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SHORT COMMUNICATION

DiC14-amidine cationic liposomes stimulate myeloid dendritic cells through Toll-like receptor 4

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DiC14-amidine cationic liposomes were recently shown to promote Th1 responses when mixed with allergen. To further define the mode of action of diC14-amidine as potential vaccine adjuvant, we characterized its effects on mouse and human myeloid dendritic cells (DC). First, we observed that, as compared with two other cationic liposomes, only diC14-amidine liposomes induced the production of IL-12p40 and TNF-α by mouse bone marrow-derived DC. DiC14-amidine liposomes also activated human DC, as shown by synthesis of IL-12p40 and TNF-α, accumulation of IL-6, IFN-β and CXCL10 mRNA, and up-regulation of membrane expression of CD80 and CD86. DC stimulation by diC14-amidine liposomes was associated with activation of NF-κB, ERK1/2, JNK and p38 MAP kinases. Finally, we demonstrated in mouse and human cells that diC14-amidine liposomes use Toll-like receptor 4 to elicit both MyD88-dependent and Toll/IL-1R-containing adaptor inducing interferon IFN-β (TRIF)-dependent responses.

Key words: Adjuvants · Cationic liposome · Dendritic cells · Toll-like receptors

Supporting Information available online

Introduction

Cationic liposomes are extensively used as vectors for nucleic acid transfection [1]. They were also shown to stimulate humoral as well as cell-mediated immune responses and are therefore considered as potential adjuvants for protein-based or DNA-based vaccines [2–7]. In this context, we previously demonstrated that mice injected with diC14-amidine (3-tetradecylamino-*tert*-butyl-*N*-tetradecylpropionamidine) cationic liposomes combined with a recombinant allergen (Der p 1) developed a Th1-biased immune response and were protected against subsequent induction of a Th2-type allergen-specific response [8]. Another study demonstrated that potent anti-tumor immunity could be generated by incorporating a tumor antigen into 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) cationic liposomes [9, 10]. Since dendritic cells (DC) represent a major target of vaccine adjuvants, these observations led to the hypothesis that cationic liposomes might stimulate DC. Indeed, cationic liposomes were shown to induce increased expression of the co-stimulatory molecules CD80 and CD86 in DC [11]. Moreover, DOTAP cationic

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liposomes were recently found to induce ERK activation and synthesis of chemokines in bone marrow-derived mouse DC (BMDC) [12].

Here, we first compared the ability of three cationic liposomes to elicit IL-12p40 and TNF-α synthesis by myeloid DC. The observation that diC14-amidine liposomes were the most potent in inducing cytokine synthesis led us to study the activation of signal transduction pathways and the involvement of Toll-like receptors (TLR) in the DC response to diC14-amidine liposomes.

Results and discussion

Di-C14 amidine liposomes stimulate mouse and human DC

In a first series of experiments we compared the ability of three cationic liposomes to induce the production of IL-12p40 and TNF-α in mouse BMDC. As shown in Fig. 1A, only diC14-amidine liposomes elicited IL-12p40 and TNF-α synthesis. The effect of diC14-amidine was dose dependent as shown in Fig. 1B. The diC14-amidine preparation contained less than 0.001 EU/mL endotoxin as assessed by the chromogenic Limulus amebocyte lysate assay. To further exclude a role for endotoxin contamination in the induction of cytokine synthesis by diC14-amidine liposomes, we performed additional experiments using polymyxin B. Whereas addition of polymyxin B (10 μg/mL) abrogated as expected LPS-induced production of IL-12p40, it did not affect diC14-amidine-induced IL-12p40 secretion in BMDC (see Supporting Information Table S1).

Human monocyte-derived DC also responded to diC14-amidine liposomes as evidenced by the induction of IL-12p40 and TNF-α synthesis (Fig. 1C), the accumulation of IFN-β, IL-6 and CXCL-10 (IP-10) mRNA (Fig. 1D) and the increased expression of CD80 and CD86 (Fig. 1E), indicating that these cationic liposomes induce maturation of human DC.

