Simultaneous *In Vivo* Time Course and Dose Response Evaluation for TCDD-Induced Impairment of the LPS-stimulated Primary IgM Response

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent suppressor of humoral immunity but the specific molecular mechanisms responsible for immunosuppression by TCDD are poorly understood. *In vivo* and *in vitro* studies of the primary humoral IgM response demonstrated that the B cell is a sensitive cell type to modulation by TCDD. We hypothesized that *in vivo* administration of TCDD disrupts expression of transcription factors controlling B cell to plasma cell differentiation. Female C57BL6 mice were treated with a single dose of TCDD (3, 10, or 30 μg/kg) and/or vehicle (sesame oil). On day 4 post-TCDD administration mice were sensitized with 25 μg lipopolysacchride (LPS) by intraperitioneal injection to stimulate an immune response. Splenocytes were isolated on subsequent days following LPS, up to 3 days post-LPS, and the expression of IgM, XBP-1, PAX5, BCL-6, and Blimp-1 was assessed. TCDD treatment dose-dependently suppressed LPS-induced IgM antibody-forming cell number, which was correlated with decreased frequency of CD19⁺ CD138⁺ cells. Gene expression analysis revealed that TCDD caused a dose-dependent suppression of Igμ chain, Igκ chain, IgJ chain, XBP-1, and Blimp-1. TCDD also dose-dependently suppressed LPS-stimulated increases in Blimp-1 protein expression in CD19⁺ B cells. The deregulation of Blimp-1 expression by TCDD provides a partial explanation for the concomitant suppression of the IgM response and confirms previous observations established *in vitro*.

Key Words: LPS; TCDD; differentiation; B cell; Blimp-1; *in vivo*.

Decades of research have established 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the prototypical aryl hydrocarbon receptor (AHR) ligand, as a health hazard. The immune system is highly sensitive to TCDD, as demonstrated by significant disruption of a range of immune responses in animal models at doses as low as 4 ng/kg (Burleson *et al.*, 1996; Clark *et al.*, 1983; House and Lauer, 1990). Humoral immune responses have been found to be especially sensitive to TCDD, as evidenced by suppression of both *in vivo* and *in vitro* primary IgM responses to T-cell dependent, T-cell independent, and polyclonal B-cell activators (reviewed in Holsapple *et al.*, 1991;

Kerkvliet, 2002), with maximal suppression of the primary anti-sheep erythrocytes (sRBC) IgM response observed at approximately 30 μg/kg TCDD in C57BL6 mice (Vecchi *et al.*, 1980). *In vitro* studies have demonstrated that B cells, primary as well as certain cell lines, are highly sensitive to direct impairment by TCDD (Dooley and Holsapple, 1988; Sulentic *et al.*, 1998) and that the ensuing suppression of the primary IgM response is dependent on activation of the AHR (Sulentic *et al.*, 1998; Vorderstrasse *et al.*, 2001).

Robust humoral immunity requires large amounts of immunoglobulin (Ig) secretion, a function chiefly attributed to the antibody-forming cell (AFC). Differentiation into an AFC results in significant changes in morphology, phenotype, and gene expression profile. At the molecular level, cellular control of plasmacytic differentiation is accomplished by multiple transcription factors, including BCL-6, Blimp-1, Pax5, and XBP-1 (reviewed in Calame and Lin, 2003; Igarashi and Ochiai, 2007). Regulatory interaction of transcription factors is conceptualized in Figure 1, illustrating the interconnected and reciprocal regulation controlling plasmacytic differentiation. In the case of the primary IgM antibody response, the end product of plasmacytic differentiation is an AFC synthesizing and secreting large amounts of pentameric IgM, formed through Ig J chain (IgJ) coupling five individual IgM antibodies together, with each single IgM antibody made of two Ig light chains and two Ig heavy chains.

Numerous methods of immune system activation initiate differentiation of B cells into AFCs, with LPS one of the most broadly applied polyclonal B cell stimulators resulting in IgM secretion. LPS stimulates B cells through Toll-like Receptor 4 (Hoshino *et al.*, 1999) and RP105/CD180 (Ogata *et al.*, 2000), initiating signaling cascades that converge on the transcription factors depicted in Figure 1, triggering the differentiation process culminating in the plasma cell phenotype.

We hypothesized that changes in gene and protein expression associated with *in vitro* TCDD-mediated suppression of IgM secretion would extend to the *in vivo* humoral IgM response. To test this hypothesis an extensive simultaneous analysis of dose response and kinetics during the humoral response for multiple outcomes, in the absence and presence of

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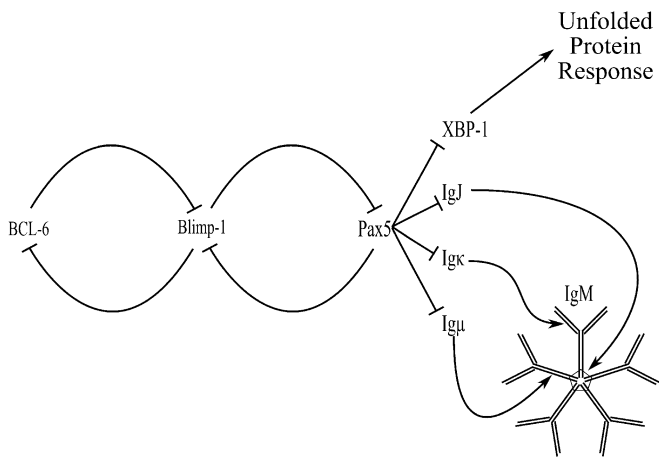
FIG. 1. Schematic representation of proteins involved in plasmacytic differentiation.

TCDD, was undertaken. The experimental design allows systematic assessment of TCDD effects on multiple regulatory proteins controlling B-cell differentiation, *in vivo* under physiologically relevant conditions. Our results verify and extend previous *in vitro* observations regarding the mechanism for TCDD suppression of the primary IgM response. Specifically, TCDD suppressed all phenotypic outcomes associated with the AFC response examined, implying TCDD-mediated disruption of one or more critical events prior to antibody production. In examining steps that precede antibody secretion, we observed TCDD disruption of LPS-induced changes in *Pax5* mRNA abundance. TCDD decreased induction of both *Blimp-1* mRNA and frequency of CD19^{+} Blimp-1[elevated] cells following LPS treatment. In addition, TCDD reduced expression of surface major histocompatibility complex (MHC) Class II induced by LPS. Cumulatively, these observations point to TCDD-mediated deregulation of either Blimp-1 expression or some proximal event responsible for controlling Blimp-1 expression.

