2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) Alters the Regulation and Posttranslational Modification of p27<sup>kip1</sup> in Lipopolysaccharide-Activated B Cells

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters B-cell differentiation, as evidenced by a marked decrease in immunoglobulin M (IgM) secretion and in the number of antibody-forming cells (AFC) induced by antigenic stimulation. The objective of the present studies was to evaluate the effect of TCDD on the level of p27<sup>kip1</sup>, a cyclin-dependent kinase inhibitor that is a critical regulator of cellular differentiation. In the well-characterized B-cell line, CH12.LX, a modest decrease in p27<sup>kip1</sup> was observed during the initial 24-h post-LPS (lipopolysaccharide) activation, which then gradually increased above background at 48 and 72 h. Conversely, in the presence of TCDD, p27<sup>kip1</sup> was not induced and remained unchanged from LPS unstimulated cells throughout the entire 72-h period post-LPS activation. In addition, Western blotting revealed that TCDD treatment altered the profile of p27<sup>kip1</sup> migration as compared to the LPS-activated control. Time-of-addition studies demonstrated that the greatest sensitivity of p27<sup>kip1</sup> to TCDD treatment occurred within the initial 24-h post-LPS activation. Interestingly, LPS-induced Ig κ light chain and IgM secretion also exhibited the greatest period of sensitivity (i.e., inhibition) to TCDD during the first 24-h post-LPS activation. In addition, TCDD markedly suppressed the LPS-induced differentiation of CH12.LX cells into IgM secreting AFC, with a modest but cumulative effect on cell proliferation over a 72-h period. Collectively, these findings show that TCDD altered the cellular concentration and posttranslational modification of p27<sup>kip1</sup> in this activated B-cell line model, which occurred concomitantly with altered B-cell differentiation and suggests that cyclin-dependent kinase inhibitors may be an important intracellular target in TCDD-mediated inhibition of B-cell differentiation.

Key Words: TCDD; B cell; p27<sup>kip1</sup>; LPS; cyclin-dependent kinase inhibitor.

The B cell has been widely established as a sensitive immunological target of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), as evidenced by a marked suppression of immunoglobulin (Ig) production (Dooley and Holsapple, 1988; Luster et al., 1988; Morris and Holsapple, 1991; Morris et al., 1993; Tucker et al., 1986). The molecular mechanism(s) responsible for the altered B-cell function after TCDD treatment remains poorly understood due to the overall complexity of the process by which a resting B cell, which has been activated by an antigen, differentiates into an immunoglobulin-secreting plasma cell. Recent evidence from this laboratory suggests that the inhibition by TCDD of LPS (lipopolysaccharide)-activated Ig secretion (specifically IgM) is, at least in part, transcriptionally regulated through an aryl hydrocarbon receptor (AhR)–dependent decrease in μ heavy chain gene expression (Sulentic et al., 1998, 2000). The emerging view, concerning when during antigen-induced B-cell progression, TCDD acts to impair Ig production strongly suggests the involvement of at least two critical stages: cellular activation and differentiation. In contrast, B-cell proliferation has consistently been reported to be relatively refractory to the inhibitory effects of TCDD at concentrations that markedly suppress Ig production (Dooley and Holsapple, 1988; Luster et al., 1988; Morris and Holsapple, 1991; Morris et al., 1993; Tucker et al., 1986).

A critical aspect to the bridging of early cellular activation events with subsequent B-cell differentiation occurs through the appropriate regulation of the cell cycle. In response to antigen-specific and T-cell-derived activation signals, B cells undergo an initial proliferative burst followed by cell-cycle arrest and differentiation into Ig secreting cells (Paul and Seder, 1994; Vazquez et al., 1986). Cell cycle progression is controlled by a family of serine/threonine kinases called the cyclin-dependent kinases (cdk). Activation of cdk is regulated through association with regulatory subunits called cyclins. Unlike cdk, which are constitutively expressed, cyclin expression oscillates with respect to the cell cycle, allowing for controlled activation of cdk as well as substrate specificity (Fisher, 1997; Morgan, 1995). Cyclin-cdk complexes are controlled through several mechanisms, including accumulation of cyclins, positive and negative phosphorylation or dephosphorylation of critical tyrosine and serine/threonine residues, and...
specific cdk inhibitors (CKI) (Morgan 1995; Sherr and Roberts 1995). Several recent studies demonstrate that CKI proteins are important for B-cell activation and differentiation (Bouchard et al., 1997; Schrantz et al., 2000). In addition, TCDD and other AhR ligands have previously been demonstrated to alter the cellular concentration of several regulatory cell-cycle proteins, including CKI, in hepatocytes, thymocytes, and in several immature B-cell lines (Kolluri et al., 1999; Rinninger et al., 1997; Ryu et al., 2003). Based on the identification of a DRE (dioxin response element) in the p27kip1 promoter and the demonstration of p27kip1 modulation by TCDD, the objective of the present studies was to investigate the effects of TCDD on p27kip1 in activated B-cells (Kolluri, 1999; Kwon et al., 1996). For these studies, the CH12.LX B-cell line was employed due to our previous characterization, showing that this cell line closely mimics the response of primary B cells to LPS activation (i.e., robust induction of IgM secretion) and sensitivity to inhibition by TCDD (Sulentic et al., 1998, 2000). In the present study we show that the cellular concentration of p27kip1 was significantly altered by TCDD in LPS-activated CH12.LX cells and was closely associated with the disruption of Ig regulation. These findings are concordant with several recent reports demonstrating a link between p27kip1 and differentiation in other cellular models (Bouchard et al., 1997; Durand et al., 1997; Hengst and Reed, 1996: Kranenburg et al., 1995; Schrantz et al., 2000). Therefore, an altered effect on CKI regulation by TCDD may represent a critical event contributing to altered B-cell differentiation and Ig regulation.