DiC14-amidine liposomes stimulate activation of NF-κB and ERK1/2, JNK, p38 MAP kinases in DC

Since NF-κB and MAP kinases are known to contribute to DC activation in several systems, we studied the impact of diC14-amidine liposomes on these signaling pathways in mouse BMDC. As shown in Fig. 2A, electrophoretic mobility shift assay (EMSA) using a DNA probe containing the κB binding site of the human IL-8 promoter revealed that diC14-amidine induced the appearance in DC nuclei of DNA binding activity to this site, which is indicative of NF-κB activation. Furthermore, Western blot experiments established that diC14-amidine induces activation of MAP kinases as shown by a marked increase in the phosphorylation of ERK, JNK and p38, which was maximal at 30 min and began to decline at 60 min (Fig. 2B). Experiments using inhibitors of these signaling pathways provided additional evidence that NF-κB and JNK activation both contribute to the induction of IL-12p40 synthesis (see Supporting Information Table S2).

Stimulation of DC by diC14-amidine liposomes depends on TLR4

The pattern of DC activation observed in previous experiments is reminiscent of responses elicited by ligands of TLR. As most TLR are coupled to the MyD88 adaptor protein [13], we compared the production of IL-12p40 in response to diC14-amidine in wild-type and MyD88-deficient BMDC. As shown in Fig. 3A, the production of IL-12p40 by BMDC stimulated by diC14-amidine was blunted in MyD88-deficient BMDC, indicating the involvement of TLR in the DC response to these cationic liposomes. We then evaluated the responses of BMDC deficient in TLR2, TLR4 and MD2, the latter protein being closely associated with TLR4 [14]. Whereas TLR2-deficient DC responded as efficiently as wild-type DC to diC14-amidine, neither TLR4-deficient nor MD2-deficient BMDC were responsive (Fig. 3A). Since TLR4-mediated signaling depends on both MyD88 and TIR-containing adaptor inducing interferon IFN-β (TRIF) adaptor proteins, we evaluated the response of TRIF-deficient LPS2 mice [15] to diC14-amidine. As shown in Fig. 3A, the production of IL-12p40 was blunted in TRIF-deficient BMDC, indicating that both MyD88-dependent and TRIF-dependent signaling pathways are engaged upon exposure to diC14-amidine and are required for efficient activation of BMDC.

To confirm that diC14-amidine liposomes use TLR4/MD2 to exert their stimulatory action, we tested their effect on HEK293 cells stably expressing human TLR2, TLR3 or TLR4/MD2 and co-transfected with an NF-κB-dependent reporter plasmid. As shown in Fig. 3B, NF-κB activation in response to diC14-amidine was detected only in HEK cells expressing TLR4/MD2 (Fig. 3B). Furthermore, we confirmed in this system that the TLR4-dependent response induced by diC14-amidine was not affected by the addition of polymyxin B, whereas the LPS response was profoundly inhibited as expected (Fig. 3B).

Eritoran is an analog of LPS that antagonizes its activity by binding to the TLR4–MD-2 complex. In the crystal structure of the TLR4–MD-2 complex, the four saturated acyl chains of Eritoran fully occupy the MD-2 hydrophobic pocket, suggesting a prominent role of C12–C14 acyl chains in the binding mechanism [16]. Crystal structures of MD-2 and its complex with the tetra-acylated lipid A core of LPS have been determined at 2-Å resolution [17]. The four saturated C12–C14 acyl chains of the ligand fully occupy MD-2a large hydrophobic cavity. Interestingly, diC14-amidine structure is characterized by a hydrocarbon tail region with saturated C14 hydrocarbon chains, which are also found in the lipid A active moiety of LPS but not present in the other cationic lipids investigated in the present study and which did not activate TLR4 cascades. All together, these data strongly suggest that the saturated acyl chains might be involved in TLR4 engagement by diC14-amidine through binding to MD-2. Molecular dynamics simulations of the insertion of diC14-anidine in MD2 cavity revealed that two amidine molecules do occupy a

volume identical to that of one tetra-acylated lipid A molecule in the cavity (M. Lensink, personal communication)