MATERIAL AND METHODS

Animals. Female 6- to 8-week-old C57BL6 mice were purchased from the National Cancer Institute and housed in accordance with Michigan State University Institutional Animal Care & Use Committee policy. Mice were administered TCDD (3, 10, or 30 µg/kg) and/or vehicle (sesame oil) by a single oral gavage according to individual body weights 4 days prior to induction of an immune response. On day 0, mice received 25 µg *Salmonella typhosa* LPS or vehicle (phosphate buffered saline [PBS]) by intraperitoneal injection to initiate a primary humoral immune response. Each time point and treatment group consisted of six mice. Tissue samples were collected from mice that received only TCDD or vehicle treatment alone on day 0 of the study to establish baseline effects of TCDD on all outcomes measured, then on subsequent days tissues were collected from all treatment groups. Mice were euthanized by carbon dioxide asphyxiation and spleens removed for processing into single cell suspensions by mechanical disruption. Splenocytes from individual mice were divided into separate aliquots for flow cytometry, RNA and DNA isolation, protein isolation, and IgM AFC response enumeration.

Chemicals. TCDD was purchased from Accustandard (New Haven, CT) and prepared in sesame oil (Sigma-Aldrich, St Loius, MO). *S. typhosa* LPS (Sigma-Aldrich) was prepared immediately prior to administration in PBS.

Flow cytometric analysis. Spleen cell preparations were depleted of erythrocytes by ammonium chloride lysis. FcγIII/II (CD16/CD32) receptors were blocked with BD Biosciences Fc Block (2.4G2, San Jose, CA) followed by incubation with phycoerythrin-labeled anti-CD19 antibodies (2D4, BD Biosciences) to identify B cells. Cells were washed two times to remove excess antibody and fixed with 1× BD Cytofix. For detection of intracellular proteins cells were permeabilized with BD Biosciences 1× Perm/Wash according to manufacturer's instructions. Sources of primary and secondary antibodies used are listed in Supplemental Table S1. Excess antibody was removed with two washes before cells were resuspended in 1× FCM (flow cytometry) buffer (1× Hank's Balanced Salt Solution with 1% bovine serum albumin and 0.09% sodium azide). Cell analysis was performed on 20,000 CD19^{+} events collected using a BD FACSCalibur flow cytometer (BD Biosciences) with CellQuest Pro for acquisition. Data were analyzed using FlowJo 7.2 (Treestar Software, Ashland, OR).

Gene expression analysis. RNA was isolated from splenocytes using Trizol (Sigma-Aldrich) according to manufacturer's protocol. RNA pellets obtained following isopropanol precipitation and ethanol wash were resuspended in Promega SV RNA Lysis Solution and then processed according to the manufacturer's protocol to further purify RNA (Promega, Madison, WI). cDNA was generated using Applied Biosystems High Capacity Archive kit according to manufacturer's instructions. TaqMan primer/probe sets were used for all gene expression analysis (listed in Supplemental Table S2), and quantified by $\Delta\Delta C_t$ method using 18S ribosomal RNA for normalization (Livak and Schmittgen, 2001). Real-time PCR was performed using ABI 7900HT or Prism 7000 real-time PCR machines.

IgM AFC response. Detailed methods used for enumeration of AFC can be found in Holsapple and Tucker (1984). Trinitophenol (TNP)-haptenated sRBC were prepared as described in (Rittenberg and Pratt, 1969). In brief, single cell suspensions of splenocytes were combined with a solution of warm 0.5% agar and TNP-haptenated sRBC. Guinea pig complement (Cedar Lane Labs, Burlington, NC) was added, then vortex mixed briefly and aliquots placed onto a Petri dish. Samples were overlaid with a glass coverslip and incubated at 37°C in 5% carbon dioxide overnight. Hemolytic plaques were counted under magnification and normalized to cell number as determined using Z1 series Coulter Counter (Beckman Coulter, Fullerton, CA) to determine IgM AFC per 10^6 cells.

Statistical analysis. Results were analyzed by one-way ANOVA using Neuman-Keuls *post hoc* analysis to determine statistically significant differences among treatment groups within individual days using Graphpad Prism 4.05 (Graphpad Software, San Diego, CA). *p* Values less than or equal to 0.05 were considered significant. For legibility of figures, only treatment groups which differ significantly from sesame oil + PBS or sesame oil + LPS are indicated on graphs where space considerations do not allow for depiction of all statistically significant differences.

RESULTS

Suppression of the In Vivo LPS-Activated Primary IgM Response by TCDD

The rationale for using LPS in this study is that a strong polyclonal B-cell activator may drive a proportionally larger fraction of B cells *in vivo* than more specific antigens such as sRBC, resulting in a potentially greater sensitivity at early time points for detection of TCDD effects. To examine the *in vivo* effects of TCDD on the LPS-activated primary IgM response
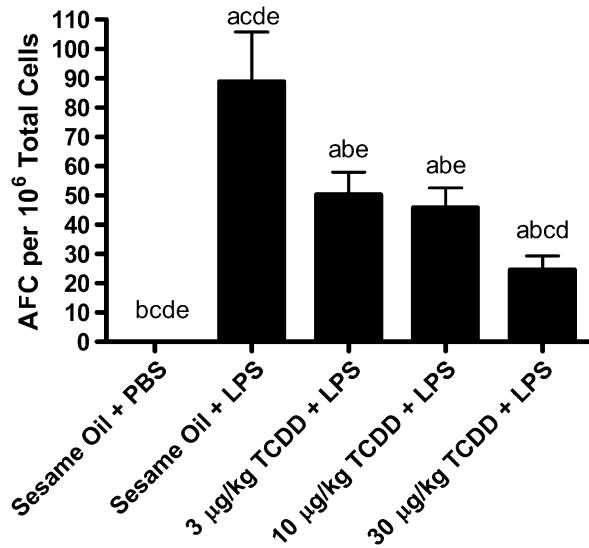
FIG. 2. Suppression by TCDD of the LPS-activated IgM AFC response. Four days prior to stimulation of the immune response six mice per treatment group were administered a single dose of TCDD (3, 10, or 30 µg/kg) and/or vehicle (sesame oil) by oral gavage. On day 0 mice received single intraperitoneal injections of either PBS or 25 µg LPS. IgM AFCs were enumerated on day 3 following LPS. Results are depicted as mean AFC per 10^6 splenocytes ± SE of the mean. [a]$p < 0.05$ compared with sesame oil + PBS, [b]$p < 0.05$ compared with sesame oil + LPS, [c]$p < 0.05$ compared with 3 µg/kg TCDD + LPS, [d]$p < 0.05$ compared with 10 µg/kg TCDD + LPS, and [e]$p < 0.05$ compared with 30 µg/kg TCDD + LPS.

