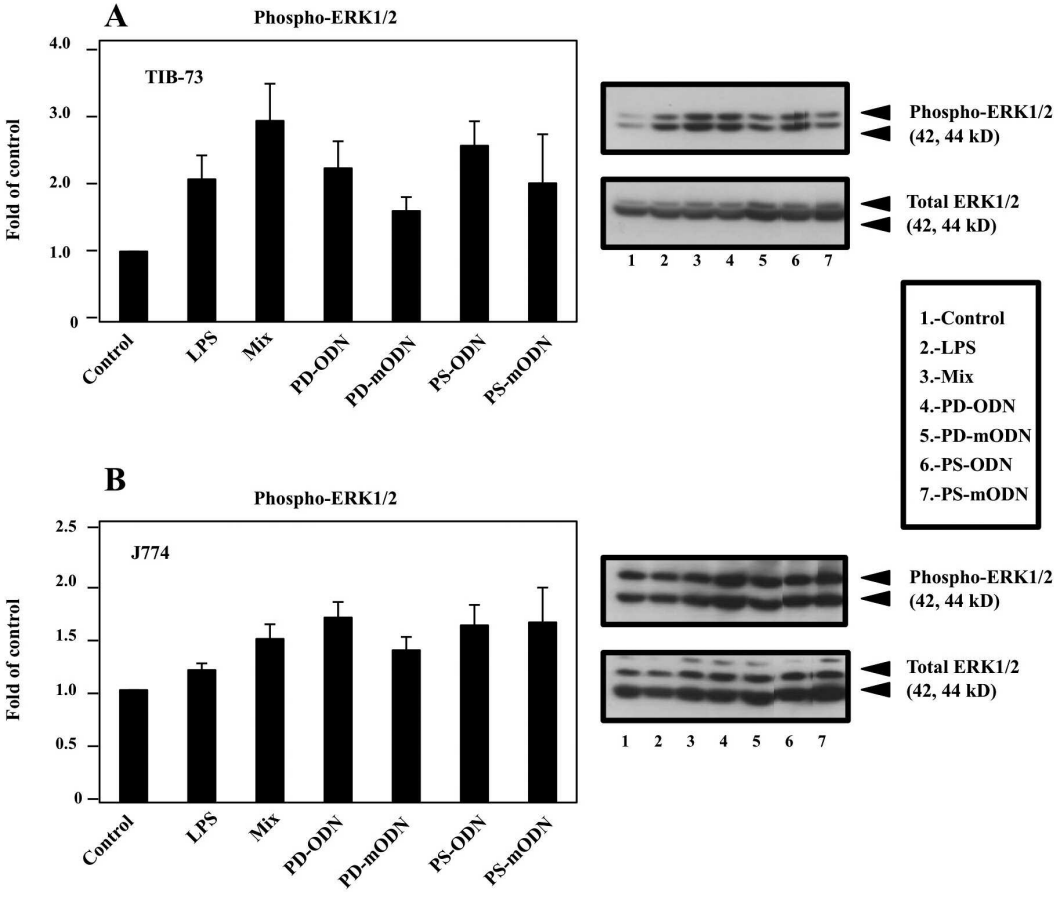


Fig. 3. Phosphorylation of ERK 1/2 (Thr 202/Tyr 204) in TIB-73 hepatocytes and J774 macrophages. TIB-73 hepatocytes **(A)** and macrophage cell line J774 **(B)** on basal state (Control) and after 30 minutes stimulation with a mixture of IFNγ, IL-1ß and LPS (Mix), PD-ODN, PD-mODN, PS-ODN or PS-mODN, all of them at 100 μg/ml. Histograms represent quantification of ERK 1/2 phosphorylation relative to total ERK 1/2. Graphs represent the mean ± standard error from three different experiments. *: P<0.05; **: P<0.01. Lanes that were not contiguous in the original blot have been placed adjacent on the shown figure to maintain the order of the different stimuli used.

