Differential expression of Toll-like receptors in murine peritoneal macrophages in vitro on treatment with cisplatin

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Abstract
In the present study, we have investigated the differential expression of Toll-like receptors [(TLRs) 1–9] in murine peritoneal macrophages in vitro, on treatment with cis-diaminedichloroplatinum (II) (cisplatin). It is demonstrated that cisplatin induces the expression of TLRs and is a potent activator of Toll-signaling pathway. The enhanced expression of TLR2, -3, -4, -5, -6, -7, -8 and -9 is observed at different time intervals after 5 μg ml⁻¹ cisplatin treatment. The expression of downstream signaling molecules of TLR-signaling pathway—myeloid differentiation factor 88 (MyD88), IRAK1, tumor necrosis factor receptor-associated factor 6 and transcription factors IRF3 and nuclear factor-κB (NF-κB)—has also been investigated. The expression of TLR2, -3, -4 and -9 was down-regulated in cisplatin-treated macrophages in the presence of inhibitors of mitogen-activated protein kinases and NF-κB pathways, suggesting a role of these pathways in cisplatin-induced TLR expression. It is also observed that pretreatment of macrophages with cisplatin and subsequent incubation with TLR ligands significantly enhanced the production of pro-inflammatory cytokines (tumor necrosis factor-α, IFN-γ, IL-1β and IL-12) and iNOS expression in macrophages. The data suggest that treatment of macrophages with cisplatin renders them more susceptible to subsequent induction of pro-inflammatory cytokines and iNOS expression by different TLR ligands. It is proposed that the pharmacological reagents like cisplatin can be used to manipulate the innate immune responses, which may be effectively used for the development of novel therapeutic approaches.

Introduction
Toll-like receptors (TLRs) are a family of mammalian homologues of Drosophila Toll protein and play an important role in host defense (1, 2). TLRs are pattern recognition receptors that have key roles in detecting microbes and initiating inflammatory responses. A host of new microbial products that activate specific TLRs have been defined and different components that mediate intracellular signaling have been identified (3). TLRs mediate recognition of microbial targets in several organisms including humans, mice and flies (1, 2, 4). TLRs recognize a wide variety of pathogen-associated molecular patterns from bacteria, viruses and fungi, as well as some host molecules (5, 6). Remarkably, individual TLRs interact with several structurally unrelated ligands of exogenous and endogenous origin (7, 8).

The intracellular signaling pathways activated by TLRs share with IL-1Rs signaling owing to their conserved Toll-IL-1R (TIR homology) domains (9, 10). Activation of signaling through TIR domain results in the recruitment of the cytoplasmic adaptor molecule myeloid differentiation factor 88 (MyD88), activation of serine/threonine kinases of the IL-1R-associated protein kinases (IRAKs) family and ultimately degradation of I-κB and translocation of nuclear factor-κB (NF-κB) to the nucleus (11).

cis-Diaminedichloroplatinum (II) (cisplatin) is a potent anti-cancer compound, which is active against a wide variety of tumors (12, 13). Previous studies have demonstrated that cisplatin activates macrophages in vitro to enhanced tumoricidal state and up-regulates their antigen-presenting ability (14). Cisplatin also significantly increases the production of reactive oxygen intermediates, nitric oxide (NO), IL-1 and tumor necrosis factor-α (TNF-α) both in vitro and in vivo (15–17). The role of calcium, tyrosine kinase and phosphatase in the in vitro activation of macrophages with cisplatin has also been reported (18–20). Since cisplatin induces the activation of macrophages in vitro and that macrophages play a central role in innate immune response and...
Cisplatin induces expression of TLRs in macrophage

Immunosurveillance against microbes and are known to express pattern recognition receptors on their surface (21), we investigated if cisplatin induced the expression of TLRs. It is reported for the first time that cisplatin induced enhanced expression of TLRs and its associated adaptors/signaling molecules in macrophages on in vitro treatment. It is well documented that Toll-signaling pathway leads to the activation of mitogen-activated protein (MAP) kinases and nuclear translocation of NF-κB (22–24). Present studies show that cisplatin in addition to increasing the expression of TLRs also activates MAP kinases and induces phosphorylation of IκB. It is further observed that cisplatin-induced expression of TLRs is dependent on these pathways and the expression of TLR2, -3, -4 and -9 was down-regulated in the presence of inhibitors of MAP kinase and NF-κB pathways.