we selected a dose of 30 µg/kg TCDD, which is established to cause near-maximal suppression of the sRBC-activated primary IgM response *in vivo* (Vecchi *et al.*, 1980), then selected half-log decreasing doses to profile the TCDD-mediated effects. *In vivo* LPS treatment induced a significant increase in the IgM AFC response, which was significantly suppressed by all concentrations of TCDD in a dose-dependent manner (Fig. 2). This may be the first study to demonstrate that *in vivo* exposure to the polyclonal B cell activator, LPS, results in an increase in IgM AFC response that is inhibited by TCDD.

LPS treatment induced a significant increase in spleen size, as reflected in the spleen weight/body weight ratio, which was significantly attenuated by TCDD treatment (Fig. 3A). Treatment with TCDD alone did not significantly alter spleen weight/body weight ratio, and no treatments caused significant changes in body weight (data not shown). TCDD attenuation of LPS-induced increases in spleen weight/body weight ratio was associated with a commensurate decrease in total cellularity, suggesting a modest suppression in spleen cell proliferation.

An extensive phenotyping of splenic B cells from all treatment groups was performed in which individual mice were evaluated for all outcomes detailed in this manuscript to maximize the statistical strength of this study. FCM was used to profile cell surface and intracellular protein abundance. In the normal course of an immune response LPS-activated B cells upregulate expression of several cell surface proteins, including MHC Class II to aid in antigen presentation. Surface
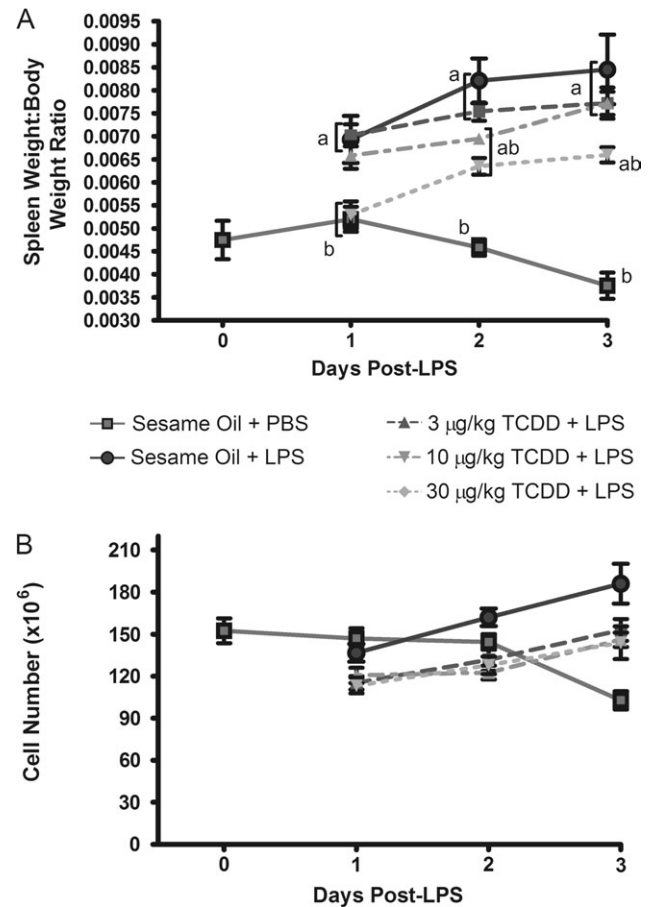
FIG. 3. Suppression by TCDD of LPS-induced spleen cell proliferation. Spleen weight, body weight, and total cell recovery per spleen were determined after mice were euthanized. (A) Ratio of spleen weight to total body weight. (B) Total splenocytes recovered per spleen following initial tissue processing into single cell suspensions. Results from six mice per group are depicted as mean ± SE of the mean. [a]$p < 0.05$ compared with sesame oil + PBS and [b]$p < 0.05$ compared with sesame oil + LPS.

MHC Class II expression on $CD19^+$ B cells increased significantly in response to LPS, peaking on day 1 and modestly declined by day 3. As B cells proceed toward the AFC phenotype cell surface expression of MHC Class II declined, which is consistent with our observation of peak MHC Class II expression 1 day after LPS treatment and which then declined on days 2 and 3 (Fig. 4B). Administration of 30 µg/kg TCDD significantly attenuated the LPS-induced MHC Class II on the surface of $CD19^+$ B cells at all time points evaluated, whereas lower doses of TCDD marginally attenuated LPS-induced cell surface MHC Class II upregulation.