MATERIALS AND METHODS

Chemicals. TCDD was purchased from AccuStandard, Inc. (New Haven, CT) in 100% dimethyl sulfoxide (DMSO). The certificate of product analysis stated that the purity of TCDD was 99.1%, as determined by AccuStandard using GC/MS. DMSO and LPS were purchased from Sigma-Aldrich (St. Louis, MO).

Cell line. The CH12.LX B cell line derived from the murine CH12 B-cell lymphoma (Arnold et al., 1983) has been previously characterized by Bishop and Haughton, (1986), and was a generous gift from Dr. Geoffrey Haughton (University of North Carolina). CH12.LX cells were grown in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with heat-inactivated 10% bovine calf serum (Hyclone, Logan, UT), 13.5 mM HEPES, 23.8 mM sodium bicarbonate, 2.0 U/ml penicillin, 100 µg/ml streptomycin, 2.0 mM L-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 50 µM β-mercaptoethanol. Cells were maintained at 37°C in an atmosphere of 5% CO2. The day before treatment, CH12.LX cells (2.5 × 10^6 cells/ml) were cultured in treatment media (growth media as stated above but with 5% heat-inactivated bovine calf serum [BCS]) overnight at 37°C in an atmosphere of 5% CO2.

Western blot analysis. Western blot analysis was performed on cell lysates from CH12.LX cells. Cell lysates were prepared in HEDG (25 mM HEPES, 2 mM EDTA, 1 mM DTT, and 10% glycerol), sonicated 3 times for 5 s to break open the nuclei, and centrifuged at 100,000 × g for 1 h at 4°C. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA). Where indicated, cell lysates were incubated with 400 U/ml protein phosphatase (New England Biolabs, Beverly, MA) for 30 min at 30°C. Cell lysate proteins were resolved by denaturing SDS-PAGE (Life Science Products, Inc., Denver, CO). The percent acrylamide, which ranged between 10 and 14%, is indicated in the figure legends. The proteins were transferred to nitrocellulose following electrophoresis (Amersham Pharmacia Biotech, Arlington Heights, IL). Protein blots were blocked in BLOTTO buffer (4% low-fat dry milk/1% BSA in 0.1% Tween-20 TBS) for 1–2 h at room temperature. Rabbit anti-mouse p27kip1 and rabbit anti-mouse phospho-Ser10 p27kip1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). In addition, a second rabbit anti-mouse phospho-Ser10 p27kip1 antibody was purchased from Zymed (San Francisco, CA). Sheep HRP-conjugated anti-mouse IgG and donkey HRP-conjugated anti-rabbit IgG were purchased from Amersham Pharmacia Biotech. Immunooehanical staining was performed as previously described (Williams et al., 1996). Detection was performed using the ECL method (Amersham Pharmacia Biotech). Where indicated, protein blots were stripped and reprobed. Stripping was performed by submerging the membrane in stripping buffer [100 mM 2-ME, 2% SDS, and 62.5 mM Tris (pH 6.7)] for 30 min at 50°C. Protein blots were then washed, blocked, and reprobed as stated above. Optical density for the protein of interest was measured by densitometry using a model 700 imaging system (Bio-Rad). All blots were stripped and normalized by reprobing for β-actin, using an anti-mouse β-actin antibody (Sigma-Aldrich).