It is also reported that the increased expression of TLRs in macrophages on treatment with cisplatin makes them functionally more responsive to TLR ligands.

Methods

Mice

Inbred strains of BALB/c mice of either sex at 8–10 weeks (20–22 g) of age were used for obtaining peritoneal macrophages.

Cell cultures and reagents

Macrophage monolayers were cultured in RPMI 1640 medium supplemented with heat-inactivated FCS (10%), penicillin (100 U ml⁻¹), streptomycin (100 U ml⁻¹) and gentamycin (20 µg ml⁻¹) at 37°C in humidified air containing 5% CO₂. Medium RPMI 1640, Tri reagent, Zymosan A and LPS were purchased from Sigma–Aldrich Chemicals, St Louis, Mo, USA. FCS was from the Biological Industries, Israel and cisplatin was obtained from Cadila Pharmaceuticals, India. Positive ligands for TLR3 and TLR9, PolyI:C and CpG DNA were kind gift from Bruce Beutler, Scripps Research Institute, La Jolla, CA, USA. Polyclonal antibodies specific for TLR1, -2, -3, -4, -6, -7, -8, -9, MyD88, IRAK1, tumor necrosis factor receptor-associated factor 6 (TRAF6), IRF3, phospho-IκB-α, phospho-JNK, phospho-p38, phospho-p42/44, iNOS, actin and HRP-conjugated anti-rabbit, anti-mouse and anti-goat IgGs were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. TLR5 antibody was purchased from Immunex, San Diego, CA, USA. One-step reverse transcription (RT)–PCR kit was from Qiagen, Germany. Mouse primers for TLRs 1–9 were purchased from GENSET Singapore Biotech. Pvt Ltd, Singapore. SP600125, SB202190 and PD98059 were purchased from Calbiochem, La Jolla, CA, USA. Curcumin was a gift from Unico Pharmaceuticals, Ludhiana, India. ELISA kits were purchased from BD PharMingen, San Diego, CA, USA. One-step reverse transcription (RT)–PCR kit was from GENSET Singapore Biotech. Pvt Ltd, Singapore. SP600125, SB202190 and PD98059 were purchased from Calbiochem, La Jolla, CA, USA. Curcumin was a gift from Unico Pharmaceuticals, Ludhiana, India. ELISA kits were purchased from BD PharMingen, San Diego, CA, USA. One-step reverse transcription (RT)–PCR kit was from GENSET Singapore Biotech. Pvt Ltd, Singapore. SP600125, SB202190 and PD98059 were purchased from Calbiochem, La Jolla, CA, USA. Curcumin was a gift from Unico Pharmaceuticals, Ludhiana, India. ELISA kits were purchased from BD PharMingen, San Diego, CA, USA. One-step reverse transcription (RT)–PCR kit was from GENSET Singapore Biotech. Pvt Ltd, Singapore. SP600125, SB202190 and PD98059 were purchased from Calbiochem, La Jolla, CA, USA. Curcumin was a gift from Unico Pharmaceuticals, Ludhiana, India. ELISA kits were purchased from BD PharMingen, San Diego, CA, USA. One-step reverse transcription (RT)–PCR kit was from GENSET Singapore Biotech. Pvt Ltd, Singapore. SP600125, SB202190 and PD98059 were purchased from Calbiochem, La Jolla, CA, USA. Curcumin was a gift from Unico Pharmaceuticals, Ludhiana, India. ELISA kits were purchased from BD PharMingen, San Diego, CA, USA. One-step reverse transcription (RT)–PCR kit was from GENSET Singapore Biotech. Pvt Ltd, Singapore. SP600125, SB202190 and PD98059 were purchased from Calbiochem, La Jolla, CA, USA. Curcumin was a gift from Unico Pharmaceuticals, Ludhiana, India. ELISA kits were purchased from BD PharMingen, San Diego, CA, USA. One-step reverse transcription (RT)–PCR kit was from GENSET Singapore Biotech. Pvt Ltd, Singapore. SP600125, SB202190 and PD98059 were purchased from Calbiochem, La Jolla, CA, USA. Curcumin was a gift from Unico Pharmaceuticals, Ludhiana, India. ELISA kits were purchased from BD PharMingen, San Diego, CA, USA. One-step reverse transcription (RT)–PCR kit was from GENSET Singapore Biotech. Pvt Ltd, Singapore. SP600125, SB202190 and PD98059 were purchased from Calbiochem, La Jolla, CA, USA. Curcumin was a gift from Unico Pharmaceuticals, Ludhiana, India. ELISA kits were purchased from BD PharMingen, San Diego, CA, USA.