The IgM AFC response has been widely used to enumerate antibody-secreting plasma cells. Our results show that intraperitoneal administration of LPS induced a significant increase in splenic AFCs, which was suppressed in a dose-dependent manner by TCDD treatment. Using the IgM AFC response as a phenotypic anchor, splenocytes were further characterized for CD138 expression, also termed syndecan-1,
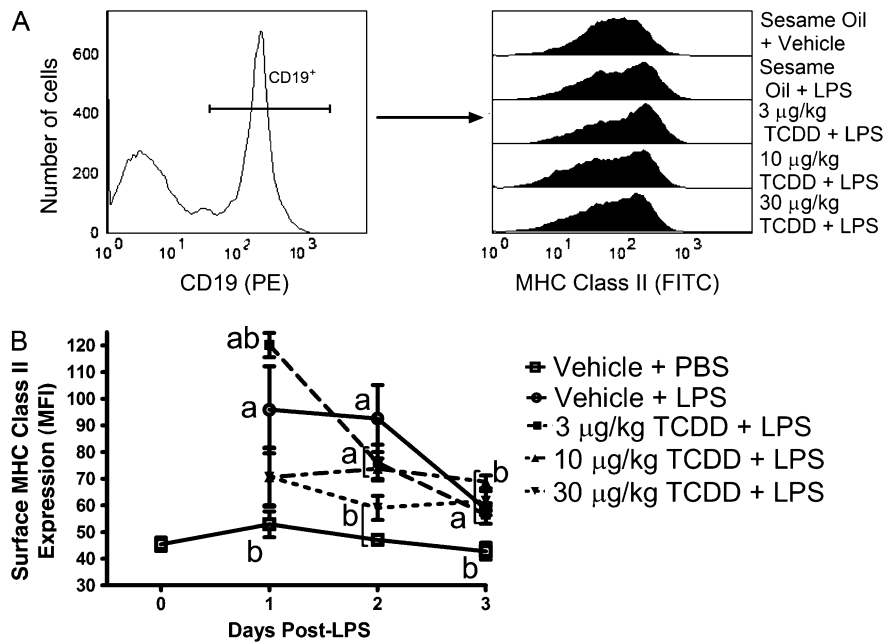
FIG. 4. Suppression by TCDD of LPS-induced cell surface MHC Class II expression. Isolated splenocytes were incubated with FcBlock, labeled with antibodies directed against CD19 and MHC Class II, then fixed with BD Cytofix. Cells were subsequently analyzed by FCM. Populations were gated by first identifying (A) CD19^{+} events, then (B) the individual median fluorescence (MFI) for MHC Class II. Results from six mice per group are depicted as mean MFI ± SE of the mean. [a]$p < 0.05$ compared with sesame oil + PBS and [b]$p < 0.05$ compared with sesame oil + LPS.

by FCM as a complementary marker of plasma cells. Figure 5A shows LPS induced an increase in frequency of CD19^{+} CD138^{+} cells, which was attenuated by TCDD treatment, an indication that suppression of the IgM AFC response was not simply due to suppression of antibody secretion, but also involved a blockade in LPS-activated B cell differentiation into AFCs. Extending the observation that appearance of CD19^{+} CD138^{+} cells was impaired by TCDD, LPS-induced generation of CD19^{+} Igκ^{high} cells (Fig. 5D) and CD19^{+} IgJ^{high} cells (Fig. 5E) in TCDD-treated mice was also impaired, suggesting that TCDD blocks B-cell differentiation prior to the large increase in intracellular IgJ and Igκ associated with the plasma cell phenotype. Collectively, these results demonstrate TCDD-mediated suppression of the LPS-induced primary IgM response involves failure of individual cells to not only secrete IgM, but to express cell surface markers indicative of the AFC phenotype.

TCDD-Mediated Suppression of LPS-Induced Changes in Gene and Protein Expression Involved in B Cell to AFC Transition

Differentiation of a resting B cell into an AFC is regulated by a network of reciprocally acting transcription factors, conceptualized in Figure 1. Previous *in vitro* studies established that TCDD impaired expression of several latter components of the plasma cell phenotype, including expression of IgJ, Igκ, Igμ, Pax5, and Blimp-1 (Schneider *et al.*, 2008, (2009). High levels of IgJ, Igκ, Igμ, and XBP-1 are

characteristic of antibody-secreting plasma cells, and splenocytes isolated from LPS-treated mice showed increasing levels of *IgJ*, *Igκ*, and *Igμ* mRNA peaking 2–3 days post-LPS (Figs. 6A, 6C, and 6D, respectively), with the largest fold increase in the expression of *IgJ*. TCDD impaired LPS-induced increases of *IgJ*, *Igκ*, and *Igμ* mRNA levels with *IgJ* significantly impaired relative to LPS treatment at all time points evaluated. *Igκ* and *Igμ* have a relatively high level of constitutive expression in B cells because they form the B cell antigen receptor, whereas *IgJ* is required only for the production of secreted pentameric IgM and dimeric IgA. In this study *IgJ* is likely to be the best marker of Ig destined for secretion, and thus an indicator of antibody-secreting plasma cells. The expression profile for total and spliced *XBP-1* followed a similar pattern to *IgJ*, *Igκ*, and *Igμ*, peaking in response to LPS on days 2 and 3. Although not statistically significant, TCDD treatment did appear to partially impair induction of *XBP-1*, similar again to the patterns observed with *Igκ* and *Igμ* mRNA levels.