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Quantitative RT-PCR was performed as previously described (Williams et al., 1996) with several modifications. Briefly, total RNA from each sample was isolated using Tri Reagent (Sigma-Aldrich). RNA samples were first analyzed for DNA contamination by PCR analysis without reverse transcriptase. Total DNA-free RNA (100 ng) and internal standard (rcRNA) were reverse transcribed simultaneously in the same reaction tube. Final reaction concentrations for the kip1 PCR reaction were 4 nM MgCl2, and 2.5 units Taq DNA polymerase (Promega, Madison, WI). Samples were cycled 30 times, with each cycle consisting of 94°C for 15 s, 57°C for 30 s, and 72°C for 45 s. PCR products were visualized by ethidium bromide staining. Quantitation was performed by assessing the optical density for both the target gene and internal standard using a Gel Doc 1000 video imaging system (BioRad). The number of transcripts was calculated from a standard curve generated from the density ratio between the gene of interest and a specific internal standard concentration. Primer sequences for kip1 are as follows: FP, CCGAGGAGGAATGTCAAACG; RP, CCGAGGCTTTAGTCTGAAAAG, with a product size of 223 bp.

DNA synthesis. CH12.LX cells were pre-incubated overnight at 37°C in 5% CO2, then treated with TCDD (1–30 nM) or vehicle (0.02% DMSO) followed by LPS (5 µg/ml) stimulation at 37°C in 5% CO2. Cells were pulsed with 1 Ci of [3H]-thymidine (NEN, Boston, MA) after 18 h of stimulation and were harvested at 24 h. [3H]-thymidine incorporation was measured using a Arebion scintillation analyzer and expressed as mean counts per min (CPM) ± SD.

Antibody-forming-cell (AFC) assay and cell proliferation. One milliliter of CH12.LX cells at a density of 2.5 × 10^5 cells/ml was added to each well of a 24-well culture plate and pre-incubated overnight at 37°C in 5% CO2. The next day the cells were treated with TCDD (0.01–10 nM) or vehicle (0.01% DMSO) and stimulated with LPS (5 µg/ml) in quadruplicate, for each treatment group, to determine the cell counts and the number of AFC. To determine the number of viable cells, 22,500 pronase units (CalBioChem, La Jolla, CA) were added to each sample and incubated for 10 min to break up cell clumps and remove nonviable cells. Ten milliliters of Isoton II (Coulter Corp., K. Aleah, FL) was then added to each sample and the cells were enumerated on a model Z1 Coulter Particle Counter (Coulter Corp.). Cell viability was monitored throughout the course of the entire experiment by trypan blue (Sigma-Aldrich) exclusion. Since CH12.LX cells secrete antibodies directed against an epitope on the sheep erythrocyte (Bishop, 1986; Mercolino, 1986) 72 h post-LPS treatment, the number of AFCs was assayed using a modification of the Jerne plaque assay as previously described (Delaney and Kaminski 1993).

ELISA (enzyme-linked immunosorbent assay). After a 48-h incubation at 37°C in 5% CO2, supernatants were harvested from naive or LPS (5 µg/ml)-activated CH12.LX cells that were treated with 10 nM TCDD or vehicle (0.01% DMSO). Supernatants were analyzed for IgM by sandwich ELISA, as
RESULTS

TCDD Alters the Cellular Concentration and Posttranslational Modification of p27\textsuperscript{kip1} in LPS-Activated B Cells

As demonstrated in primary B cells (Schrantz, et al., 2000), LPS stimulation of the CH12.LX B cells caused an initial downregulation of p27\textsuperscript{kip1} for the first 24 h, which, at 48 and 72 h, was induced above the unstimulated time-0 control (Fig. 1). The mean change from naïve non-LPS activated cells (i.e., normalized to 1.0) from four separate experiments for vehicle versus TCDD-treated cells were 0.60 ± 0.10 vs. 1.44 ± 0.09 at 24 h; 3.04 ± 0.72 vs. 1.40 ± 0.12 at 48 h; and 6.11 ± 1.1 vs. 1.674 ± 0.06 at 72 h. Clearly, TCDD altered the LPS-induced change in p27\textsuperscript{kip1} throughout the 72-h time period, resulting in a similar p27\textsuperscript{kip1} concentration as measured in CH12.LX cells not activated with LPS (Fig. 1). The effect of TCDD on LPS-induced modulation of p27\textsuperscript{kip1} was concentration-dependent as measured at 24 h (data not shown) and 48 h (Fig. 2). Although, the cellular concentration of p27\textsuperscript{kip1} may be regulated, in part, at the level of transcription, current evidence supports posttranslational modification via phosphorylation as a primary mechanism of p27\textsuperscript{kip1} regulation. Indeed, the effects of TCDD and LPS on kip1 expression, as determined by quantitative RT-PCR analysis, were modest but followed a similar trend to that observed with p27\textsuperscript{kip1} protein (compare Figs. 1 and 3).