(2.5 μg/ml); PD-ODN, PD-mODN, PS-ODN or PS-mODN, all of them at 100 μg/ml was also determined by Western blot analysis. Figure 6A and 6B show the regulatory trend of TLR9 protein expression in TIB-73 hepatocytes compared to J774 cells. Thus, although variability among assays prevents us from obtaining significant differences, we can observe that TLR9 showed an opposite regulation between both cell lines. Thus, TLR9 expression was slightly downregulated, this effect being higher after 24 h incubation in the presence of PS-ODN, and almost undetectable under LPS incubation (Fig. 6A). On the contrary, TLR9 expression on J774 cells was upregulated after 24 h incubation in presence of every stimulating agent assayed (Fig. 6B).

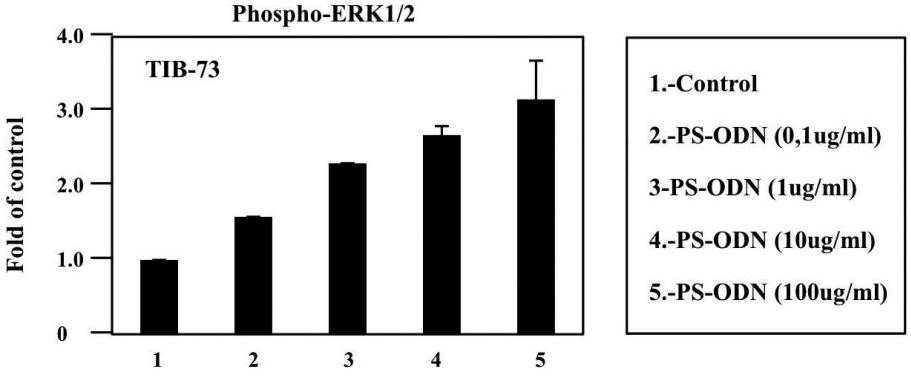


Fig. 4. Dose-response of ERK 1/2 (Thr 202/Tyr 204) phosphorylation to PS-ODN stimulation in TIB-73 hepatocytes. Histograms represent ERK 1/2 phosphorylation relative to basal state following 30 minutes treatment with different PS-ODN doses. Graphs represent the mean ± standard error from three different experiments. *: P<0.05; **: P<0.01.