FCM analysis of Blimp-1 protein in CD19^{+} B cells showed LPS treatment induced an increase in the number of B cells expressing Blimp-1, consistent with activation that precedes the AFC stage. Expression of Blimp-1 protein is quantitatively associated with the AFC phenotype, increasing as B cells differentiate into plasma cells (Kallies and Hasbold, 2004), a finding supported by our own observation. The peak *in vivo* AFC response occurs 3–4 days post-LPS (data not shown), correlating with peak frequency for CD19^{+} Blimp-1[elevated]
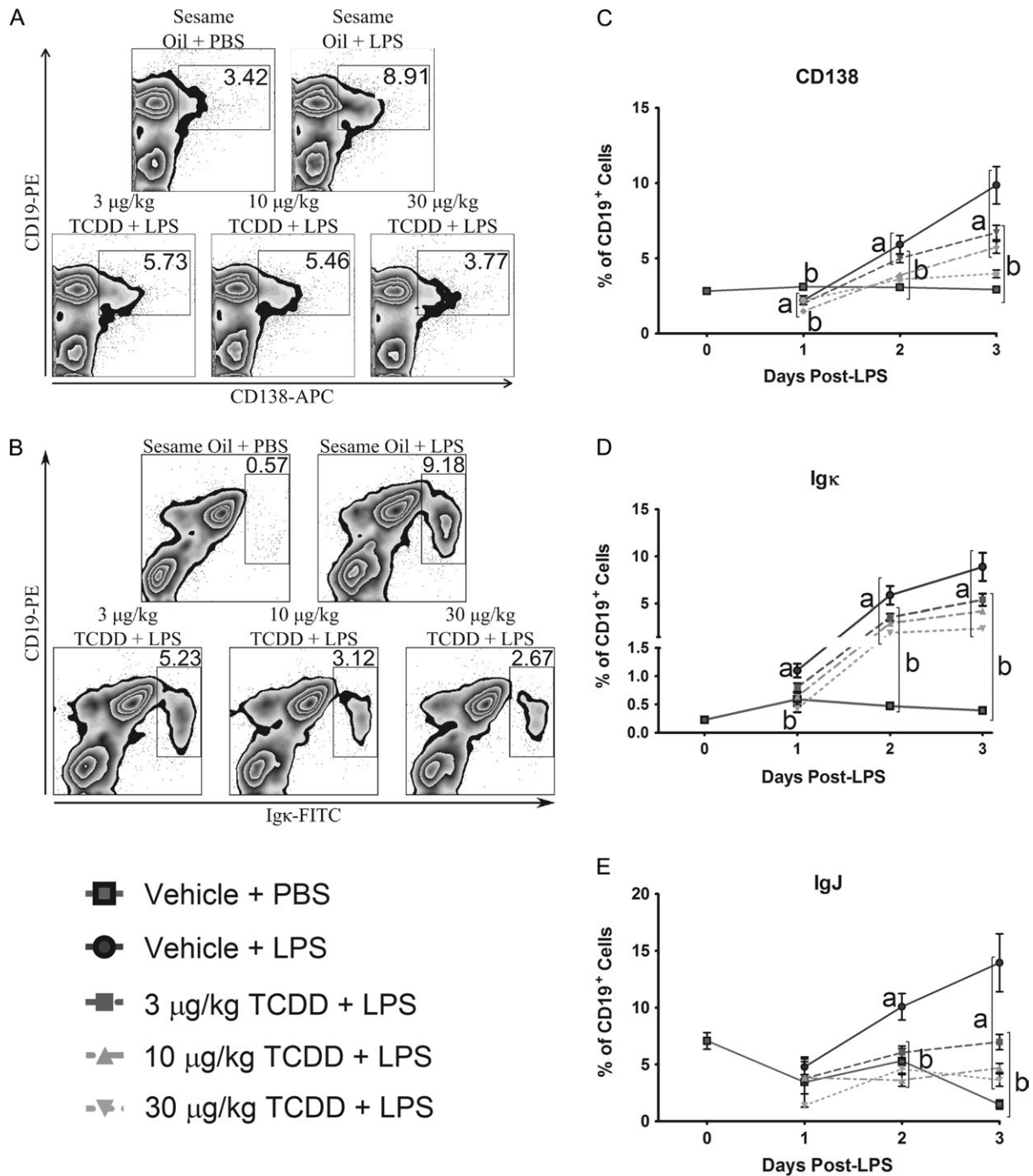
FIG. 5. TCDD treatment impaired expression of phenotypic indicators for plasmacytic differentiation. To assess B cells for CD138 expression, isolated splenocytes were incubated with FcBlock, labeled with antibodies directed against CD19 and CD138, then fixed with BD Cytofix. For detection of total Igκ or IgJ, cells were blocked, labeled with CD19 antibodies, and fixed with BD Cytofix. At the completion of the time course, cells were permeabilized with BD Perm/Wash, incubated with antibodies directed against either IgJ or Igκ and analyzed by FCM. Populations were gated by first identifying CD19^{+} events as depicted for (A) CD138 and (B) Igκ, and then gated on populations that expressed elevated levels of (C) CD138, (D) Igκ, or (E) IgJ. Results from 6 mice per group are depicted as mean frequency of CD19^{+} cells ± SE of the mean for respective measurements. [a]$p < 0.05$ compared with sesame oil + PBS and [b]$p < 0.05$ compared with sesame oil + LPS.

cells. TCDD treatment impaired generation of CD19^{+} Blimp-1[elevated] B cells in LPS-treated mice (Fig. 7). In agreement with protein expression, measurements of *Blimp-1* mRNA levels in isolated splenocytes showed that LPS-treated mice possessed significantly increased *Blimp-1* mRNA levels on days 2 and 3. For example, 10 and 30 μg/kg TCDD treatment caused significant dose-dependent suppression of LPS-induced *Blimp-1* mRNA levels over the time course (Fig. 8B).
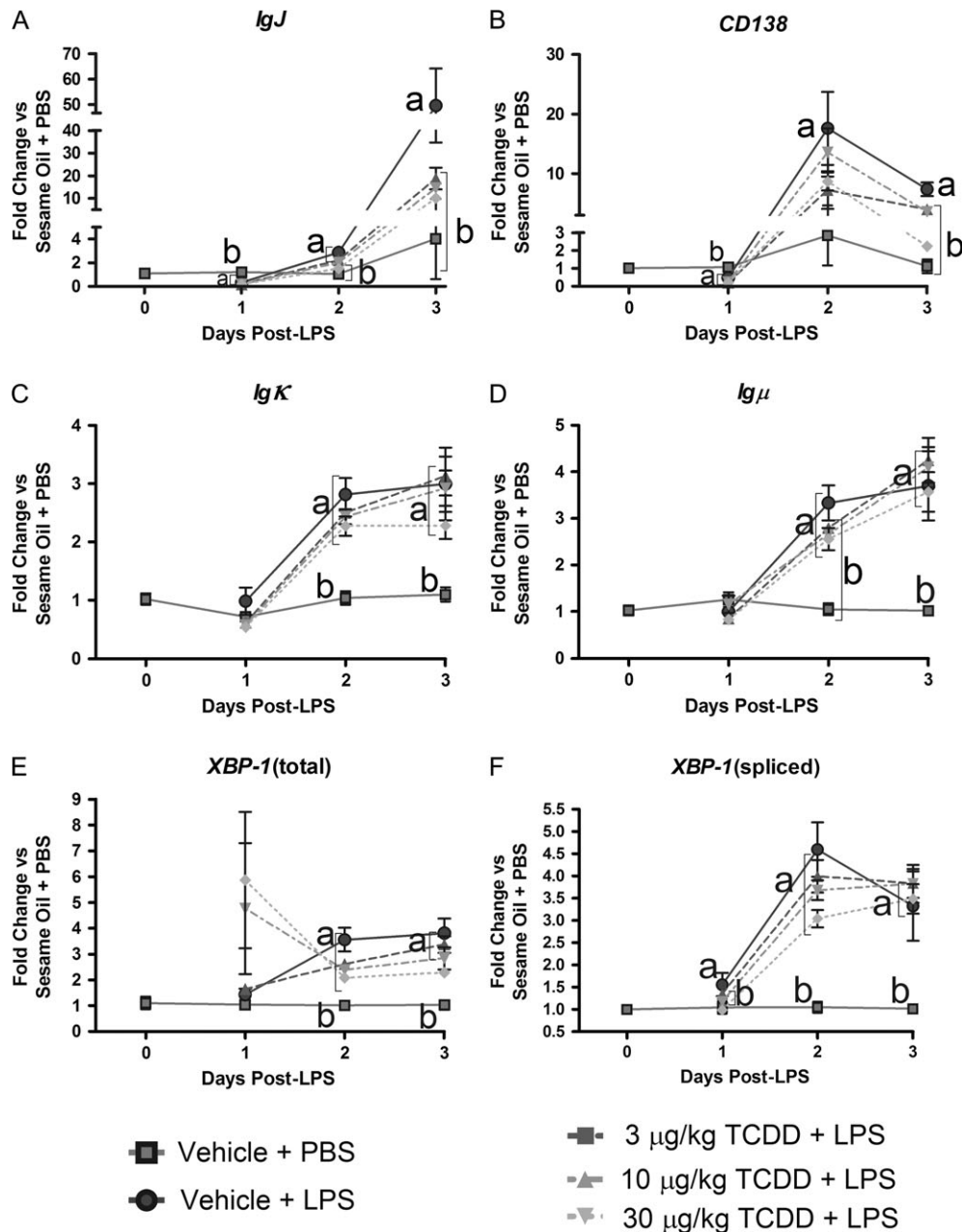
FIG. 6. Gene expression profiles for LPS-induced plasmacytic differentiation in the presence and absence of TCDD treatment. Total RNA from splenocytes was isolated and cDNA synthesized for gene expression analysis. Target gene mRNA levels were normalized to 18S and the fold change was calculated by the $\Delta\Delta C_t$ method. (A) *IgJ*, (B) *CD138*, (C) *Igκ*, (D) *Igμ*, (E) total *XBP-1*, or (F) spliced *XBP-1*. Results from six mice per group are depicted as mean fold change ± SE of the mean. [a]$p < 0.05$ compared with sesame oil + PBS and [b]$p < 0.05$ compared with sesame oil + LPS.