RNA isolation, RT and PCR

Total RNA was isolated from the macrophages by Tri reagent in accordance with the supplier's instructions. The RNA was reverse transcribed using a one-step RT–PCR kit and amplified by PCR using the specific murine primers indicated in the Results. The thermo cycle conditions were 28 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, after which an additional extension step at 72°C for 10 min was included. Electrophoresis of amplified cDNA was carried out on a 2% agarose gel and stained with ethidium bromide. The murine primer sequences are as follows: TLR1, forward 5′-GGTATTCATTAGGCAGATC-3′; TLR1, reverse 5′-GGTATTCATTAGGCAGATC-3′; TLR2, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR2, reverse 5′-GGATTCCTTCTATGTTGTA-3′; TLR3, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR3, reverse 5′-GGATTCCTTCTATGTTGTA-3′; TLR4, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR4, reverse 5′-GGATTCCTTCTATGTTGTA-3′; TLR5, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR5, reverse 5′-GGATTCCTTCTATGTTGTA-3′; TLR6, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR6, reverse 5′-GGATTCCTTCTATGTTGTA-3′; TLR7, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR7, reverse 5′-GGATTCCTTCTATGTTGTA-3′; TLR8, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR8, reverse 5′-GGATTCCTTCTATGTTGTA-3′; TLR9, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR9, reverse 5′-GGATTCCTTCTATGTTGTA-3′; TLR10, forward 5′-GGATTCCTTCTATGTTGTA-3′; TLR10, reverse 5′-GGATTCCTTCTATGTTGTA-3′.

Isolation and activation of macrophages

Macrophage monolayers were prepared as described previously (15). Peritoneal exudate cells were harvested from peritoneal lavage using chilled serum-free RPMI 1640 medium and added to wells of 24-well tissue culture plates (Nunc, Denmark). After 2 h of incubation at 37°C in an atmosphere of 5% CO₂, the non-adherent cells were removed by washing (three times) with warm serum-free medium and the adherent cells were further incubated in complete medium overnight to form macrophage monolayers. More than 95% of the adherent cell population was macrophages as determined by morphology and non-specific esterase staining. Cisplatin (2, 5 or 10 µg ml⁻¹) treatment was given for different time intervals in fresh medium. Cisplatin (5 µg ml⁻¹) treatment was observed to be most optimum, as determined for the expression of TLR2, TLR4, MyD88, IRAK1, TRAF6 and IRF3. Similarly, for positive controls, the macrophage monolayers were treated with ligands for TLRs: Zymosan A (10 µg ml⁻¹) (25), PolyI:C (25 µg ml⁻¹) (26), LPS (10 µg ml⁻¹) (27) and CpG DNA (1.5 µM) (28).

To study the expression of phospho-IκB, phospho-JNK, phospho-p38 and phospho-p42/44, macrophage monolayers were serum starved for 6 h. Thereafter, cisplatin (5 µg ml⁻¹) treatment was given for indicated time periods.

In another set of experiments, the macrophage monolayers were pre-incubated with JNK inhibitor SP600125 (10 µM), p38 inhibitor SB202190 (10 µM), p42/44 inhibitor PD98059 (10 µM) and NF-κB inhibitor curcumin (10 µM) for 1 h (29, 30). Thereafter, medium was removed, cells were washed with warm medium and further incubated in fresh medium with cisplatin (5 µg ml⁻¹) for 12 h. Western blotting of samples was done as described. The concentrations of different inhibitors used were most optimum. At these concentrations the inhibitor did not affect the cell viability of macrophages checked up to 48 h of treatment.