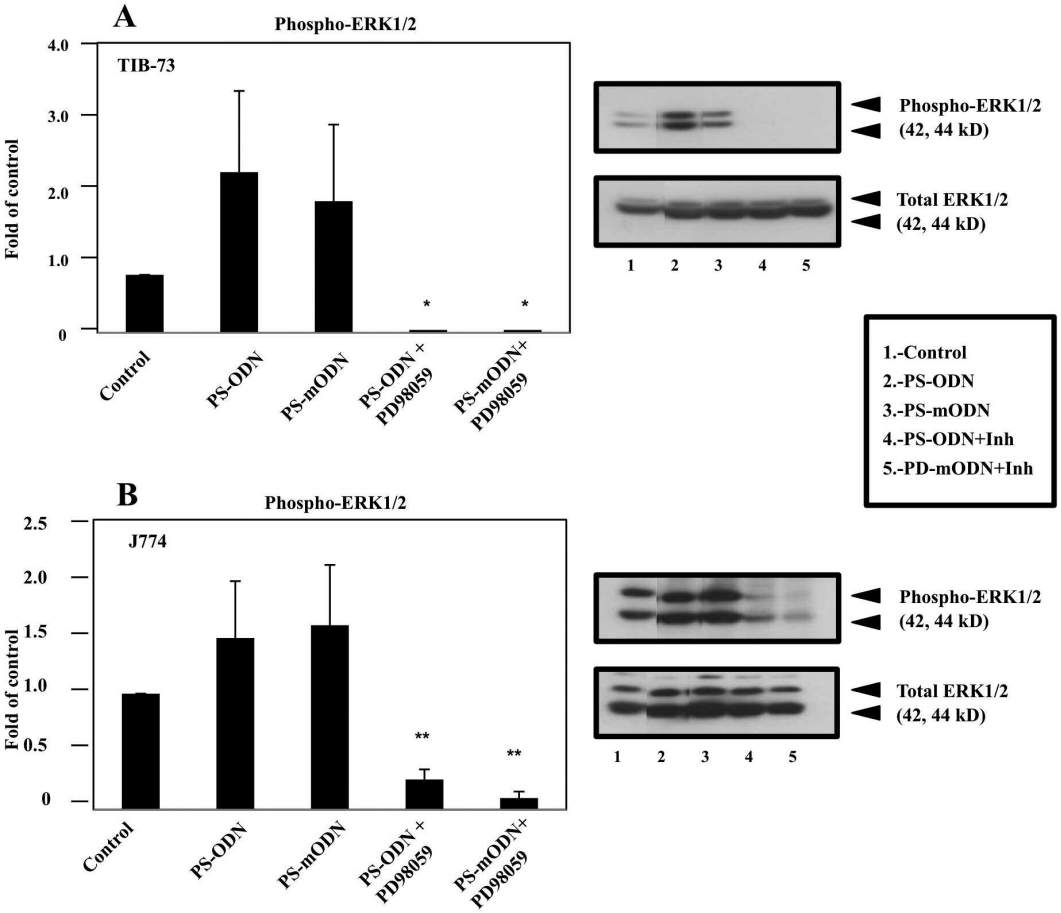


Fig. 5. Phosphorylation of ERK 1/2 (Thr 202/Tyr 204) MAPK in cells treated with PS-ODN or PS-mODN and MEK1 inhibitor (PD98059). Phosphorylation levels of ERK 1/2 were analyzed in TIB-73 hepatocytes **(A)** and J774 cells **(B)** following 30 min treatment with PS-ODN or PS-mODN (50 μg/ml), alone or after pre-incubation with the MEK1 inhibitor, PD98059 (100 μM) for 1 hour. Histograms represent quantification of ERK 1/2 phosphorylation relative to total ERK 1/2 MAPK. Graphs represent the mean ± standard error from three different experiments. *: P<0.05; **: P<0.01. Lanes that were not contiguous in the original blot have been placed adjacent on the shown figure to maintain the order of the different stimuli used.