Previous *in vitro* studies with LPS-activated CH12.LX, a murine B cell line, and mouse splenocytes suggest that TCDD treatment impaired downregulation of Pax5 (Schneider *et al*., 2008; Yoo *et al*., 2004), a necessary regulatory event for plasmacytic differentiation (Lin and Angelin-Duclos, 2002; Nera *et al*., 2006). In agreement with prior *in vitro* observations, splenocytes from mice treated with LPS showed the greatest decrease in mRNA abundance of *Pax5* on day 2 of the time course. Mice treated with TCDD prior to LPS had

elevated *Pax5* mRNA levels on day 1 of the time course, with 10 and 30 μg/kg TCDD doses preventing the *Pax5* down-regulation associated with LPS treatment observed most clearly on day 2 of the time course (Fig. 8A).

BCL-6, acting in concert with Pax5, is a negative regulator of Blimp-1 gene expression (Shaffer *et al*., 2000; Tunyaplin *et al*., 2004). B cells constitutively express BCL-6 under resting conditions, with initiation of the immune response causing a decrease in BCL-6 expression (Ohkubo and Arima,
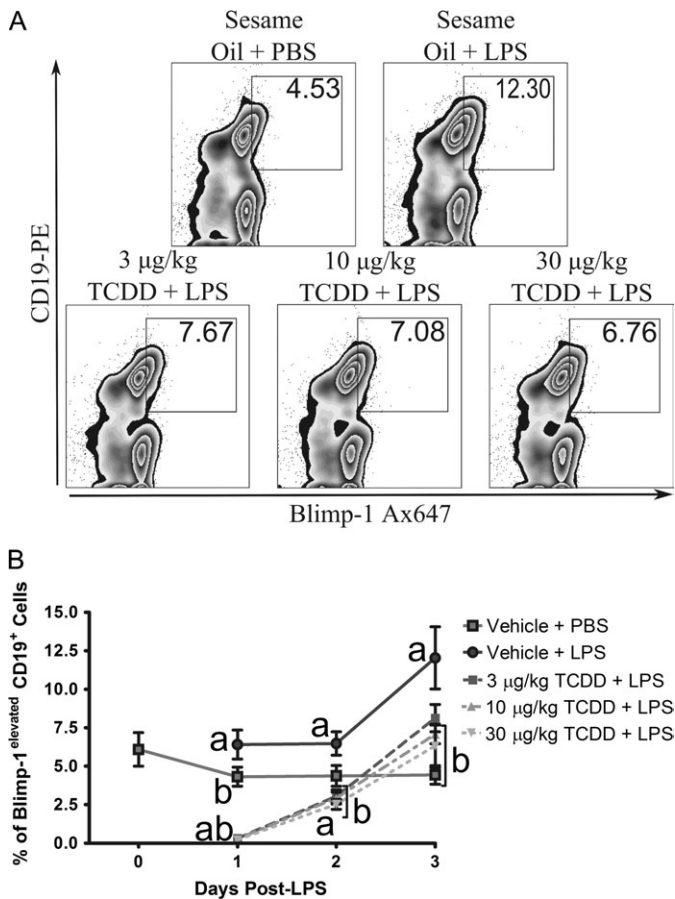
FIG. 7. TCDD treatment suppressed LPS-stimulated generation of CD19$^+$ Blimp-1^{elevated} cells. Isolated splenocytes were incubated with FcBlock, labeled with antibodies directed against CD19, then fixed with BD Cytofix. At the completion of the time course cells were permeabilized with BD Perm/Wash and incubated with antibodies directed against Blimp-1. Cells were subsequently analyzed by FCM. (A) Representative plots from individual mice 3 days post-LPS showing the CD19$^+$ Blimp-1^{elevated} cell population. (B) Time course and dose response for generation of CD19$^+$ Blimp-1^{elevated} cell population. Results from six mice per group are depicted as mean frequency of CD19$^+$ Blimp-1^{elevated} cells ± SE of the mean. $^a p < 0.05$ compared with sesame oil + PBS and $^b p < 0.05$ compared with sesame oil + LPS.

2005), in turn allowing Blimp-1 expression to rise. *In vivo* LPS treatment decreased *BCL-6* mRNA levels on days 1 and 2, but concomitant treatment with TCDD did not appear to alter BCL-6 gene expression (Fig. 8C).

