2,3,7,8-Tetrachlorodibenzo-p-Dioxin Induces Transcriptional Activity of the Human Polymorphic hs1,2 Enhancer of the 3′Igh Regulatory Region

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2,3,7,8-Tetrachlorodibenzo-p-Dioxin Induces Transcriptional Activity of the Human Polymorphic hs1,2 Enhancer of the 3′Igh Regulatory Region

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2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is an environmental toxicant known to inhibit Ab secretion and Ig expression. Inhibition of Ig expression may be partially mediated through repression of the 3′Igh regulatory region (3′IghRR). TCDD inhibits mouse 3′IghRR activation and induces aryl hydrocarbon receptor binding to dioxin response elements within the 3′IghRR enhancers hs1,2 and hs4. The human hs1,2 enhancer (hu-hs1,2) is polymorphic as the result of the presence of one to four invariant sequences (ISs), which have been correlated with several autoimmune diseases. The IS also contains a dioxin response element core motif. Therefore, the objective was to determine whether hu-hs1,2 activity is sensitive to TCDD. Using a mouse B cell line (CH12.LX), we compared the effects of TCDD on mouse hs1,2 versus hu-hs1,2 activity. TCDD inhibited mouse hs1,2 similarly to the mouse 3′IghRR. In contrast, hu-hs1,2 was activated by TCDD, and antagonist studies supported an aryl hydrocarbon receptor-dependent activation, which was replicated in a human B cell line (IM-9). Absence of Pax5 binding sites is a major difference between the human and mouse hs1,2 sequence. Insertion of the high-affinity Pax5 site in hu-hs1,2 markedly blunted reporter activity but did not alter TCDD’s effect (i.e., no shift from activation to inhibition). Additionally, deletional analysis demonstrated a significant IS contribution to hu-hs1,2 basal activity, but TCDD-induced activity was not strictly IS number dependent. Taken together, our results suggest that hu-hs1,2 is a significant target of TCDD and support species differences in hs1,2 regulation. Therefore, sensitivity of hu-hs1,2 to chemical-induced modulation may influence the occurrence and/or severity of human diseases associated with hu-hs1,2. The Journal of Immunology, 2012, 188: 3294–3306.
involving inhibition of the 3′IghRR and Igh expression in the B cell dysfunction induced by TCDD and other AhR ligands. Furthermore, AhR protein expression is markedly upregulated in activated mouse and human B cells, suggesting the potential for enhanced susceptibility of activated B cells to TCDD toxicity (15, 21, 22).

Interestingly, the human 3′IghRR has been associated with several human immune-related disorders, including Burkitt’s lymphoma, celiac disease, IgA nephropathy, and, more recently, systemic sclerosis, plaque psoriasis, psoriatic arthritis, dermatitis herpetiformis, and rheumatoid arthritis (23–28). With the exception of Burkitt’s lymphoma (which was not evaluated), incidence and/or severity of these diseases appears to correlate with a polymorphism within the human hs1,2 (hu-hs1,2) enhancer of the α1 3′IghRR. This polymorphism is characterized by an ∼38-bp invariant sequence (IS) that is repeated in tandem up to three times and contains potential binding sites for NF-κB, AP-1, Sp1, and NF1 (29–32) (Fig. 1B). Using an EMSA with an NF-κB consensus oligo and Abs against Sp1 as competitors, Frezza et al. (33) verified binding of NF-κB and Sp1 within the IS of hu-hs1,2. Additionally, we identified a DRE-like site within the IS that contains the DRE-core motif and is well conserved with the functional DRE previously evaluated by EMSA in the mouse hs1,2 (4). Therefore, the objective of the current study was to determine whether, like the mouse 3′IghRR, the polymorphic hu-hs1,2 enhancer is sensitive to TCDD-induced modulation.