Phosphorylation of p27\textsuperscript{kip1} can lead either to rapid degradation through ubiquitination or increased stability, depending on the specific amino acid phosphorylated on p27\textsuperscript{kip1} (Ishida et al., 2002, 2000; Montagnoli et al., 1999). To better resolve, the potential differences in posttranslational modification of p27\textsuperscript{kip1} by Western blotting and therefore determine the effect of LPS and TCDD on p27\textsuperscript{kip1} regulation, the percent acrylamide in the SDS–PAGE gel was decreased as compared to the initial Western-blot analysis displayed in Figure 1. Under these modified conditions, two distinct p27\textsuperscript{kip1} immunoreactive bands were visualized (Fig. 4A). A slower-migrating 26.0 kDa form of p27\textsuperscript{kip1} similar to the previously identified Ser\textsuperscript{10} phosphorylated-p27\textsuperscript{kip1} was observed (Ishida et al., 2000). At 48 and 72 h, LPS stimulation caused an increase in the slower-migrating 26.0 kDa form of p27\textsuperscript{kip1}; whereas, TCDD cotreatment produced an increase in the faster-migrating 23.6 kDa form of p27\textsuperscript{kip1} and a marked decrease in the slower-migrating 26.0 kDa form (Figs. 4A and 4C). In an attempt to confirm that the slower-migrating p27\textsuperscript{kip1} immunoreactive band was in fact a Ser\textsuperscript{10} phosphorylated form of p27\textsuperscript{kip1}, Western analysis was performed using two different anti-phospho-Ser\textsuperscript{10} p27\textsuperscript{kip1} antibodies. Although both antibodies reacted with bands that were...

![FIG. 1. TCDD alters cellular p27\textsuperscript{kip1} protein in LPS-activated CH12.LX cells. CH12.LX cells were treated with TCDD (10 nM) or vehicle (0.01% DMSO) and then activated with LPS (5 μg/ml). Cell lysates were isolated at 0, 24, 48, or 72 h. Proteins (25 μg/lane) were resolved on a 14% SDS–PAGE gel and probed with an anti-p27\textsuperscript{kip1} antibody. As a loading control, blots were stripped and reprobed for β-actin. Immunoblots were quantified for p27\textsuperscript{kip1} by densitometry. The adjusted volume (OD × area) for all samples was normalized to the loading control (β-actin) and expressed as fold change from time 0. Results are representative of four separate experiments.](https://academic.oup.com/toxsci/article-abstract/75/2/333/165879/2-3-7-8-Tetrachlorodibenzo-p-dioxin-TCDD-Alters)
significantly larger than 27 kDa, and likely ubiquitin-tagged Ser10 phosphorylated-p27kip1, neither antibody recognized the slower-migrating 26.0 kDa form of p27kip1, originally suspected of being phosphorylated Ser10 (data not shown). Western blotting for p27kip1 was also performed on nuclear and cytosolic fractions prepared from CH12.LX cells at 24 and 48 h post-LPS activation in the presence and absence of 10 nM TCDD (data not shown). The cellular concentration of p27kip1 in the cytosolic fractions was similar to that observed in the whole cell preparations shown in Figure 4. Western blotting for nuclear p27kip1 revealed multiple bands, again likely reflecting multiple forms of phosphorylated and ubiquitin-tagged p27kip1. To further investigate whether the change in the migration of the p27kip1 immunoreactive band was possibly due to changes in the phosphorylation state of p27kip1, the cell lysates were subjected to phosphatase treatment and resolved by SDS-PAGE. Phosphatase treatment of the samples derived from LPS-activated cells at 48 h resulted in a complete disappearance of the 26.0 kDa form and its shift toward a faster migrating 24.7 kDa form of p27kip1. Phosphatase treatment had no effect on the faster-migrating 23.6 kDa form of p27kip1.

These findings confirm the presence of phosphorylated residues in the slower-migrating 26.0 kDa form of p27kip1 (Fig. 4B). These results also suggest that the 26.0-kDa form of p27kip1 possesses either other posttranslational modifications that distinguish it from the 23.6-kDa form of p27kip1 or that there are additional phosphorylated residues that are resistant to λ phosphatase activity.