DISCUSSION

This study demonstrates that *in vitro* events previously implicated in TCDD-mediated suppression of the primary IgM response occur in the context of an *in vivo* T-independent humoral immune response. TCDD modestly attenuated LPS-induced increases in splenocyte number, accompanied by profound suppression of the IgM response at all concentrations of TCDD evaluated. Assessment of multiple phenotypic

endpoints associated with AFCs confirms that TCDD prevents LPS-induced increases in XBP-1 and all components of pentameric IgM. The observations that CD138 expression, IgJ, and Igκ production are suppressed advance our understanding of TCDD's mechanism of action, as previous studies have not directly shown suppression of intracellular antibody production on a single cell basis, and as such could never conclusively establish whether TCDD suppression of the primary IgM response resulted from suppression of antibody secretion and production or the differentiation of B cells into AFC. TCDD-impaired LPS-stimulated upregulation of Blimp-1, a transcription factor established to be a central regulator of B-cell differentiation. Collectively, these results strongly support our hypothesis that TCDD impairs B cell to plasma cell differentiation.

Different methods of B-cell activation result in distinctly different cell signaling programs and cumulative responses (Donahue and David, 2007). In order to maximize the validity of *in vivo* and *in vitro* comparisons it was deemed important to utilize similar modes of B-cell activation as in previous studies. TCDD suppression of the T-cell–dependent anti-sRBC IgM antibody response has been reported by many laboratories (Dooley and Holsapple, 1988; Luster and Germolec., 1988; Smialowicz *et al.*, 1994; Tucker *et al.*, 1986; Vecchi *et al.*, 1980; Vorderstrasse *et al.*, 2001), but to our knowledge no studies have evaluated TCDD-mediated suppression of the *in vivo* primary IgM response in the context of the commonly used polyclonal B-cell activating stimulus, LPS. Moreover, no published study to date has attempted to correlate alteration in the expression of regulatory transcription factors with *in vivo* TCDD suppression of the IgM response to any stimulus.

In prior studies by Smialowicz and coworkers only 30 μg/kg (single dose) TCDD, not 10 or 3 μg/kg TCDD, suppressed the T-cell–independent IgM AFC response. Unlike the present study, TCDD was delivered intraperitoneally followed by intravenous sensitization 7 days later with TNP-LPS (Smialowicz *et al.*, 1996). Significant suppression of the IgM response at 3 μg/kg TCDD and higher in the present study is likely due to modest differences in the experimental design, including the strain of mouse used, time interval between TCDD administration and B cell activation, the B-cell activator (LPS vs. TNP-LPS) employed, route of TCDD administration, and methods for detection of humoral immune response.

In agreement with moderate attenuation of LPS-stimulated increases in cell number observed in this *in vivo* study, TCDD treatment modestly decreased proliferation, as measured by [^{3}H] thymidine incorporation, in isolated splenocytes activated *in vitro* with LPS (Morris *et al.*, 1993). A minor decrease in proliferation is insufficient to explain the magnitude of suppression in the IgM response observed in the present study, especially because the IgM AFC response was normalized to cell number. However, the observed effect on decreased spleen cellularity was consistent with the possibility that an event upstream of antibody production is disrupted by TCDD.
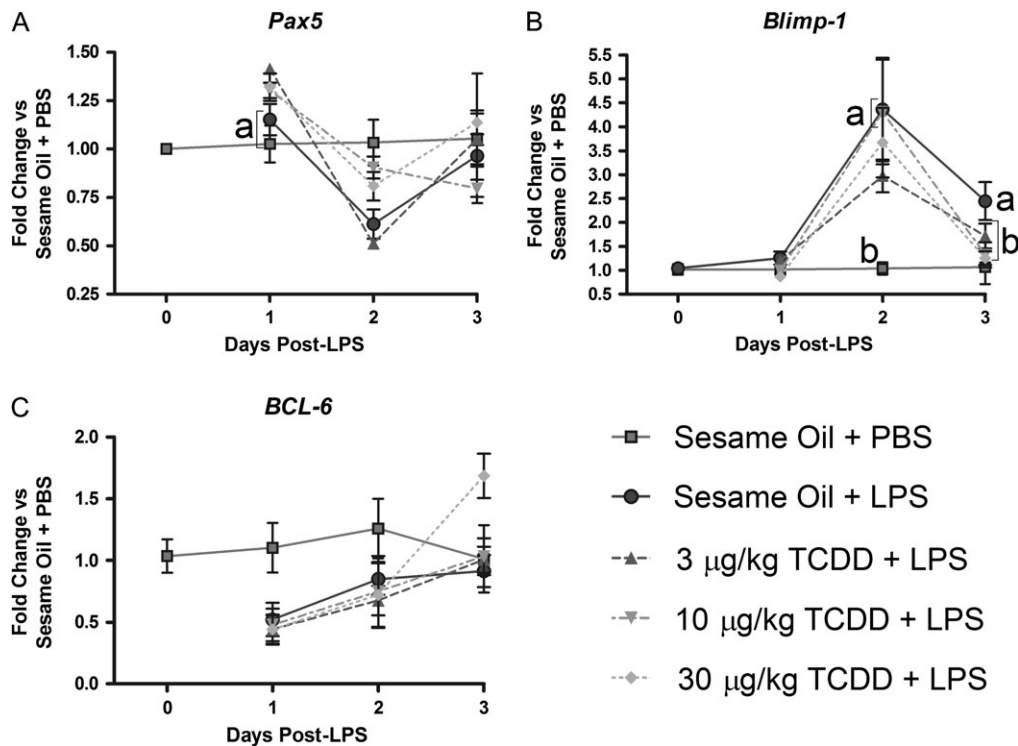
FIG. 8. Gene expression profiles for transcription factors controlling plasma cell fate are influenced by LPS and TCDD treatments. Total RNA from splenocytes was isolated and cDNA synthesized for gene expression analysis. Target gene mRNA levels were normalized to 18S and the fold change was calculated by the $\Delta\Delta C_t$ method. (A) *Pax5*, (B) *Blimp-1*, or (C) *BCL-6*. Results from six mice per group are depicted as mean fold change ± SE of the mean. [a]$p < 0.05$ compared with sesame oil + PBS and [b]$p < 0.05$ compared with sesame oil + LPS.