For iNOS protein expression, murine peritoneal macrophage monolayers were pre-treated with cisplatin (5 µg ml⁻¹) for 12 h. The cells were washed and further incubated for 24 h with ligands for TLRs: Zymosan A, PolyI:C, LPS, CpG DNA. Western blotting of samples was done as described.
TLR8, reverse 5′-GACCCAGAAGTCTCATGGA-3′; TLR9, forward 5′-CCAGAGCTTCTCAGAACC-3′; TLR9, reverse 5′-GTATAGAAGCTGCGTTGTG-3′; GAPDH, forward 5′-CCTGCGATATTGTTG-3′ and GAPDH, reverse 5′-AACA-
CAGATTGCGATGAA-3′.

The expression of housekeeping gene GAPDH was checked for each set of RT-PCR experiment. The possible contamination of any PCR component was excluded by performing a PCR with these components in the absence of RT product in each set of experiment (negative control).

Preparation of cell lysates and immunoblotting
The macrophage monolayers with or without treatment with cisplatin were washed with ice cold PBS containing 1 mM Na₂VO₄, and then lysed in 50 µl of lysis buffer [20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1 mM Na₃VO₄, 2 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 20 µM leupeptin and 0.15 U ml⁻¹ aprotinin] for 20 min at 4°C. The lysates were centrifuged at 10 000 × g for 10 min and the supernatants were incubated with 5 µl of anti-TLR2 antibody overnight at 4°C with constant shaking. Protein A (5 µl) was added and the samples were incubated for 4 h at 4°C and centrifuged at 10 000 × g. Thereafter, pellets were washed five times with 1 ml lysis buffer by centrifugation. The pellets were suspended in 50 µl of lysis buffer and added equal volume of sample buffer. Samples were boiled for 3 min at 95°C, centrifuged at 10 000 × g and the supernatants were run on 10% SDS–PAGE. Western blotting of samples for TLR1 or TLR6 was done as described.

Immunoprecipitation
The macrophage monolayers treated with cisplatin (5 µg ml⁻¹) were lysed with lysis buffer and 100 µl cell lysates were incubated with 4 µg protein A and incubated at 4°C for 1 h. The cell lysate was then centrifuged at 10 000 × g for 10 min. Supernatants were incubated with 5 µl of anti-TLR2 antibody overnight at 4°C with constant shaking. Protein A (5 µg) was added and the samples were incubated for 4 h at 4°C and centrifuged at 10 000 × g. Thereafter, pellets were washed five times with 1 ml lysis buffer by centrifugation. The pellets were suspended in 50 µl of lysis buffer and added equal volume of sample buffer. Samples were boiled for 3 min at 95°C, centrifuged at 10 000 × g and the supernatants were run on 10% SDS–PAGE. Western blotting of samples for TLR1 or TLR6 was done as described.

Assays for TNF-α, IFN-γ, IL-1β and IL-12
Murine peritoneal macrophages were pre-treated with cisplatin (5 µg ml⁻¹) for 12 h. The cells were washed and further treated for 16 h with TLR2 ligand, Zymosan A (10 µg ml⁻¹); TLR3 ligand, PolyI:C (25 µg ml⁻¹); TLR4 ligand, LPS (10 µg ml⁻¹) or TLR9 ligand, CpG DNA (1.5 µM). Supernatants were collected and TNF-α, IFN-γ, IL-1β and IL-12 were measured by commercial ELISA kits from BD PharMingen.

Statistical analysis
Statistical analysis between different groups was analyzed by analysis of variance.

Results
Dose kinetics of cisplatin
The macrophage monolayers (10⁶ cells per well) were treated with 2, 5 or 10 µg ml⁻¹ of cisplatin for 3–18 h. It is observed that 5 µg ml⁻¹ of cisplatin was most optimum, as it induced a sustained gene and protein expression of TLR2 and TLR4 from 3 to 18 h (Figs 1 and 2). With 2 µg ml⁻¹ of cisplatin treatment, TLR2 gene expression was only observed at 3 h and for TLR4 3–6 h (Fig. 1). The protein expression was observed at 12–18 h for TLR2 and only at 18 h for TLR4 (Fig. 2). With 10 µg ml⁻¹ of cisplatin treatment, the gene expression for TLR2 and TLR4 was only observed at 3 h (Fig. 1). TLR2 and TLR4 proteins were expressed at basal levels in macrophages treated with 10 µg ml⁻¹ (Fig. 2).