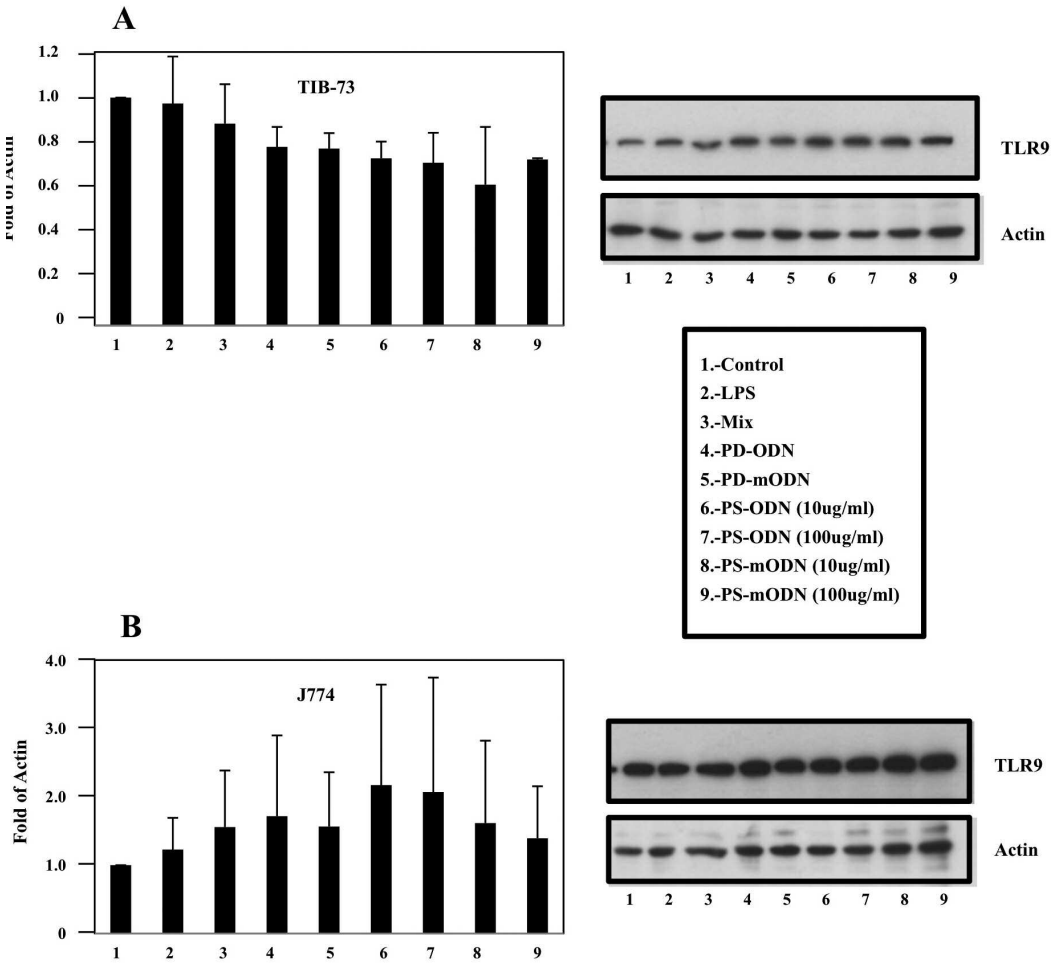


Fig. 6. Western blot analysis of TLR9 expression in TIB-73 hepatocytes and J774 macrophages after 24 hour incubation under different stimulatory conditions. Total lysates of TIB-73 hepatocytes **(A)** and macrophage cell line J774 **(B)** on basal state (Control) and after 24 hours stimulation with LPS, a mixture of IFNγ, IL-1ß and LPS (Mix), PD-ODN, PD-mODN, PS-ODN or PS-mODN, were blotted with specific antibodies. Histograms represent quantitation of TLR9 relative to expression levels of actin in TIB-73 hepatocytes **(A)** and J774 macrophages **(B)** incubated under the same experimental conditions. Results are presented as the mean ± error from three different experiments. *: P<0.05; **: P<0.01.

Discussion

The liver can be considered an immunocompetent organ playing a crucial role in the innate immune response to microbial infections (Volpes et al., 1992; Weiler-Normann and Rehermann, 2004; Gao et al., 2008). It produces both inflammatory cytokines and acute-phase reactants and it is involved in removing pathogens and microbial products from the blood (Tanikawa et al., 1998). The liver is significantly involved in the clearance of injected ODN, since it has been described that intravenously injected PS-ODN is cleared with a half-life of 23.3 min, and at 90 min more than 90% is concentrated in the liver (Bijsterbosch et al., 1997). Additionally, following subcutaneous administration, the highest percentage of administered dose was found in the liver (32% at 4 h) (Noll et al., 2005). Because of the prominent role in DNA degradation the overall importance of ODN on the immune effector and/or regulatory function of hepatocytes in response to foreign and self DNA has been probably underscored. In support of this point of view, our previous work showed that hepatocytes can respond directly to synthetic ODN by increasing the generation of both peroxides and nitrites and inhibiting the growth of intracellular *Salmonella typhimurium* (Sanchez-Campillo et al., 2004). This effect is additive with a mix of LPS and pro-inflammatory cytokines. We also demonstrated the induced expression of hepatocyte Mn-SOD by stimulation with CpG ODN; LPS; the mix of IFNγ, IL-1ß and LPS; and their combinations (Sanchez-Campillo et al., 2004). Accordingly, we undertook the present study to further analyse the mechanisms involved in the expression of that biological response to synthetic ODN in the hepatocyte cell line TIB-73. Although TLR9 is currently admitted to be almost exclusively expressed in certain immune cells such as dendritic cells, B cells and macrophages (Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006), we show here definitive evidence that TIB-73, a murine hepatocyte cell line, constitutively expresses TLR9 protein analysed by confocal microscopy and Western blot. In this line of evidence, a few groups had previously reported that primary-cultured murine

hepatocytes and hepatocyte cell lines express TLR1-9 mRNA, as well as MyD88 and MD-2 transcripts (Liu et al., 2002; Preiss et al., 2008), CD14 mRNA and protein (Liu et al., 1998; Vodovotz et al., 2001; Preiss et al., 2008), TLR2 mRNA (Vodovotz et al., 2001), a very low level of TLR2 protein that is up-regulated by the cytokines IL-1α, IL-1ß or TNF-α (Liu et al., 2000; Matsumura et al., 2003), TLR4 and MD-2 proteins in the *Propionibacterium acnes*-sensitized liver that render hepatocytes more susceptible to LPS-induced apoptosis and liver damage (Romics et al., 2004), TLR3 mRNA which induces hepatocyte apoptosis (Khvalevsky et al., 2007) and TLR9 mRNA in our previous work (Sanchez-Campillo et al., 2004). Thus, as far as we know, this is the first confirmed description of TLR9 protein expression in a hepatocyte cell line.