Indeed, our findings might lead to the development of diC14-amidine as a vaccine adjuvant since there is growing interest in the use of TLR4 ligands to induce T cell-mediated immunity. Interestingly, monophosphoryl lipid A, which is the TLR4 ligand in the most advanced phase of clinical development, was recently shown to be rather inefficient in inducing MyD88-dependent responses, whereas it elicits MyD88-independent TRIF-dependent cytokine production [18]. Since it has been shown that MyD88-mediated signaling is required for the induction of Th1-type immune responses [19], activation of the MyD88-dependent pathway might be required for adjuvants included in vaccines against intracellular pathogens or tumors and in anti-allergy immunotherapies. Indeed, we previously found that diC14-amidine liposomes combined with the allergen Der p 1 induced

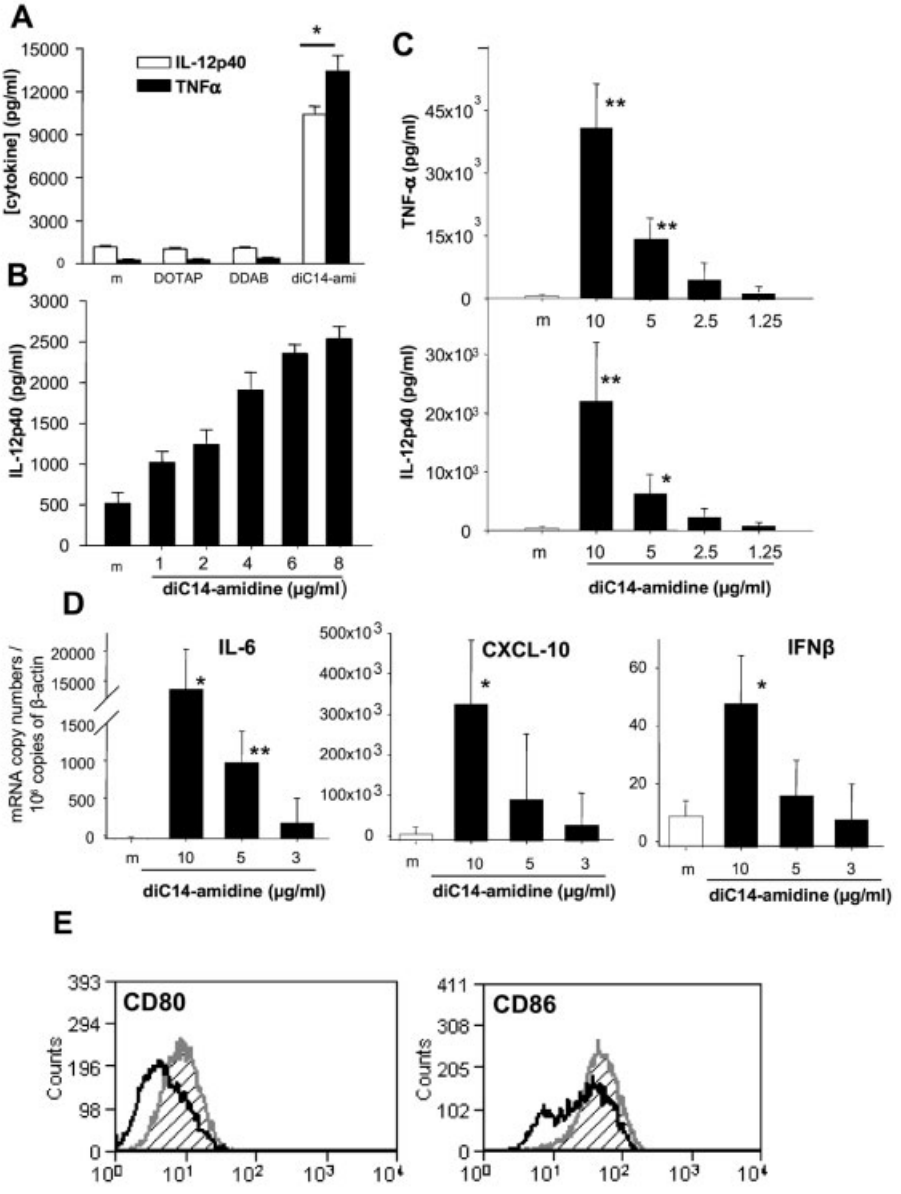

Figure 1. DiC14-amidine activates mouse and human DC. (A) BMDC were incubated in medium alone (m) or containing diC14-amidine (diC14-ani), dimethyldioctadecylammonium bromide (DDAB) , or DOTAP liposomes at a concentration of 4 μg/mL; after 17 h, culture supernatants were harvested and assayed for mouse IL-12p40 and TNF-α; *$p<0.001$ as compared with other cationic liposomes. (B) BMDC were cultured for 17 h in medium alone (m) or containing graded concentrations of diC14-amidine liposomes followed by determination of IL-12p40 levels in culture supernatants. (C, D) Human DC were incubated for 20 h in medium alone (m) or medium containing indicated concentrations of diC14-amidine liposomes followed by determinations of IL-12p40 and TNF-α levels in culture supernatants by ELISA (C) and IL-6, CXCL10, and IFN-β mRNA by real time RT-PCR (D); data are shown as mean + SEM from five independent experiments performed in triplicate; *$p<0.05$, **$p<0.01$ as compared with DC in medium alone. (E) Cell surface expression of CD80 and CD86 as determined by flow cytometry; the black line represents DC in medium alone and the gray line with hatched histogram DC exposed to diC14-amidine liposomes (5 μg/mL). One representative experiment out of four is shown.