Surprisingly, MyD88, IRAK1, TRAF6 and IRF3 were not expressed in macrophages treated with 2 or 10 µg ml⁻¹ of cisplatin (Figs 3 and 4). Whereas with 5 µg ml⁻¹ of cisplatin treatment, a sustained expression of MyD88, IRAK1, TRAF6 and IRF3 is observed (Figs 3 and 4).

Viability of macrophages as measured with MTT was almost 100% with 2 and 5 µg ml⁻¹ of cisplatin for 24 h, whereas with 10 µg ml⁻¹ of cisplatin treatment the viability was reduced by 15–20% (data not shown). It is therefore
Cisplatin induces expression of TLRs in macrophage

Concluded that 5 μg ml⁻¹ of cisplatin is the most optimum dose and was used in all further experiments.

Expression of TLR6 in macrophages after cisplatin (5 μg ml⁻¹) treatment

Maximum expression of TLR6 mRNA was seen at 12 h (Fig. 5a) and TLR6 protein at 18 h of cisplatin treatment (data not shown).

To check whether, in addition to enhanced expression of TLR2, cisplatin also induces dimerization of TLR2 with TLR1 or with TLR6, immunoprecipitation experiments were done. Cell lysates were immunoprecipitated with anti-TLR2 and immunoblotted with either anti-TLR1 or anti-TLR6 antibodies. Only the expression of TLR6 was observed in the TLR2 immunoprecipitates by western blotting. Maximum expression was observed at 18 h (Fig. 5b).

Expression of TLR3, -5, -7, -8 and -9 in macrophages after cisplatin (5 μg ml⁻¹) treatment

The maximum expression of TLR5, -7 and -8 mRNA was observed at 3–6 h (Fig. 6), whereas the maximum expression of TLR3 and TLR9 mRNA was seen at 12 h (Figs 7 and 8). Maximum expression of TLR3 protein was at 12 h (Fig. 7), TLR7 from 3 to 12 h (Fig. 6) and TLR5, -8 and -9 between 12 and 18 h (Figs 6 and 8). Specific known TLR ligands were used as positive control.

Immunoprecipitation and western blot analysis of TLR4–MyD88

The association of TLR4 and MyD88 was also checked by immunoprecipitation. Maximum expression of MyD88 was observed at 18 h (Fig. 9) in cell lysates of cisplatin (5 μg ml⁻¹)-treated macrophages immunoprecipitated with anti-TLR4 antibody.

Activation of phospho-IκB, phospho-JNK, phospho-p38 and phospho-p42/44

The expression of phospho-IκB was observed at 30–60 min (Fig. 10) in cisplatin (5 μg ml⁻¹)-treated macrophages. The expression of phospho-JNK and phospho-p38 was maximum between 15 and 30 min, whereas the expression of phospho-p42/44 was consistent from 5 to 60 min (Fig. 11). LPS was used as positive control.

TLR2, -3, -4 and -9 protein expression on treatment with SP600125, SB202190, PD98059 and curcumin

It was observed that there is a significant decrease in the expression of TLR2, -3, -4 and -9 in macrophages pre-treated with SP600125, SB202190, PD98059 and curcumin and subsequently incubated for 12 h in the presence of cisplatin (5 μg ml⁻¹) (Fig. 12).

Expression of iNOS protein on treatment with TLR ligands after cisplatin priming

There is significantly enhanced expression of iNOS protein when macrophages were pre-treated with cisplatin (5 μg ml⁻¹) for 12 h and then subsequently exposed to TLR ligands: Zymosan A, Poly:1:C, LPS and CpG DNA (Fig. 13).

Expression of pro-inflammatory cytokines on treatment with TLR ligands after cisplatin priming

There is significantly enhanced production of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β and IL-12) when macrophages were pre-treated with cisplatin (5 μg ml⁻¹) for 12 h and then subsequently exposed to TLR ligands: Zymosan A, Poly:1:C, LPS and CpG DNA (Fig. 14).