Using stable expression of a transgene or transient expression of luciferase reporter constructs in a well-characterized mouse B cell line model (CH12.LX), we identified a marked difference in the effects of TCDD on human versus mouse hs1,2 activity. TCDD significantly inhibited LPS-induced activation of the mouse hs1,2 enhancer (mo-hs1,2). This is consistent with the inhibitory effect of TCDD on mouse 3′IghRR activation. In contrast, hu-hs1,2 was significantly activated by TCDD, and this activation was enhanced with LPS stimulation. Additionally, we observed a similar TCDD-induced activation of hu-hs1,2 in a human B cell line (IM-9). A major difference between the human and mouse hs1,2 enhancers is the presence of a high-affinity Pax5 binding site within mo-hs1,2 that is not present in hu-hs1,2 (34). In our study, insertion of the high-affinity Pax5 binding site within hu-hs1,2 suppressed basal reporter activity and overall activity induced by TCDD and/
or TCDD but did not change TCDD’s effect (i.e., no shift from activation to inhibition). Furthermore, using deletion analysis, we found that the IS significantly contributed to hu-hs1,2 activity, but TCDD-induced hs1,2 activation was not strictly dependent on IS number. Moreover, AhR appears to play a prominent role in the effects of TCDD on hu-hs1,2 activity based on the antagonism of TCDD-induced activity with pretreatment of an AhR antagonist. Taken together, our results suggest that hu-hs1,2 is a significant target of TCDD and support species differences in regulation of the hs1,2 enhancer. TCDD represents a large class of AhR ligands found in the environment, diet, and pharmaceuticals, potential exposure to a broad range of chemicals could modulate hu-hs1,2 activity and influence the occurrence and/or severity of human diseases associated with the hu-hs1,2.

Materials and Methods

Human buccal cell genomic DNA isolation, nested PCR, and sequence analysis for hs1,2 allelic determination

Genomic DNA from buccal cells (approved by the Wright State University Institutional Review Board under protocol SC 3584) was extracted using the GenElute Mammalian Genomic DNA Kit according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Genomic DNA (1 μg) was used as the template in the first round of a two-round nested-PCR assay, which allows for selective amplification within the αs, rather than αs, 3′IghRR (Fig. 1A), as previously described (32). Briefly, the 5.4-kb fragment of αs 3′IghRR was selectively amplified by the hu-αs 3′IghRR primers (Table I). The first-round PCR reaction conditions were 1.5 U Taq Platinum High Fidelity (Invitrogen, Carlsbad, CA), 1X Platinum High Fidelity Buffer, 0.2 mM 2′-deoxynucleoside 5′-triphosphates, 1.5 mM MgCl2, 15 pmol primers, and water to a final volume of 50 μl. The reaction cycles were 94˚C for 2 min, followed by 10 cycles at 94˚C for 30 s, 59˚C for 30 s, 68˚C for 5 min, followed by 20 more cycles at 94˚C for 30 s, 57˚C for 30 s, 68˚C for 5 min, and one final extension at 72˚C for 10 min. The second-round, nested PCR reaction selectively amplified the polymorphic region of the αs, hs1,2 enhancer using the hu-αs, hs1,2 primers, as previously identified (32) (Table I). For the nested PCR, the template was 10 μl from the first-round PCR reaction and the reaction conditions were 1 U Hot-Master Taq DNA polymerase (5 PRIME, Gaithersburg, MD), 1X reaction buffer, 0.2 mM 2′-deoxynucleoside 5′-triphosphates, 15 pmol primers, and water to a final volume of 50 μl. The reaction cycles were 94˚C for 2 min, followed by 30 cycles at 94˚C for 30 s, 56˚C for 30 s, 72˚C for 1 min, followed by a final extension at 72˚C for 5 min. The second-round PCR products (30 μl) were separated on an 8% polyacrylamide gel, stained with SYBR Green I Nucleic Acid for 40 min, and visualized using a LAS3000 Fuji imager (Fujiﬁlm Medical Systems USA, Stamford, CT). The αsA, αsC, αsI, and αsD alleles produce PCR product sizes of 297, 350, 403, and 456 bp, respectively. Allele assignments were conﬁrmed by sequencing (Retrogen, San Diego, CA).

Chemicals and reagents

TCDD in 100% DMSO was purchased from AccuStandard (New Haven, CT). The certicate of product analysis stated the purity of TCDD to be 99.1%, as determined by AccuStandard using gas chromatography/mass spectrometry. The previously characterized AhR antagonist CH-221919 (35) was purchased from Calbiochem (San Diego, CA) and dissolved in 100% DMSO. DMSO and LPS (Escherichia coli) were purchased from Sigma-Aldrich. LPS was dissolved in 1X PBS.