Th1-biased responses and prevented Th2-type allergic responses [8]. This might be related to the ability of di-C14 amidine to trigger MyD88 signaling since MyD88-deficient DC exposed to diC14-amidine produced only low levels of IL-12 as compared with DC of wild-type animals. On the other hand, the blunted response of TRIF-deficient BMDC to diC14 amidine demonstrates that the action of these cationic liposomes also depends on TRIF signaling. This is consistent with the up-regulation of the expression of costimulatory molecules and the induction of IFN-β and CXCL-10 gene transcription induced by diC14 amidine in human DC since both phenomena were shown to require TRIF signaling [18, 20, 21].

Concluding remarks

DiC14-amidine liposomes represent a novel type of TLR4 agonists that engage both MyD88-dependent and TRIF-dependent TLR4 signaling pathways. We suggest that vaccine formulations based on diC14 amidine liposomes should be further explored to elicit Th1-type immune responses.

Materials and methods

Reagents and cell lines

The MEK1/2 inhibitor U0126 was obtained from Cell Signaling (Danvers, USA). The p38 MAP kinase inhibitor SB203580, the JNK inhibitor SP 6001125, the MG132 proteasome inhibitor MG-132 and the IκBα phosphorylation inhibitor BAY-11–7082 were purchased from Calbiochem (Nottingham, UK). Polymyxin B was from Sigma (Bornem, Belgium). Dimethyldioctadecylammonium bromide (DDAB) and DOTAP were from Avanti Polar Lipids (Alabaster, USA). Murine recombinant GM-CSF was from R&D Systems (Abingdon, UK). Human recombinant IL-4 and human recombinant GM-CSF were purchased from BruCells SA (Brussels, Belgium). Lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4, the TLR3 ligand polyinosine-polycytidylic acid (polyI:C), and the TLR2 ligand Pam2CGDPKHPKSF diacylated lipoprotein (FSL-1) were from InvivoGen (San Diego, USA). Fugene6 was purchased from Roche (Vilvoorde, Belgium). Endotoxin levels were determined using the limulus assay (Limulus amebocyte lysate QCL 1000, Cambrex, East Rutherford, USA). Human embryonic kidney (HEK) cells stably transfected with TLR4 and MD2 or with TLR2 or TLR3 (termed here HEK-TLR4/MD2, HEK-TLR2 and HEK-TLR3 cells, respectively) were kindly provided by D. Golenbock (University of Massachusetts Medical School, Worcester, USA) [22].