Discussion

In this paper, we report the enhanced expression and up-regulation of TLRs and its associated proteins in macrophages on treatment with anti-cancer drug, cisplatin. The effect of cisplatin on expression of TLR2, TLR4 and downstream signaling molecules was checked with different doses of the drug—2, 5 and 10 μg ml⁻¹. Five micrograms per milliliter dose was found to be most optimum. With 5 μg ml⁻¹ dose, maximum transcription of TLR2 gene was at 3 h and TLR4 gene at 3–6 h. The transcription of TLR2 and TLR4 genes was sustained, though with gradual decrease from 3 to 18 h (Fig. 1). In contrast, with 2 and 10 μg ml⁻¹ dose of cisplatin, the transcription of TLR2 and TLR4 genes was only between 3 and 6 h (Fig. 1). TLR2 and TLR4 protein expression was observed between 3 and 18 h of cisplatin (5 μg ml⁻¹) treatment (Fig. 2). With 2 μg ml⁻¹ dose, TLR2 protein was expressed between 12 and 18 h, while TLR4 was only expressed at 18 h of treatment (Fig. 2). Ten micrograms per milliliter of cisplatin did not induce enhanced expression of TLR2 or TLR4 protein (Fig. 2).

Fig. 3. Western blot analysis of MyD88 (a) IRAK1 (b) and TRAF6 (c) expression in macrophages after 2, 5 and 10 μg ml⁻¹ of cisplatin treatment. Lane-1, untreated macrophages; lane-2, macrophages treated for 3 h; lane-3, macrophages treated for 6 h; lane-4, macrophages treated for 12 h; lane-5, macrophages treated for 18 h; lane-6, macrophages treated for 24 h and lane-7, macrophages treated with LPS. The figure is representative of three independent experiments with similar result.
Surprisingly, the macrophages treated with 2 and 10 μg ml⁻¹ of cisplatin did not show the expression of downstream signaling molecules, MyD88, IRAK1, TRAF6 and IRF3, whereas the macrophages treated with 5 μg ml⁻¹ of cisplatin.

Fig. 4. Western blot analysis of IRF3 expression in macrophages after 2, 5 and 10 μg ml⁻¹ of cisplatin treatment. Lane-1, untreated macrophages; lane-2, macrophages treated for 18 h; lane-3, macrophages treated for 24 h and lane-4, macrophages treated with LPS. The figure is representative of three independent experiments with similar result.

Fig. 5. (a) RT-PCR analysis of TLR6 mRNA expression in macrophages after cisplatin (5 μg ml⁻¹) treatment. Lane-1, untreated macrophages; lane-2, macrophages treated for 3 h; lane-3, macrophages treated for 6 h; lane-4, macrophages treated for 12 h; lane-5, macrophages treated for 18 h and lane-6, macrophages treated with Zymosan A. The figure is representative of three independent experiments with similar result. (b) Western blot analysis of TLR6 protein expression in macrophages after cisplatin (5 μg ml⁻¹) treatment on immunoprecipitation with TLR2 antibody. Lane-1, untreated macrophages; lane-2, macrophages treated for 3 h; lane-3, macrophages treated for 6 h; lane-4, macrophages treated for 12 h; lane-5, macrophages treated for 18 h and lane-6, macrophages treated with Zymosan A. The figure is representative of three independent experiments with similar result.

Fig. 6. RT-PCR/western blot analysis of TLR5, -7 and -8 mRNA/protein expression in macrophages after cisplatin (5 μg ml⁻¹) treatment. Lane-1, untreated macrophages; lane-2, macrophages treated for 18 h; lane-3, macrophages treated for 12 h and lane-5, macrophages treated for 18 h. The figure is representative of three independent experiments with similar result.

Fig. 7. RT-PCR/western blot analysis of TLR3 mRNA/protein expression in macrophages after cisplatin (5 μg ml⁻¹) treatment. Lane-1, untreated macrophages; lane-2, macrophages treated for 3 h; lane-3, macrophages treated for 6 h; lane-4, macrophages treated for 12 h; lane-5, macrophages treated for 18 h and lane-6, macrophages treated with PolyI:C. The figure is representative of three independent experiments with similar result.