Cell lines

The CH12.LX B cell line was derived from the mouse CH12 B cell lymphoma (36), which arose in B10.H-2H-4p/Wts mice (B10.A × B10.129). The CH12.LX cell line was characterized by Bishop and Haughton (37) and was generously donated by Dr. Geoffrey Haughton (University of North Carolina, Chapel Hill, NC). Since its initial characteriza- tion, the CH12.LX cell line has been extensively used to study a variety of cellular processes specific to B cells and has provided a useful model in studying the effects of TCDD on B cell function, because it expresses a functional AhR-signaling pathway and exhibits TCDD-induced effects on SFR expression that mirror effects seen in vivo and in primary B lymphocytes (4, 6, 18, 38). Additionally, the CH12.LX cell line is known to spontaneously class switch (39), and the CH12.LX clone used in these studies is an IgA-expressing variant of the original line, as verified by flow cytometry and ELISA (7). Using these cells and the y2b mini-locus model, developed and generously provided by Dr. Laurel Eckhardt (Hunter College, The University of New York City, New York, NY) (40), we also generated the CH12-y2b-3′Igh cell line, which stably expresses a single copy of an inducible y2b Igh transgene under the regulation of the 3′IghRR, with loxP sites flanking the hs3B and hs4 enhancer pair (7, 40). Because CH12.LX cells have class switched to IgA, CH12-y2b-3′Igh cells do not endogenously express y2b Igh or secrete IgG2b (7). As described by Shi and Eckhardt (40), we transiently transfected a CRE recombinase GFP expression plasmid in CH12-y2b-3′Igh cells, sorted the cells for GFP expression, and isolated deleitional derivative clones (CH12-y2b-hs3A/hs1,2) that only expressed a y2b transgene regulated by the hs3A and hs1,2 enhancer pair (Fig. 2B). The hs3A and hs1,2 enhancers originated from a 1.1-kb XbaI fragment containing the hs3A enhancer and a 3.6-kb XbaI-HindIII fragment containing the hs1,2 enhancer, both of which were isolated from the λ phage clone M2 containing BALB/c ge- nomic DNA (40). Clones were characterized by PCR analysis for successful recombination and deletion of the hs3B and hs4 enhancer pair (data not shown). The CH12-y2b-hs3A/hs1,2 and CH12.LX cell lines were grown as previously described (7).

The human IM-9 B cell line originated from an EBV-transformed B lymphoblast isolated from a white female patient with multiple myeloma and was purchased from American Type Culture Collection (ATCC no. CCL-159; Manassas, VA). IM-9 cells were shown to express a functional AhR-signaling pathway (41), which is an important characteristic for an appropriate cellular model to study the effects of TCDD on B cell function. IM-9 cells were grown in RPMI 1640 media (Mediatech, Herndon, VA) supplemented with 10% bovine calf serum (HyClone, Logan, UT), 10 mM HEPES, 20 mM sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM 1-glutamine, 10 mM sodium pyru- vate, and 50 μM 2-ME. All cells were maintained at 37˚C in an atmosphere of 5% CO2. Additionally, cell viability for all cell lines was measured by trypan blue exclusion and was minimally affected by LPS, DMSO, or TCDD treatment (data not shown).

FIGURE 2. TCDD inhibits LPS-induced mo-hs1,2 enhancer activity. (A) CH12.LX cells were transiently transfected with the mo-hs1,2 reporter plasmid. Cells were either cultured for 24 h with media alone (NA) or with varying concentrations of TCDD (0–10.0 nM) in the presence of LPS (1.0 μg/ml) stimulation. Luciferase enzyme activity (mean ± SEM, n = 3) is represented on the y-axis, a RUU040-1 clone was transiently transfected with a CRE recombinase expressing a y2b transgene under the regulation of the hs3A/hs1,2 enhancer pair, were either cultured for 48 h in media alone (NA) or treated with various concentrations of TCDD (0–10.0 nM) in the presence of LPS (1.0 μg/ml) stimulation. y2b protein expression (mean ± SEM, n = 3) is also shown. The cell lysate was determined by sandwich ELISA and standardized to ng/1 μl of total protein, as shown on the y-axis. Results are representative of at least three independent experiments. **p < 0.01, versus the corresponding vehicle control (0.01% DMSO [0 nM TCDD]), one-way ANOVA, followed by Dunnett post hoc test. C, LPS stimulation alone.
Table I. Primers used for real-time PCR, two-round nested PCR, and site-directed mutagenesis