Disruption in the production of any single Ig component, or protein important in antibody secretion, impairs the primary IgM response. With that knowledge, we assayed expression of all immunoglobulin components. Expression of Igµ and Igκ was more modestly inhibited than the IgJ chain, and may provide insight on why the IgM response is highly sensitive to suppression by TCDD. B cells must express antigen receptors on their surface to receive survival signals (Rajewsky, 1996), hence the high constitutive levels of both Igµ and Igκ. TCDD inhibition of B-cell antigen receptor components may result in deletion of some B cells, preventing detection of some affected B cells using current techniques. Additionally, suppression of Igµ and Ig light chain expression can be expected to impair expression of all forms of immunoglobulin, whereas the IgJ chain is essential only for IgM and IgA classes of immunoglobulin. TCDD strongly attenuates IgJ chain expression, and can therefore be expected to markedly impair the assembly of pentameric IgM, an outcome verified by TCDD-mediated decrease in the AFC response. It is also plausible that less striking effects of TCDD on LPS-induced Igκ and Igµ expression are due to the high constitutive level of both Igκ and Igµ.

Impaired expression of Ig components disrupts antibody synthesis, but B cells must also resolve endoplasmic reticulum stress resulting from increased Ig protein synthesis necessary for robust secretion of antibody. Following activation B cells upregulate expression of *XBP-1* mRNA, which can be spliced to an alternative mRNA translating to full length XBP-1, a transcription factor essential for resolution of the unfolded protein response (Reimold *et al.*, 2001). Consistent with an interpretation that TCDD blocks plasmacytic differentiation upstream of antibody production and secretion, TCDD treatment reduced both total and spliced *XBP-1* expression in LPS-treated mice (Figs. 6E and 6F).

Separate from their function in antibody synthesis and secretion, B cells change surface protein expression during transition into AFCs. Abundance of surface CD19, MHC Class II, and B-cell antigen receptor all decrease as B cells become plasma cells, whereas surface expression of CD138 is upregulated (reviewed in Fairfax *et al.*, 2008). CD138 has been commonly used as a marker to enumerate plasma cells, and is a complementary phenotypic marker not directly involved in antibody secretion. LPS and TCDD cotreated mice were impaired in their ability to generate CD19^{+} CD138^{+} cells compared with mice treated with LPS in the absence of TCDD. Viewed cumulatively, TCDD affects expression of several proteins required for the synthesis and secretion of antibody, as well as CD138, and thus likely disrupts a step in B-cell differentiation preceding antibody production and establishment of the plasma cell phenotype *in vivo*.

Observations in this study indicate that TCDD interferes with LPS-induced Blimp-1 upregulation, and taken collectively, point toward TCDD-mediated disruption of a fundamental control

switch during the primary IgM response. Blimp-1 expression is required for the generation of plasma cells (Shapiro-Shelef *et al.*, 2003), thus failure to upregulate Blimp-1 expression leads to inhibition of plasma cell appearance, consistent with TCDD-mediated suppression of both IgM AFC response and frequency of CD19$^+$ CD138$^+$ B-cell frequency. Direct evidence for deregulation of Blimp-1 expression *in vivo* comes from the observations that TCDD treatment dose-dependently decreased the frequency of CD19$^+$ Blimp-1^{elevated} B cells and *Blimp-1* mRNA abundance in LPS-treated mice. Previous results from this laboratory demonstrated TCDD-activated AHR could directly regulate the Igμ 3′ α enhancer (Sulentic *et al.*, 2004a, b), an event in the IgM response distal to Blimp-1 upregulation during plasmacytic differentiation, implying the cumulative mechanism for TCDD-mediated suppression of the IgM is multifaceted. Suppression of both initiation of plasmacytic differentiation and Ig expression provides a partial explanation for the high sensitivity of B cells to TCDD.

Importantly, recent studies of Blimp-1 conditional knockout mice and identification of XBP-1 as a regulator of hepatic lipid metabolism provide interesting, indirect links for two well-recognized human TCDD toxicities, chloracne and hepatosteatosis, to Blimp-1 expression. Magnúsdóttir and coworkers described a keratinocyte-specific knockout of Blimp-1 in mice resulting in a phenotype remarkably similar to the most consistently observed human health outcome produced by high dose TCDD exposure, chloracne (Zugerman, 1990). Blimp-1 knockout in keratinocytes resulted in a sequela termed by the investigators as "a cyst-like structure that proved to be filled with lipid" in the skin of adult mice (Magnusdottir *et al.*, 2007). If TCDD inhibits Blimp-1 expression in keratinocytes similarly to B cells, then a shared mechanism for TCDD toxicity involving deregulation of Blimp-1 in different tissues becomes more plausible. Along these lines, XBP-1, a transcription factor that is indirectly controlled by Blimp-1 in B cells, is a regulator of lipid homeostasis in the mouse liver (Lee and Scapa, 2008). Human serum dioxin-like chemical levels are associated with hepatosteatosis (Lee and Yao, 2006), and furthermore, TCDD induces hepatosteatosis in mice (Boverhof *et al.*, 2005). Coupling the knowledge TCDD reduced XBP-1 expression, and is downstream of Blimp-1, it is tempting to speculate that TCDD-induced deregulation of Blimp-1 is a key event in some of the toxicities produced by TCDD.

Our study did not detect a consistent TCDD-induced alteration in BCL-6 mRNA levels for LPS-treated mice. It is possible the mechanism of TCDD-induced suppression of the primary IgM response does not involve alteration in BCL-6 gene expression, or owing to high constitutive levels of BCL-6 and multiple cell types in the spleen expressing BCL-6 (Zhou and Ono, 2005), it may be difficult to detect TCDD effects on LPS-induced decreases in BCL-6 expression for B cells. In contrast, it is possible to observe effects of TCDD on *Pax5* because B cells are the predominant source of *Pax5* mRNA in splenocytes, thus provided greater sensitivity for detection than *BCL-6*.

Results of this study provide compelling evidence for TCDD impairment of the *in vivo* primary IgM response via a block in plasmacytic differentiation at, or upstream of, Blimp-1 expression, not simply at the level of antibody production. Upregulation of several phenotypic indicators for AFCs, including CD138 and intracellular levels of Igκ and IgJ, were impaired by TCDD exposure. Collectively, these results validate and enhance the value of mechanistic *in vitro* studies establishing TCDD-induced deregulation of the Igμ 3′ α enhancer (Sulentic *et al.*, 2004a, b), Pax5 (Schneider *et al.*, 2008), and Blimp-1 (Schneider *et al.*, 2009). Future studies are planned to examine TCDD alteration of events preceding Blimp-1 upregulation in B cells.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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