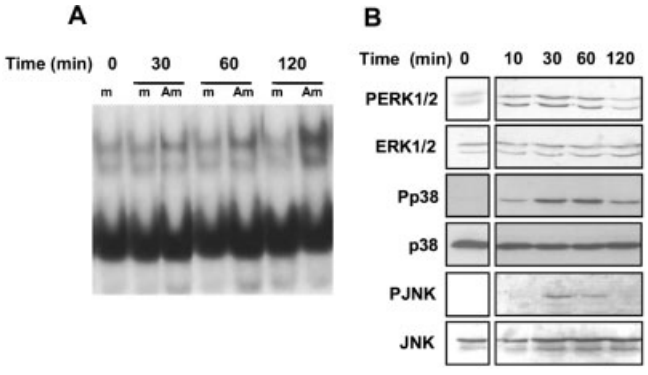

Figure 2. NF-κB and MAPK activation by diC14-amidine liposomes. (A) BMDC were incubated in medium alone (m) or diC14-amidine liposomes (4 μg/mL) (Am) for the indicated periods of time. NF-κB activation was determined by EMSA using a radiolabeled probe corresponding to the NF-κB binding site of the human IL-8 promoter; one representative experiment out of three is shown. (B) BMDC were incubated with diC14-amidine liposomes (4 μg/mL) for the indicated periods of time. MAPK activation was analyzed by Western blotting with anti-phospho-ERK1/2, anti-phospho-p38 or anti-phospho-JNK antibodies. Blots were reprobed with anti-ERK1/2, anti-p38 or anti-JNK antibodies to assess equal loading.

Liposome preparation

DiC14-amidine liposomes (3-tetradecylamino-*N*-tert-butyl-*N'*-tetradecylpropionamidine) were synthesized as described previously [23]. The liposomes used in this study were prepared as previously described [8].

Animals

Female BALB/c mice (7 weeks old) and wild-type (WT) C57BL/6 mice (6 weeks old) were obtained from Harlan (AD Horst, The Netherlands). C57BL/6 mice genetically deficient in the expression of TLR2, TLR4, MD2 or MyD88 were kindly provided by F. Trottein (Institut Pasteur de Lille, France), K. Miyake (Tokyo University, Japan) and M. Moser (IBMM, ULB, Belgium). LPS2 mice, which carry a mutation in the *TRIF* gene were obtained from B. Beutler (Scripps Research Institute, La Jolla, CA) [15].

Preparation of mouse BMDC

Mouse BMDC were prepared as described[24] with minor modifications. Briefly, BM cells isolated from femurs and tibias were cultured at 1.2×10^6 cells/mL for 8 days in RPMI 1640 supplemented with 10% FBS and 10 ng/mL mouse rGM-CSF. On day 9, cells were collected, centrifuged, and resuspended in fresh medium. The purity of BMDC preparations was above 97% as estimated by CD11c staining.

Activation of BMDC

On day 9, BMDC from WT, TLR4-, TLR2-, MD2-, MyD88- or TRIF-deficient mice (10^6 cells/well) were washed twice by serum-free RPMI 1640, and cells were incubated with different concentrations

of diC14-amidine in serum-free RPMI 1640 for 4 h. BMDC were then washed twice with serum-free RPMI 1640, and incubated with complete medium containing 20 ng/mL rmGM-CSF. As controls, BMDC were also incubated with LPS (final concentration 1 μg/mL). To address the putative influence of endotoxin contamination in the cell activation, BMDC were pretreated for 30 min with polymyxin B (10 μg/mL). When appropriate, BMDC were treated at 37°C for 1 h with the following MAPK- and NF-κB-specific inhibitors prior to activation of BMDC by amidine: U0126 (25 μM), SB203580 (25 μM), SP6001125 (25 μM), the MG132 (25 μM) and BAY-11–7082 (25 μM). Negative control BMDC were pre-incubated with equivalent amounts of DMSO. Culture supernatants were collected, and then centrifuged at 10 000 × *g* for 5 min. Finally, culture supernatants were stored at –80°C until use for assay.