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward (F)</th>
<th>Reverse (R)</th>
<th>Primer Sequences</th>
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<td>R: 5′-TCAGCCAGCTGAAGAGACTCCGC-3′</td>
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<td>Igl-Ck</td>
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<td>b-actin</td>
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<td>R: 5′-CTCTTCCAGGAGAGAAAAGAGAT3′</td>
<td></td>
</tr>
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<td>hu-α1 3′IghRR</td>
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<td>R: 5′-GACCTCTTCTGAGGAGATG-3′</td>
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</tr>
<tr>
<td>hu-α1 hs1.2</td>
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<td>R: 5′-GACTCTTCTGAGGAGATG-3′</td>
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<td>R: 5′-GACCTCTTCTGAGGAGATG-3′</td>
<td></td>
</tr>
<tr>
<td>α1BIS1</td>
<td>F: 5′-GACCTCTTCTGAGGAGATG-3′</td>
<td>R: 5′-GACCTCTTCTGAGGAGATG-3′</td>
<td></td>
</tr>
<tr>
<td>α1α + Pax5</td>
<td>F: 5′-AGGCCACACTTGGTAGGAGGAAAGATG-3′</td>
<td>R: 5′-AGGCCACACTTGGTAGGAGGAAAGATG-3′</td>
<td></td>
</tr>
</tbody>
</table>

*Primer targets for real-time PCR analysis (Igh-Ca and Igl-Ck, endogenous Ig H chain and L chain gene expression; b-actin, housekeeping gene), two-round nested-PCR analysis (hu-α1 3′IghRR, first-round PCR; hu-α1 hs1.2, second-round PCR), and site-directed mutagenesis (α1AIS1, deletion of IS1 in α1A; α1BIS1, deletion of IS1 in α1B; α1α + Pax5, insertion of Pax5 binding site in α1α). (44). Human α1 hu-hs1.2 luciferase reporter plasmids were generously provided by Dr. Michel Cogné (Laboratoire d’Immunologie, Limoges, France) and included the V_{H} promoter alone and the α1α, α1β, and α1C polymorphic hu-hs1.2 plasmids. The hu-hs1.2 reporters contained the V_{H} promoter (181 bp) 5′ of the luciferase gene and the hu-hs1.2 enhancer with one (α1A), two (α1B), or three (α1C) Is3′s of the luciferase gene (29) (Fig. 4A). Sequencing of the hu-hs1.2 enhancer inserts (~542 bp, depending on the number of IS repeats) verified the presence of the 302-bp sequence previously identified by Mills et al. (34) as the hu-hs1.2 enhancer (EMBL AF013723), which includes a 135-bp sequence with core homology to the mo-hs1.2 enhancer. All reporter plasmids consisted of a 4.818-kb pGL3 basic luciferase reporter backbone (Promega, Madison, WI). Additionally, the hu-hs1.2 plasmids unexpectedly contained a transcriptionally active DRE core motif in the multiple cloning site of the pGL3 backbone that was expressed as the relative quantification value compared with cells cultured with media alone (NA). Results are representative of three separate experiments (n = 3 for each treatment group). **p < 0.01, ***p < 0.001, compared with the respective vehicle control, one-way ANOVA with a Dunnett post hoc test.

Protein isolation for γ2b analysis

CH12.y2b-hs3A/hs1.2 cells in media alone (naive [NA]) or stimulated with LPS (1 μg/ml) (alone control [C]) and treated with varying concentrations of TCDD (0–10 nM) or the vehicle control (0.01% DMSO; 0 nM TCDD) were plated into 12-well plates at a concentration of 2.5 × 10^5 cells/well and incubated for 48 h. Protein isolation and ELISA analysis for γ2b protein levels were performed, as previously described (7). Briefly, cells were centrifuged at 3000 rpm and lysed with mild lysis buffer (1% Nonidet P-40, 50 mM NaCl, 10 mM NaPO4, 2 mM EDTA) containing freshly added protease inhibitors (Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics, Indianapolis, IN). Cell lysates were centrifuged at 14,000 rpm, and supernatants were collected and stored at −80°C until analysis. To measure γ2b, cell lysates were thawed on ice, and protein concentrations were determined by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Samples were then diluted to the lowest sample concentration, 1 μg total protein was analyzed for γ2b by ELISA, as described (4), and colorimetric detection was performed every minute over a 1-h period using a Spectramax plus 384 automated microplate reader with a 405-nm filter (Molecular Devices, Sunnyvale, CA). The concentration of γ2b in each sample was calculated using SOFTmax PRO analysis software (Molecular Devices) using a standard curve generated from the kinetic rate of absorption for known γ2b concentrations. Results are represented as mean (± SE) γ2b IgG (ng)/1 μg total protein (n = 3–4).