**TCDD Targets B-Cell Differentiation**

Since p27kip1 is a cell-cycle regulatory protein, the effect of TCDD on DNA synthesis and cellular proliferation of the CH12.LX cells was examined by measuring [3H]-thymidine incorporation and by determining the actual cell number, respectively, following treatment with LPS and TCDD. LPS modestly increased [3H]-thymidine incorporation at 24 h, which appeared to be inhibited by TCDD but not in a concentration-dependent manner (Fig. 5). Though not significantly different, a similar effect on cell number was observed at 24 and 48 h (Table 1). At 72 h, LPS stimulation decreased the total number of CH12.LX cells per culture as compared to non-LPS-stimulated cells and is consistent with an increase in the number of cells differentiating into antibody-forming cells. The most pronounced effect of TCDD on cell number was observed

![Graph showing effect of TCDD on p27kip1 protein in a concentration-dependent manner in LPS-activated CH12.LX cells.](image)

**FIG. 2.** TCDD alters cellular p27kip1 protein in a concentration-dependent manner in LPS-activated CH12.LX cells. CH12.LX cells were treated with TCDD (10 nM) or vehicle (0.01% DMSO) and then activated with LPS (5 μg/ml). Cell lysates were isolated at 24 and 48 h. Proteins (25 μg/lane) were resolved on a 10% SDS–PAGE gel and probed with an anti-p27kip1 antibody. As a loading control, blots were stripped and reprobed for β-actin. Immunoblots were quantified for p27kip1 by densitometry. The adjusted volume (OD × area) for all samples was normalized to the loading control (β-actin) and expressed as fold change from time 0. The graph represents the results at the 48-h time point. Results are representative of at least three separate experiments.

**FIG. 3.** Effect of TCDD on kip1 mRNA expression in LPS-activated CH12.LX cells. CH12.LX cells were treated with TCDD (10 nM) or vehicle (0.01% DMSO) and then activated with LPS (5 μg/ml). Quantitative RT-PCR analysis for kip1 was performed on RNA extracted at 0, 8, 24, 48, or 72 h following chemical treatment. Transcripts for kip1 are identified on the y-axis as 107 molecules/100 ng total RNA. Results represent the mean ± SE of six separate RNA isolations; * represents values that are significantly different from time 0 at p < 0.05, using Dunnett’s two-tailed t test.

![Graph showing effect of TCDD on kip1 mRNA expression in LPS-activated CH12.LX cells.](image)
at 72 h, and appears to be due to a modest but cumulative increase in doubling time over the 72-h culture period (Table 1). In addition to, and independent of the effect by TCDD on cell number, TCDD also markedly suppressed the differentiation of CH12.LX cells into antibody-forming cells as measured by a plaque forming-cell assay (Table 1). A dramatic inhibition in the intracellular concentration of Ig \( \kappa \) light chain protein was also observed at 48 and 72 h after LPS activation in the TCDD-treated cells (Fig. 6).

TCDD Alters the Cellular Concentration of P27\(^{kip1} \) if Added Prior to the First 24 H after LPS-Stimulation of CH12.LX Cells

Tucker and coworkers demonstrated that inhibition of the antibody-forming cell response by TCDD occurred only when TCDD was added within the first 24 h after addition of antigen (Tucker et al., 1986). Studies were conducted to determine whether the effects of TCDD on the intracellular concentration of Ig \( \kappa \) light-chain protein, IgM secretion, and P27\(^{kip1} \) post-translational modification were also critically dependent on the time of TCDD addition to culture after activation by antigen. The present study shows that TCDD must be added to cultures within 8 h of LPS stimulation for an inhibition of Ig \( \kappa \) light chain protein and IgM secretion (Figs. 7 and 8). TCDD was unable to alter these responses if added 24 h post-LPS stimulation. An effect on P27\(^{kip1} \) posttranslational modification also followed a similar pattern in that TCDD was able to inhibit LPS-induced modification of P27\(^{kip1} \) if added to culture within 8 h of LPS stimulation (Figs. 9A and 9B). TCDD had a markedly decreased effect on the ratio of the two forms of P27\(^{kip1} \) when added at 24 h as compared to 0 and 8 h post-LPS stimulation.

DISCUSSION

The CKI, P27\(^{kip1} \), has been identified as a critical regulator of B-cell differentiation (Bouchard et al., 1997; Durand et al.,...
1997; Hengst and Reed, 1996; Kranenburg et al., 1995; Schrantz et al., 2000). In the present study, cellular p27kip1 was examined in LPS-activated CH12.LX cells by Western-blot analysis. Interestingly, in the presence of TCDD, the basal, time 0 levels of p27kip1 were maintained during LPS activation whereas LPS activation in the absence of TCDD significantly modulated the expression of p27kip1. This is in contrast to previous reports in which TCDD caused an elevation in p27kip1 in hepatocytes (Rininger et al., 1997), 5L hepatoma cells, and fetal thymus (Kolluri et al., 1999). It is notable that one significant difference between the hepatic cellular models and B-cell models is that the hepatic cellular models are terminally differentiated. In contrast, B cells require antigen activation prior to differentiation into Ig-secreting cells. Therefore, the differing effects of TCDD on p27kip1 in the CH12.LX cells vs. the hepatocytes and fetal thymus may relate to the cell type, the specific differentiation process, the state of differentiation the cells are in, and/or the presence of a biological modifier (i.e., LPS). It is notable that Rye and coworkers also observed a decrease in cellular p27kip1 in several pro/pre-B-cell lines after treatment with 7,12-dimethylbenz(a)anthracene, another ligand for the AhR (Ryu et al., 2003).