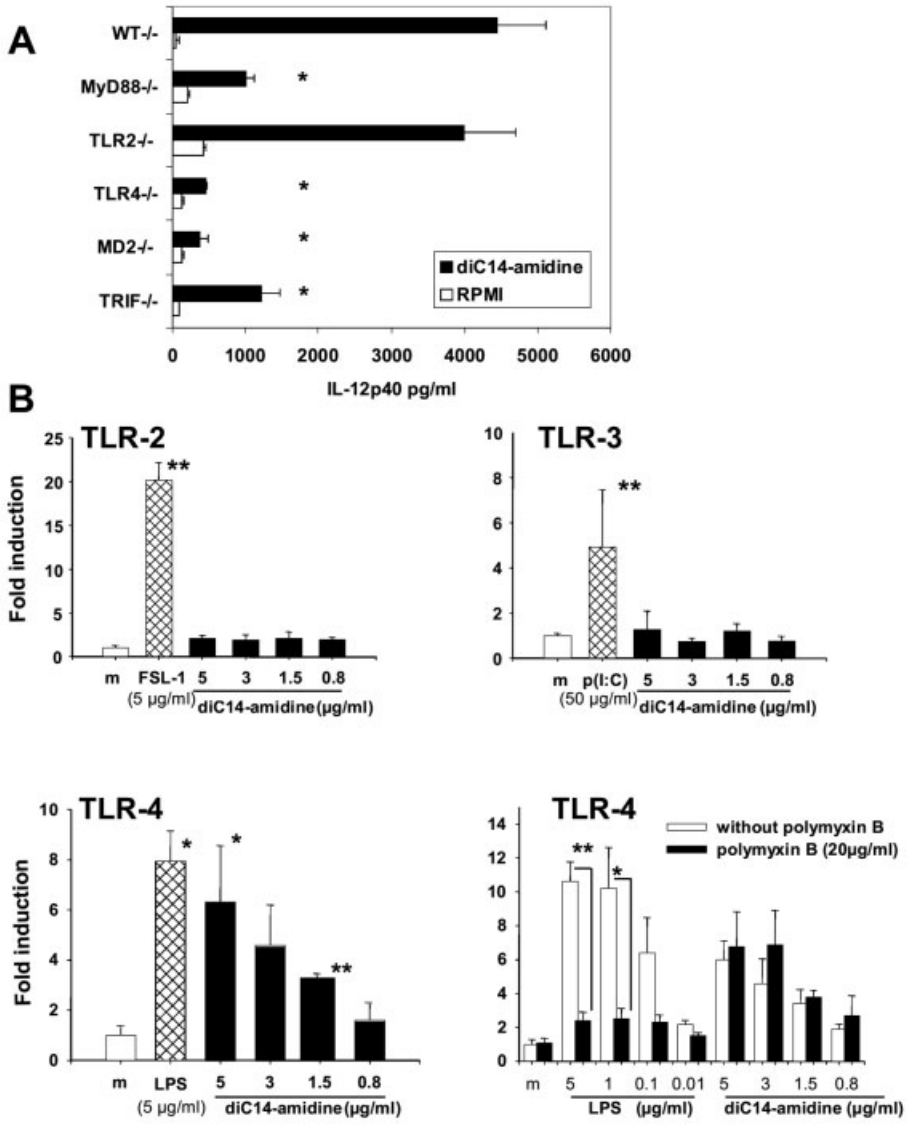

Figure 3. DC activation by diC14-amidine liposomes depends on TLR4. (A) BMDC isolated from either WT mice or mice deficient in MyD88, TLR2, TLR4, MD2 or TRIF were cultured for 17 h in medium alone or containing diC14-amidine liposomes (4 μg/mL) before determination of IL-12p40 levels in culture supernatants; data are shown as mean + SEM from at least three independent experiments performed in triplicate; $^{*}p<0.01$ as compared with WT cells treated with diC14-amidine. (B) HEK293 cells stably transfected with plasmids encoding human TLR2, TLR3 or TLR4/MD-2 (labelled TLR-4) were transiently transfected with plasmids encoding firefly luciferase driven by the NF-κB promoter of human IL-8; after incubation in medium alone (m) or containing positive controls (FSL-1 5 μg/mL as TLR2 ligand, polyI:C (pI:C) 50 μg/mL as TLR3 ligand, LPS 5 μg/mL as TLR4 ligand) or diC14-amidine liposomes, dual-glo luciferase assays were performed to assess NF-κB activation; $^{*}p<0.05$, $^{**}p<0.01$ as compared with DC incubated in medium alone (m); in a parallel experiment, HEK TLR4/MD-2 cells were incubated in medium alone or containing either LPS or diC14-amidine liposomes in presence or absence of polymyxin B (20 μg/mL); data are shown as mean + SEM of three independent experiments; $^{*}p<0.05$, $^{**}p<0.01$ as compared with DC stimulated in absence of polymyxin B.