RNA isolation, cDNA synthesis, and real-time PCR

CH12.LX cells in media alone (NA) or stimulated with LPS (1 μg/ml; C) and treated with 10 nM TCDD or the vehicle control (0 nM TCDD) were plated into 12-well plates at a concentration of 2.5 × 10^5 cells/well and incubated for 0, 24, 36, or 48 h. Total RNA was isolated using TRI Reagent (Sigma-Aldrich), according to the manufacturer’s protocol. The RNA concentration was determined using a NanoDrop (Thermo Scientific, Wilmington, DE), and 200 ng total RNA was reverse transcribed to cDNA using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA) in a 10 μl reaction volume. The reaction cycles were 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. The expression of β-actin (endogenous control to normalize cDNA concentrations) and the endogenous H chain and L chain (i.e., αC region, Ca, and κ C region, Ck, respectively) genes were quantified by real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems), as previously described (42). Primers for Ca, Ck, and β-actin span an intron and are listed in Table I. The results of the PCR amplification were analyzed using the 7500 system SDS software to determine relative quantification values (i.e., fold change).

**FIGURE 3.** TCDD inhibits endogenous Ig H chain and L chain genes. CH12.LX cells (2.5 × 10^5 cells/ml) stimulated with LPS and treated with 0 nM TCDD or the vehicle control (0.01% DMSO) (0 nM TCDD) were plated into 12-well plates and incubated for 24, 36, or 48 h prior to total RNA isolation. Total RNA (200 ng) was reverse transcribed to cDNA and used to amplify the endogenous H chain and L chain genes (Ca (A) and Ck (B), respectively) via SYBR Green real-time PCR. The results are expressed as the relative quantification value compared with cells cultured with media alone (NA). Results are representative of three separate experiments (n = 3 for each treatment group). **p < 0.01, ***p < 0.001, compared with the respective vehicle control, one-way ANOVA with a Dunnett post hoc test.

Site-directed mutagenesis

Mutations of the $\alpha_{1A}$ and $\alpha_{1P}$ plasmids were generated using PCR-based site-directed mutagenesis, according to the manufacturer’s instructions (QuickChangeXL Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA). Briefly, deletion of the IS within the $\alpha_{1A}$ plasmid ($\alpha_{1A}\Delta IS1$), deletion of the first IS from the $\alpha_{1B}$ plasmid ($\alpha_{1B}\Delta IS1$), and addition of the high-affinity Pax5 binding site within the $\alpha_{1A}$ ($\alpha_{1A} + \text{Pax5}$) were accomplished using primers listed in Table I. For the $\alpha_{1A} + \text{Pax5}$ plasmid, the high-affinity Pax5 site was inserted downstream of the AP-1/Ets, Oct, and IS in the $\alpha_{1A}$-hu-hs1,2 (specifically 26 bp 3' of the Sp1 site) to most closely resemble the mo-hs1,2 where the Pax5 is present downstream of the DRE, NF-κB, AP-1/Ets, and Oct binding sites (Fig. 1B).

PCR reaction conditions were 95˚C for 1 min, followed by 18 cycles at 95˚C for 50 s, 60˚C for 50 s, 68˚C for 5.5 min (1 min/kb of plasmid length) and then 68˚C for 7 min. Following the PCR reaction, the parental plasmid was digested by incubating with Dpn I (1 μl/50 μl reaction) for 1 h at 37˚C. Clones of mutated plasmids were replicated in bacteria and screened for the correct mutation by DNA sequencing (Retrogen, San Diego, CA).

Transient transfection and luciferase assay

For transient transfections, CH12.LX or IM-9 ($1.0 \times 10^7$) cells were resuspended in 200 μl culture media with 10 μg plasmid and transferred to a 2-mm gap electroporation cuvette (Molecular BioProducts, San Diego, CA). Cells were electroporated using an electro cell manipulator (ECM 630; BTX, San Diego, CA) with the following voltage, capacitance and resistance: 250 V, 150 μF, and 75 Ω for CH12.LX cells and 150 V, 1700 μF, and 75 Ω for IM-9 cells. For each reporter plasmid, multiple transfections were pooled, and cells were aliquoted in triplicate into 12-well plates at 2.0 $\times$ 10^5 cells/ml for CH12.LX cells and 1.0 $\times$ 10^6 cells/ml for IM-9 cells. Immediately after transfection, cells were treated for 24 h with TCDD (0–30 nM) in the absence or presence of LPS (0–1.0 μg/ml) stimulation. The vehicle control for TCDD was 0.01% DMSO (0 nM TCDD).