An involvement of p27kip1 in cellular differentiation has been well established in a variety of cell types, including B cells (Bouchard et al., 1997; Durand et al., 1997; Hengst and Reed, 1996; Kranenburg et al., 1995; Schrantz et al., 2000). In primary B cells, activation stimuli (LPS or CD40 ligand plus IL-10 and IL-2) initially produce a marked inhibition in the intracellular concentration of p27kip1, allowing for entry into the cell cycle. An initial proliferative burst is required for B-cell differentiation and is then followed by an increase in p21Waf1, which is associated with an arrest in the cell cycle allowing for B-cell differentiation (Bouchard et al., 1997; Schrantz et al., 2000). In CH12.LX cells, p21Waf1 was not detected (data not shown). Instead, p27kip1 appears to play a primary role in the differentiation process as suggested by the fact that it was downregulated during the first 24 h post-LPS stimulation and then upregulated during the differentiation phase (24 to 72 h).

### TABLE 1

Effects of TCDD on Cell Number and the Antibody-Forming Cell Response

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>Doubling time (h)</th>
<th>Antibody-forming cells, 10^5 recovered cells</th>
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<tr>
<td>Naive</td>
<td>2.24 ± 0.15</td>
<td>7.12 ± 0.16</td>
<td>16.08 ± 0.60*</td>
<td>15.62 ± 0.35</td>
<td>9.2 ± 1.6*</td>
</tr>
<tr>
<td>Naive + LPS</td>
<td>3.37 ± 0.71</td>
<td>8.30 ± 0.32</td>
<td>14.83 ± 0.33</td>
<td>16.14 ± 0.05</td>
<td>277.0 ± 11.6</td>
</tr>
<tr>
<td>Vehicle + LPS</td>
<td>2.31 ± 0.08</td>
<td>7.04 ± 0.27</td>
<td>13.07 ± 0.32</td>
<td>16.08 ± 0.23</td>
<td>295.4 ± 33.4</td>
</tr>
<tr>
<td>TCDD (nM) + LPS</td>
<td>0.01</td>
<td>2.34 ± 0.29</td>
<td>7.40 ± 0.84</td>
<td>12.60 ± 1.36</td>
<td>16.08 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.53 ± 0.26</td>
<td>7.13 ± 0.23</td>
<td>12.60 ± 0.20</td>
<td>16.44 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.04 ± 0.26</td>
<td>6.65 ± 0.60</td>
<td>9.70 ± 0.50*</td>
<td>17.83 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.06 ± 0.34</td>
<td>5.31 ± 0.73</td>
<td>8.23 ± 1.20*</td>
<td>20.05 ± 0.47</td>
</tr>
</tbody>
</table>

*Values that are significantly different from the vehicle + LPS at p > 0.05.

Note. Results are representative of at least two experiments.

FIG. 6. Effect of TCDD on the amount of Ig κ light chain in LPS-activated CH12.LX cells. CH12.LX cells were treated with TCDD (10 nM) or vehicle (0.01% DMSO) and then activated with LPS (5 μg/ml). Cell lysates were isolated at 0, 24, 48, or 72 h. Proteins (25 μg/lane) were resolved on a 14% SDS–PAGE gel and probed with an anti-IgG antibody that cross reacts with the Ig κ light chain. As a loading control, blots were stripped and reprobed for β-actin. Immunoblots were quantified for Ig κ light-chain by densitometry. The adjusted volume (OD × area) for all samples was normalized to the loading control (β-actin) and expressed as fold change from time 0. Results are representative of at least four separate experiments.
post-LPS stimulation). The absence of p21Waf1 expression in the CH12.LX cells clearly distinguishes this cell line model from primary cells and may be due to the continuous cycling of CH12.LX cells and constitutive repression of certain cell-cycle regulatory proteins. That p27kip1 serves as the primary CKI in CH12.LX cells was in fact advantageous in addressing one of our study objectives, which was to determine whether an association could be established between the inhibition of B cell differentiation into an antibody-secreting cell by TCDD and changes in the regulation of CKIs. In primary cells, a number of CKIs perform distinct, as well as overlapping functions, making it significantly more complex to study their function.