Our results also show that synthetic ODN trigger intracellular MAPK activation pathways in TIB-73 hepatocytes independent of CpG motifs. TIB-73 uptake of ODN may involve some of the described ODN-binding proteins, most of them are multifunctional proteins, including Mac-1, Scavenger receptors and albumin that bind ODN at high doses (Bijsterbosch et al., 1997) and more recently, HMGB1 and RAGE (Tian et al., 2007). Also, Diesbach et al., 2000 have described a new membrane protein in another hepatocyte cell line, HepG-2 cells, recognizing PD and PS ODNs of variable sequence. Additionally, it has been described that the immune stimulatory effects of synthetic ODN require binding to an intracellular (or endosomal) receptor. As TLR9 is the known receptor detecting CpG-ODN (Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006), it is hard to reconcile the mechanism by which non-CpG ODN functions here with the described expression of TLR9. However, many groups have recently reported that ODN lacking CpG motifs can activate the innate immune system in a TLR9-dependent manner (Vollmer et al., 2004; Roberts et al., 2005; Ishii and Akira, 2006; Preiss et al., 2008). This especially occurs when the ODN has a phosphorothioate backbone, which is the case here (Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006; Preiss et al., 2008). Thus, our results of TIB-73 activation with PS-ODN could be compatible with a mechanism mediated by TLR9. Furthermore, in our previous work and also here, we found that non CpG PD-ODN can also activate TIB-73 hepatocytes, albeit with lower potency than PS-ODN. In this case a TLR9-mediated mechanism does not seem feasible. However Yasuda et al., 2006 have described that PD-ODN lacking CpG motifs activate TLR9 provided an enforced endosomal translocation, and binding experiments utilizing surface plasmon resonance revealed low TLR9 binding to single-stranded (ss) PD-ODN lacking CpG motifs. However, at higher concentrations their TLR9 binding activity compared well with TLR9 binding of canonical PD CpG-ODN (Yasuda et al., 2006). On the opposite side, several reports have recently demonstrated that synthetic ODN intended for antisense applications (Senn et al., 2005) and DNA from both pathogens and host genomic are recognized independently of TLR9 (Suzuki et al., 1999; Akira and Takeda, 2004; Ishii and Akira, 2006), likely through some cytosolic receptor member of the RIG family (Ishii et al., 2005). Hence, at this time there is accumulating evidence of the existence of TLR9-dependent and TLR9-independent mechanisms for detecting not only pathogen DNA, but also abnormal composition, structure or chemical features of any kind of DNA (Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006). To further complicate the picture, published observations vary depending on the type of DNA assayed (Gürsel et al., 2002), the cell system used and the effector function measured (Häcker et al., 1999; Yi et al., 2002; Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006). At this time we cannot confirm whether the reported effects of synthetic ODNs on hepatocyte cell lines and whole liver described here and in our previous work (Sanchez-Campillo et al., 2004) are totally or partially TLR9-dependent.