Fig. 8. RT-PCR/western blot analysis of TLR9 mRNA/protein expression in macrophages after cisplatin (5 μg ml⁻¹) treatment. Lane-1, untreated macrophages; lane-2, macrophages treated for 3 h; lane-3, macrophages treated for 6 h; lane-4, macrophages treated for 12 h; lane-5, macrophages treated for 18 h and lane-6, macrophages treated with Cpg DNA. The figure is representative of three independent experiments with similar result.

Fig. 9. Western blot analysis of MyD88 protein expression in macrophages after cisplatin (5 μg ml⁻¹) treatment on immunoprecipitation with TLR4 antibody. Lane-1, untreated macrophages; lane-2, macrophages treated for 3 h; lane-3, macrophages treated for 6 h; lane-4, macrophages treated for 12 h; lane-5, macrophages treated for 18 h and lane-6, macrophages treated for 24 h and lane-7, macrophages treated with LPS. The figure is representative of three independent experiments with similar result.

Fig. 10. Western blot analysis of phospho-I-κB expression in macrophages after cisplatin (5 μg ml⁻¹) treatment. Lane-1, untreated macrophages; lane-2, macrophages treated for 30 min; lane-3, macrophages treated for 60 min and lane-4, macrophages treated with LPS. The figure is representative of three independent experiments with similar result.
induced consistently high expression of MyD88, IRAK1, TRAF6 and IRF3 (Figs 3 and 4). This observation suggests that TLRs downstream signaling pathways are not activated in macrophages treated with 2 and 10 \( \mu \)g/ml of cisplatin, at least with reference to TLR2 and TLR4.

TLRs are pattern recognition receptors that have key role in detecting microbes and initiating inflammatory responses (3). Research over past decades has shown that TLRs recognize a much larger repertoire of molecules than previously envisaged (5, 6). However, it is not clear how a restricted family of receptors recognize such a large repertoire of stimuli. Conversely, it has also been shown that the same ligand may be recognized by more than one TLR. For example, it has been reported that there is up-regulation of surface TLR2 in response to PolyI:C, LPS, R848 and CpG DNA (which are ligands for TLR3, -4, -7 and -9, respectively) in macrophages (26). Lipid-based structures are recognized by TLR2 (in combination with TLR1 or TLR6) and TLR4. Viral and/or bacterial nucleic acids are recognized by TLR3, -7, -8 and -9 (31). TLR7 and TLR8 are highly conserved proteins and recognize the same ligand in many cases (31, 32). TLR9 has shown to be up-regulated in response to LPS via the activation of NF-\( \kappa \)B, ERK and p38 MAP kinase signal pathways (33).

TLR2 has been reported to activate innate immune response through an extraordinary array of molecules including peptidoglycan from gram-positive bacteria, bacterial lipoproteins, mycobacterial cell wall lipoarabinomannan and glycosylphosphatidylinositol lipid from *Trypanosoma cruzi* (34, 35). TLR1 or TLR6 functionally associates with TLR2 to discriminate the different ligands (35).

TLR4 has been best characterized as a receptor for gram-negative bacterial LPS (36, 37). In addition, TLR4 is implicated in the recognition of a plant product—taxol, endogenous ligands such as heat shock proteins (Hsp60 and Hsp70) (38, 39), the extra domain A of fibronectin, oligosaccharides of hyaluronic acid, heparin sulphate and fibrinogen (40).

Though several microbial products appear capable of stimulating inflammatory responses through TLR2 and TLR4, the microbial targets identified for other Toll receptors remain elusive. TLR3 has shown to be a receptor for double-stranded viral RNA (26), while TLR5 and TLR9 get activated by flagellin and unmethylated CpG DNA, respectively (41–43). Murine TLR7 recognizes imidazoquinolines and
loxoribine (32). Imidazoquinolines and loxoribine are structurally related to guanosine nucleotide.

The TLR family is the essential recognition and signaling component of mammalian host defense. Toll signaling and activation of NF-κB originates from the conserved TIR domain, which mediates the recruitment of the TIR domain containing adaptor molecule, MyD88. The recruitment of MyD88 to proximal TIR domain of activated TLRs allows for the interaction and activation of the IL-1R-associated kinase (IRAK) family members and subsequent activation of TRAF6. These events result in NF-κB activation via the IκB kinase α-β-γ complex (11, 44).