In the present study, a modest decrease in the intracellular concentration of p27kip1 corresponded closely to a modest increase in [3H]-thymidine incorporation. The less-than-prominent nature of these effects on DNA synthesis are not surprising and may be due, in part, to the self-propagating characteristic of this cell line. However, the refractory nature of lymphocyte proliferation to TCDD treatment has also been widely reported in a number of previous studies utilizing primary lymphocytes (Dooley and Holsapple, 1988; Luster et al., 1988; Morris and Holsapple, 1991; Morris et al., 1993; Tucker et al., 1986). To more directly assess the effects of TCDD and LPS treatment on CH12.LX cell proliferation, cell counts were performed at 24-h intervals over a 72-h culture period. These results show that TCDD exerted a modest decrease in the rate of CH12.LX cell proliferation after LPS activation, as evidenced by the increase in doubling time and the very modest decrease in DNA synthesis. Collectively, our interpretation is that TCDD exerted a modest but progressive and cumulative decrease in the rate of CH12.LX cell division after LPS activation that is reflected by a decrease in the total number of cells per culture over time, as presented in Table 1.

Generally, the effects of LPS and TCDD on kip1 mRNA expression appeared to correlate with the changes observed on p27kip1 protein. However, the magnitude of this effect is in contrast to previous findings in other cell types. For example, TCDD treatment induced a five-fold induction in kip1 expression in 5L hepatoma cells. In that study, the authors concluded that increased kip1 expression in 5L hepatoma cells was responsible for the induction of p27kip1 protein (Kolluri et al., 1999). Interestingly, a DRE-like site is present within the kip1 promoter (Kolluri et al., 1999; Kwon et al., 1996); however,
deletion analysis of the \textit{kip1} promoter showed that TCDD induced the promoter through a fragment lacking the DRE-like site but contained several sites that resemble recognition half-sites for the AhR (Kolluri \textit{et al.}, 1999). In the CH12.LX B cells, transcriptional regulation does not appear to account fully for the effects of LPS and TCDD on the cellular concentration of $p27^\text{kip1}$, as evidenced by the overall modest effect produced by LPS and TCDD treatment on $kip1$ mRNA expression. Our findings are, in fact, consistent with the widely held belief that $p27^\text{kip1}$ regulation occurs predominately at the post-translational level through phosphorylation and ubiquitination. Thus far, at least two critical phosphorylation sites on $p27^\text{kip1}$

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure9.png}
\caption{Time of TCDD addition and modulation of $p27^\text{kip1}$ in LPS-activated CH12.LX cells. CH12.LX cells were treated with TCDD (10 nM) or vehicle (0.01% DMSO) at 0, 8, or 24 h after LPS (5 $\mu$g/ml) activation. Cell lysates were isolated at 48 h post-LPS stimulation. (A) Proteins (25 $\mu$g/well) were resolved on a 10% SDS-PAGE gel and probed with an anti-$p27^\text{kip1}$ antibody. As a loading control, blots were stripped and re-probed for $\beta$-actin. (B) Immunoblots were quantified for $p27^\text{kip1}$ by densitometry. The adjusted volume (OD x area) for the two distinct $p27^\text{kip1}$ immunoreactive bands is expressed as the ratio of the slower migrating (26 kDa) form to the faster migrating (23.6 kDa) form. Results are representative of at least three separate experiments.}
\end{figure}
have been identified, Thr\(^{187}\) and Ser\(^{10}\) (Hengst and Reed, 1996; Pagano et al., 1995). It has been reported that phosphorylation by cyclin E/cdk2 on Thr\(^{187}\) leads to a rapid degradation of p27\(^{kip1}\) by the ubiquitin-26S proteasome pathway (Sheaff et al., 1997; Tsvetkov et al., 1999). Conversely, phosphorylation of Ser\(^{10}\) leads to increased stability of p27\(^{kip1}\) (Ishida et al., 2000).