The TLR9 signalling cascade involves mitogen-activated protein kinases (MAPKs), such as ERK, p38, c-Jun NH2-terminal kinase (JNK), and NF-κB-inducing kinase pathways (Häcker et al., 1999; Yi et al., 2002; Akhtar et al, 2003; Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006; Seki and Brenner 2008). The signalling cascade culminates in the activation of several transcription factors, including NF-κB and activating protein-1 (AP-1) among others, which directly upregulate cytokine/chemokine gene expression (Häcker et al., 1999; Yi et al., 2002; Akhtar et al., 2003; Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006; Seki and Brenner 2008). On the other hand, not all cells of the innate immune system respond uniformly to CpG-DNA stimulation. In macrophages, CpG ODN rapidly and strongly activates ERK 1/2 and contributes to the production of IL-10, while in dendritic cells or B cells this activation is either marginal or non active (Häcker et al., 1999; Yi et al., 2002; Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006). The response to CpG ODN, thus, depends not only on the presence and the type of cellular receptors, but also can be further regulated by the differential activation of particular signal transduction pathways (Häcker et al., 1999; Yi et al., 2002; Akhtar et al., 2003; Akira and Takeda, 2004; Wagner, 2004; Ishii and Akira, 2006). Related to this, we show here that TIB-73 hepatocytes respond to submicrogram concentrations of CpG and non-CpG ODN with the activation of ERK1/2 MAPK pathway. Phosphorylation of ERK1/2 induced by synthetic ODN in TIB-73 cells is dose-response dependent. It was detectable at doses as low as 0.01 μg/ml. There was not any appreciable difference between 0.01 and 0.1 μg/ml on ERK1/2 activation (data not shown). However, there was an important threshold of ERK1/2 activation between 0.1 and 1 μg/ml, which slowly increased with exposure to higher doses of PS-ODN up to the highest dose assayed of 100 μg/ml. Pre-treatment of TIB-73 hepatocytes with the inhibitor of ERK MAPK pathways, PD98059, significantly abrogated the ERK 1/2

phosphorylation levels induced by both PS-ODN and PS-mODN. Our results demonstrate that biological response of TIB-73 hepatocytes to ODN stimulation previously described by our group (Sanchez-Campillo et al., 2004) (generation of intracellular peroxides, nitrites and antibacterial effect) is mediated by ERK1/2 MAPK signal pathway independent of CpG motifs. Thus, these results indicate that hepatocytes not only can sense the presence of synthetic ODN, but also can respond to it, reinforcing the role of hepatocyte as a cell type of the innate immune system.

Additionally, our results showed that TLR9 protein expression in TIB-73 and J774 cells incubated for 24h in presence of the assayed stimuli show an opposite pattern of regulatory expression in TIB-73 hepatocytes compared to J774 cells. Thus, TLR9 expression in TIB-73 tends to be maintained or slightly downregulated, this effect being higher under incubation in the presence of PS-ODN, and almost undetectable under LPS incubation. On the contrary, TLR9 expression by J774 cells was upregulated when incubated for 24 h under every stimulatory condition assayed. In our previous work we found that pre-treatment of hepatocytes for 4 h in the presence of LPS and the combination of LPS plus cytokines, IFNγ and IL-1ß, resulted in a dramatic decrease of TLR9 mRNA levels. Our results here show that this short-term (1-4 h) modulation of TLR9 gene expression is transitory and it does not lead to significant changes of TLR9 protein expression at longer times of stimulation (24 hour). In turn, it has been described that following LPS stimulation, TLR9 gene expression in the mouse macrophage cell line RAW264.7, was upregulated within 1 h, reached peak level at about 3 h, and returned to basal level after 6 h, which is compatible with data obtained here with 24 h stimulated J774 cells (An et al., 2002).

These results support that regulation of TLR by microbial factors may attenuate or regulate TLR signalling, either by interfering with their intracellular cascades or protein expression levels, which might play an important role in controlling the overall responses of immune cells to bacteria. Indeed, further work is necessary to identify and clarify the mechanisms involved in this regulatory process.

Additionally, recent findings on the response of the liver when using PS-ODN with therapeutic purposes point out the importance to further investigate the role of hepatocytes in the response to synthetic ODN. Thus, these reports attribute either beneficial (CpG PS-ODN induces early hepatic injury, but mediates long-term protection against LPS hepatotoxicity) (Slotta et al., 2006) or harmful collateral effects (CpG PS-ODN inflammation caused T-cell-mediated autoaggression against hepatocytes (Sacher et al., 2002) and induces a severe acute liver injury and shock-mediated death through the mitochondrial apoptotic pathway-dependent death of hepatocytes in D-GalN-sensitized mice) (Yi et al., 2006). Therefore, elucidating mechanisms of innate immune activation of the liver by nucleic acids will help the future development of more efficient or safer nucleic acid-based immunotherapies and gene therapies.

In conclusion, TIB-73 hepatocytes constitutively express TLR9 and respond to synthetic ODN stimulation through a high ERK1/2 phosphorylation independent of CpG motifs.

Acknowledgements: This work was supported by Grants from ISCIII (PI060006) and Fundación Séneca (CARM) (03112/PI/05)

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Accepted January 13, 2010