FIGURE 4. TCDD activates the polymorphic hu-hs1,2 enhancer in a concentration-dependent manner. (A) Schematic of human $V_{H}$ promoter alone and $\alpha_{1}$ hs1,2 allelic constructs containing the IS (denoted by a star). All luciferase reporter plasmids originated from the pGL3 Basic vector (Promega), and the polymorphic $\alpha_{1}$ hu-hs1,2 enhancers were inserted 3' of the luciferase gene. (B and C) CH12.LX cells were transiently transfected with $V_{H}$, $\alpha_{1A}$, $\alpha_{1B}$, or $\alpha_{1C}$ reporter constructs. Transfected cells were cultured for 24 h with either media alone (NA) or with varying concentrations of TCDD (0–10.0 nM) in the absence or presence of LPS (1.0 μg/ml) stimulation. (B) Luciferase enzyme activity (mean ± SEM, n = 3) is represented on the y-axis as RLU normalized to transfection efficiency and relative to the NA control (set to 1 RLU) of the $V_{H}$ reporter. LPS-stimulated samples were significantly ($p < 0.01$) elevated compared with their corresponding unstimulated samples, as determined by one-way ANOVA, followed by a Bonferroni post hoc test (not represented on graph). Results are representative of three separate experiments. (C) TCDD-induced activation is represented on the y-axis as fold change relative to the respective vehicle control (0.01% DMSO [0 nM TCDD]) and was generated from 3–11 separate experiments; each symbol (●, ▲, ▼, and ♦) signifies the $V_{H}$, $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1C}$ reporter constructs, respectively) represents the mean (n = 3) from a single experiment. Significance compared with either the corresponding vehicle control (0.01% DMSO [0 nM TCDD]) (B) or the respective $V_{H}$ reporter (C) was determined by one-way ANOVA, followed by a Dunnett post hoc test: *p < 0.05, **p < 0.01. C, LPS stimulation alone; >, synergistic activation by TCDD and LPS cotreatment compared with the activation induced by either treatment alone.
TCDD). For the AhR-antagonist studies, CH12.LX cells were treated with 15 μM CH-223191 for 1 h prior to TCDD (1 nM) and LPS (1.0 μg/ml) treatment. Based on a pretreatment time course, IM-9 cells were treated for 6 h with 15 μM CH-223191 prior to TCDD (30 nM) treatment. The vehicle control for TCDD and CH-223191 was 0.16% DMSO to account for the increased vehicle concentration of a TCDD and CH-223191 cotreatment. After the 24-h incubation period, cells were lysed with 1 × reporter lysis buffer (Promega) and immediately frozen at −80°C. As previously described, luciferase enzyme activity was measured, and the activity was normalized to transfection efficiency by real-time PCR analysis of the luciferase gene (6). Briefly, DNA was isolated in triplicate using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich), diluted 10-fold, and then analyzed by real-time PCR using SYBR Green for the luciferase gene. Plasmid number/cell was calculated with the equation: [ng of plasmid] × (molecules/ng of plasmid)/cell number] × 10 to account for the 10-fold dilution of DNA used in the PCR reaction. To determine the variance in transfection efficiency between plasmids, we first adjusted for normal experimental variation by calculating the transfection efficiency for each experiment as fold change relative to the control plasmid. The transfection efficiencies for each plasmid from several experiments were either displayed as individual data points or the average (mean ± SEM) of several experiments.

**Statistical analysis**

Luciferase activity and γ2b IgH levels are represented as the mean ± SEM for each treatment group (n = 3) from a representative experiment. For fold-change graphs, RLU values were transformed to TCDD-induced fold change (mean ± SEM) relative to the DMSO (0 nM TCDD) control set to 1. The mean (n = 3) was then determined for each treatment group, and the means generated from several experiments were either displayed as individual data points or the average (mean ± SEM) of several experiments, as indicated in the figure legends, significant differences (p < 0.05) were determined by a two-way ANOVA with a Bonferroni post hoc test, one-way ANOVA with a Dunnett post hoc test, or an unpaired t test.