In the present study we demonstrate that TCDD alters the posttranslational modification of p27\(^{kip1}\) in LPS-activated CH12.LX cells. In the absence of TCDD, LPS tended to shift p27\(^{kip1}\) from resolving as a 23.6 kDa protein to a slower migrating 26.0 kDa form, as identified by Western blotting, which was previously demonstrated in several cell types as a hallmark of Ser\(^{10}\) phosphorylation (Ishida et al., 2000). Increased protein stability due to Ser\(^{10}\) phosphorylation is consistent with the increased cellular p27\(^{kip1}\) at 48 and 72 h post-LPS stimulation. However, in spite of the fact that two different anti-phospho-Ser\(^{10}\) p27\(^{kip1}\) antibodies were employed in the present study, neither was able to confirm that the slower migrating 26.0 kDa p27\(^{kip1}\) immunoreactive band was in fact the Ser\(^{10}\) phosphorylated form of p27\(^{kip1}\). Since p27\(^{kip1}\) possesses multiple phosphorylation sites, a more general approach was employed in order to determine whether the changes in the migration of p27\(^{kip1}\) were at all associated with the phosphorylation status of the protein. Specifically, cell extracts from vehicle control CH12.LX cells were, at 48 h after LPS activation, subjected to \(\lambda\) phosphatase treatment and then resolved by SDS–PAGE. The 48-h sample was selected because of the strong shift in p27\(^{kip1}\) to a slower-migrating form at this specific time point. Interestingly, after \(\lambda\) phosphorylation treatment, the slower-migrating 26.0 kDa form of p27\(^{kip1}\) now resolved as a 24.7 kDa protein. No change was observed in the migration of the 23.6 kDa form of p27\(^{kip1}\). Collectively, these findings suggest that the change in the migration of p27\(^{kip1}\) from a 23.6 kDa protein to a 26.0 kDa protein in the LPS-activated CH12.LX cells is due, in part, to phosphorylation at sites other than Ser\(^{10}\). In addition to phosphorylation, p27\(^{kip1}\) must either still undergo some posttranslational modification, or there are some phosphorylated residues on p27\(^{kip1}\) that are resistant to \(\lambda\) phosphatase. In contrast to LPS activation alone, TCDD treatment of LPS-activated CH12.LX cells favored the faster-migrating 23.6 kDa form of p27\(^{kip1}\). Since TCDD has been previously shown to modulate the activity of protein kinases and phosphatases (Ashida et al., 2000), TCDD may inhibit LPS-induced phosphorylation of p27\(^{kip1}\) by activating a phosphatase or by inhibiting the activation of a protein kinase, thus decreasing the stability, and therefore the cellular concentration of p27\(^{kip1}\). Although the specific events responsible for p27\(^{kip1}\) regulation in response to LPS activation of B cells in the presence and absence of TCDD treatment remain to be elucidated, this study clearly shows that TCDD altered the LPS-induced posttranslational modification of p27\(^{kip1}\) in CH12.LX B cells.

Perhaps most importantly, the effects of LPS and TCDD on p27\(^{kip1}\) correlated with the observed effects on B-cell differentiation. Specifically, LPS dramatically increased the intracellular concentration of both p27\(^{kip1}\) and Ig \(\kappa\) light chain at 48 h post-LPS stimulation, while in the presence of TCDD neither of these proteins was induced. Since CH12.LX cells secrete antibodies directed against an epitope on sheep erythrocytes, it was possible to measure the effects of TCDD on the number of differentiated CH12.LX cells by performing a Jerne plaque assay. Past studies by this laboratory, using the CH12.LX cell as a model for elucidating the molecular mechanism for altered B-cell effector function by TCDD, have demonstrated a marked inhibition of IgM secretion and immunoglobulin heavy chain mRNA expression in LPS-activated CH12.LX cells. Although informative, those past experiments did not address whether TCDD simply inhibited IgM production and/or decreased the number of CH12.LX cells that ultimately differentiated into AFCs. The present study clearly shows that TCDD markedly suppressed the total number of CH12.LX cells that were induced by LPS to differentiate into AFCs. In addition, the studies once again confirm that TCDD produces its suppression of B-cell function by directly acting on the B lymphocyte.

Interestingly, previously reported time-of-addition studies with mouse splenocytes demonstrated a transient sensitivity, in that TCDD had to be added to culture within the first 24 h after antigen treatment in order to produce an inhibition of the anti-sheep erythrocyte IgM AFC response (Tucker et al., 1986). This critical time period of sensitivity for TCDD-mediated inhibition of antibody responses in primary splenocytes was also observed in the present time-of-addition studies employing LPS-activated CH12.LX cells. Addition of TCDD concomitantly with LPS, or 8 h post-LPS activation, produced a marked inhibition of both IgM secretion and the intracellular concentration of the Ig \(\kappa\) light chain protein. However, as in primary B cells, the inhibitory effect of TCDD on immunoglobulin production was temporally dependent as evidenced by a null effect when added 24 h after LPS activation. Similarly, the TCDD-mediated arrest of p27\(^{kip1}\) modulation following antigen stimulation was only achieved when TCDD was added prior to 24 h post-LPS activation. Collectively, these results suggest a relationship between B-cell differentiation and changes in the posttranslational modification of p27\(^{kip1}\) in this cell line model of B-cell differentiation. In addition, it is tempting to speculate that by dysregulating the effects of LPS on p27\(^{kip1}\), TCDD may alter the critical temporal events necessary for terminal B-cell differentiation.

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REFERENCES