Western blotting experiments

BMDC were washed in cold PBS, lysed with Laemmli sample buffer (60 mM Tris-HCl pH 7.5, 2% SDS, 5% glycerol, 100 mM DTT, 0.001% bromophenol blue) and analyzed by SDS-PAGE and Western blotting with polyclonal antibodies specific for both phosphorylated and non-phosphorylated forms of ERK 1/2, p38 MAP kinase or JNK (1:1000 dilution, Cell Signaling) as described previously [25].

Human DC preparation, stimulation and flow cytometric analysis

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (obtained from local routine blood donations) by density centrifugation on Lymphoprep (Nycomed, Oslo, Norway) and suspended in RPMI 1640 (Lonza, Basel, Switzerland). $CD14^+$ cells were then isolated by magnetic beads (Automacs, Miltenyi Biotec, Utrecht, The Netherlands) and cultured in 6-well plates (Corning Incorporated, Schiphol, The Netherlands) with complete RPMI 1640 culture medium containing 10% FCS, 800 U/mL rGM-CSF and 500 U/mL rIL-4. After 6 days, nonadherent cells corresponding to the DC-enriched fraction were harvested, washed and seeded in 24-well plates (Greiner Bio-one, Wemmel, Belgium). After 20 h of culture in absence or presence of diC14-amidine (5 μg/mL), supernatants were harvested for ELISA assays and DC were stained with phycoerythrin (PE)-conjugated anti-CD80 antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-CD86 antibodies (BD, Erembodegem, Belgium) before flow cytometry analysis (FACS Cyan ADP, Dako).

Cytokine assays

Mouse IL-12p40 and TNF-α were assayed in DC culture supernatants by ELISA using BD OptiEIA kits (BD). Human IL-12p40 and human TNF-α were assayed by ELISA using Biosources kits (Camarillo, CA, USA).

Preparation of nuclear extracts and EMSA

Nuclear extracts isolated from BMDC and EMSA using radiolabeled NF-κB (5′-probe from the human IL-8 promoter) were performed as previously described [25].

Reporter luciferase gene assay

HEK cells were cultured in complete DMEM medium supplemented with 10% FBS (Invitrogen, Merelbeke, Belgium) and were transiently transfected with reporter luciferase gene activated by NF-κB (pIL-8-κB-luc). After 2 days, cells were detached using Accutase (PAA, Linz, Austria) and were seeded in opaque 96-well plates (Nunc, Neerijse, Belgium). Promoter activities were analyzed using the dual-glo luciferase assay system (Promega, Leiden, The Netherlands) and normalized to Renilla luciferase activities using the pRL-TK plasmid (Promega, Madison, USA).

RNA purification and real-time RT-PCR

Total RNA was extracted using a MagnaPure LC RNA-High Performance Isolation kit (Roche Diagnostics). Real-time RT-PCR were then conducted using LightCycler-RNA Master Hybridization Probes (one-step procedure) on a LightCycler apparatus (Roche Diagnostics). Primers used to amplify specific gene products from human cDNA were: for β-actin : forward 5′-GGATGCAGAAGGAGATCACTG-3′, reverse 5′-CGATCCACACGGAGTACTTG-3′, probe BHQ-2-CCCTGGCACCCAGCACAATG-Pulsar650; for IL-6: forward 5′-GACAGCCACTCACCTCTTCA-3′, reverse 5′-AGTGCCTCTTTGCTGCTTTC-3′, probe FAM-CCTCGACGGCATCTCAGCCC-TAMRA; for IFN-β : forward 5′-GATTCCTACAAAGAAGCAGCAA-3′, reverse 5′-CAAAGTTCATCCTGTCCTTGAG-3′, probe FAM-TGGCAATTGAATGGGAGGCTTGA-TAMRA; and for CXCL-10: forward 5′-GAAATTATTCCTGCAAGCCAAT-3′, reverse 5′-CAGACATCTCTTCTCACCCTTCT-3′, probe FAM-TGTCCAGTGTTGAGATCATTGCTACA-TAMRA.

Statistics

Data were compared using the Student's *t*-test.

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Abbreviations: **DOTAP:** 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) · **HEK:** Human embryonic kidney · **TRIF:** Toll/IL-1R containing adaptor inducing interferon IFN-β

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