TLR3 and TLR4 can activate MyD88-dependent and MyD88-independent responses, leading to the production of IFN-β (45). The production of IFN-β requires the co-ordinate activation of IRF3, NF-κB and ATF–c-Jun (46). In the present investigations, it is observed that the cisplatin (5 μg ml⁻¹) treatment of macrophages in addition to enhancing the expression of TLRs also induces the expression of adaptor proteins MyD88, IRAK4, TRAF6 and activation of transcription factor IRF3 and NF-κB (phosphorylation of IκB). However, it is important to appreciate the sequence of events. The induction of TLR transcription is observed at 3 h and the protein expression at 12 and 18 h. The maximal expression of the adaptor proteins and transcription factor IRF3 is observed at 18–24 h of cisplatin treatment.

It is documented that TLR signaling involves the activation of MAP kinase pathway and activation of transcription factor NF-κB. Conversely, the expression of TLR genes can also be regulated by the MAP kinase and NF-κB-induced expression of TLR genes (47). The role of NF-κB and p38 MAP kinase in Gal-lectin-induced expression of TLR2 has been reported (47). To test whether the MAP kinases and NF-κB pathway have a role in cisplatin-induced expression of TLRs, the induction of MAP kinase and activation of NF-κB were studied in macrophages on cisplatin treatment. It is observed that cisplatin induced the activation of NF-κB as checked by the expression of phospho-IκB (Fig. 10). Cisplatin also induced the enhanced expression of phospho-JNK, phospho-p38 and phospho-p42/44 (Fig. 11). Macrophages pre-treated with inhibitors of MAP kinase pJNK, SP600125; p38, SB202190; p42/44, PD98059 and NF-κB, curcumin (30) when subsequently treated with cisplatin for 12 h showed significant inhibition in the expression of TLR2 and TLR4 and almost complete inhibition in the expression of TLR3 and TLR9 (Fig. 12). These observations clearly suggest the participation of MAPKs in the regulation of the cisplatin-induced expression of TLR2, -3, -4 and -9.

The expressions of iNOS protein and pro-inflammatory cytokine production were significantly enhanced when the macrophages primed with cisplatin were subsequently treated with ligands for TLRs (Figs 13 and 14). The data provide evidence that cisplatin, in addition to increasing the expression of TLRs in macrophages, primes them much more functionally responsive to TLR ligands-induced production of cytokines and NO.

The molecular mechanism of TLR-mediated recognition is one of the most challenging issues in Toll biology. TLRs have been shown to get activated by a plethora of compounds of microbial origin, but to date very few synthetic compounds...
have been shown to activate Toll-signaling pathway. The significance of the present studies is that cisplatin, which is a small inorganic compound, induces the enhanced expression of TLRs and its associated proteins. However, at the moment we find it difficult to provide any explanation why 2 and 10 μg ml⁻¹ of cisplatin do not induce the expression of downstream signaling molecules—MyD88, IRAK1, TRAF6 and IRF3.

Although present studies show that cisplatin (5 μg ml⁻¹) can stimulate expression of TLRs and its associated proteins, it is premature to hypothesize that it is also a ligand for TLRs. As to how and why cisplatin activates TLRs remains to be elucidated. Four decades after its discovery, Rosenberg et al. (48), cisplatin has shown its potential usefulness and is a drug of choice for number of different types of cancers (13, 14). The novel functions of TLRs in tumor biology (49, 50) suggest a new class of therapeutic targets of cancers (13, 14). The novel functions of TLRs in tumor biology (49, 50) suggest a new class of therapeutic targets. As to how and why cisplatin activates TLRs will be of interest to further investigate their increased susceptibility to TLR ligands in macrophages.

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Abbreviations

cisplatin cis-diaminedichloroplatinum (II)  
MAP mitogen-activated protein  
MyD88 myeloid differentiation factor 88  
NF-kB nuclear factor-kB  
NO nitric oxide  
RT reverse transcription  
TLR Toll-like receptor  
TNF-tumor necrosis factor  
TRAF6 tumor necrosis factor receptor-associated factor 6

References