**Results**

The mo-hs1,2 enhancer of the 3′IgHRR is a sensitive target for TCDD-induced inhibition

The CH12.LX mouse B cell line expresses a functional AhR-signaling pathway and exhibits TCDD-induced inhibition of IgH chain and L chain gene expression and Ab secretion that mirror effects seen in vivo and in primary B cells (4, 7, 15, 17, 18). Correspondingly, our previous reporter studies with CH12.LX cells demonstrated a marked inhibition by TCDD of LPS-induced mouse 3′IgHRR (composed of the hs3A, hs1,2, hs3B, and hs4 enhancers; Fig. 1A) activation that appears to involve AhR (4–7). In striking contrast, LPS and TCDD cotreatment resulted in a marked activation of the mouse hs4 enhancer (mo-hs4) (6), suggesting that TCDD-induced inhibition of LPS-stimulated 3′IgHRR activity is mediated at the hs1,2 enhancer. To test this hypothesis, we used a reporter gene identical to our earlier experiments, except that it was regulated by mo-hs1,2 (Fig. 2A). Like the mouse 3′IgHRR reporter, but in contrast to the mo-hs4 reporter (6), TCDD inhibited mo-hs1,2 activation in a concentration-dependent manner (Fig. 2A).

To avoid many of the limitations associated with transient transfections and to better reflect endogenous IgH regulation and chromatin accessibility, we generated a variant of the CH12.LX B cell line (CH12-γ2b-hs3A/hs1,2) that stably expresses a single copy of a γ2b transgene (40) under the regulation of the hs3A and hs1,2 enhancers. CH12-γ2b-hs3A/hs1,2 cells demonstrated an LPS-induced inhibition of LPS-stimulated 3′IgHRR activation that appears to involve AhR (4–7).
induced increase in γ2b expression that was inhibited by TCDD (Fig. 2B). Although we cannot say that the inhibitory effect of TCDD on γ2b expression is entirely due to hs1,2, these results are consistent with the effects of TCDD on the mo-hs1,2 luciferase reporter (compare Fig. 2A, 2B). Interestingly, the CH12.γ2b-hs3A/hs1,2 cell line model (γ2b-regulated by hs3A and hs1,2) appears to be more sensitive to TCDD-induced inhibition compared with the mo-hs1,2 luciferase reporter (compare Fig. 2B, 2A). This may reflect a contribution of the hs3A enhancer present in the stable reporter and/or differences in the sensitivity of transient versus stable reporters (i.e., the hs1,2 luciferase enhancer generally has low transfection efficiency and lower overall luciferase activity) (6). Nevertheless, the current results, in combination with previous studies (4–7, 15), suggest that the hs1,2 enhancer or the hs3A/hs1,2 enhancer pair mediates TCDD-induced inhibition of LPS-stimulated 3′IgHRR activity.

As stated above, previous studies identified a sensitive inhibition by TCDD of IgH chain and L chain gene expression and Ab levels in activated primary B cells and CH12.LX (IgM+) cells (4, 15, 17, 18). Because the current studies use a variant of CH12.LX cells that express IgA instead of IgM, we evaluated the effect of TCDD on LPS-induced expression of the endogenous H chain and L chain Ig genes (i.e., α C region, Ca, and κ C region, Cκ, respectively, Table I). TCDD significantly inhibited LPS-induced expression of both Ca and Cκ at 24, 36, and 48 h (Fig. 3). This correlates well with previous results demonstrating inhibition by TCDD of IgA protein levels in these cells (7) and with luciferase and γ2b reporter results demonstrating inhibition of the 3′IgHRR and the hs1,2 (and hs3B/hs1,2) enhancer (4–7, 15) (Fig. 2).

**TCDD activates the human polymorphic hs1,2 enhancer in the mouse CH12.LX B cell line and in the human IM-9 B cell line**

As mentioned previously, the polymorphic α1-hu-hs1,2 enhancer has been correlated with several autoimmune diseases in humans (23–28), and we identified a DRE binding site within the IS, which closely resembles the binding site found in mo-hs1,2 (Fig. 1B). Our previous studies identified TCDD-induced AhR binding to the DRE site within mo-hs1,2 (4), and in this study we demonstrate inhibition of mo-hs1,2 activation by TCDD (Fig. 2A, 2B). If, in fact, this inhibition is mediated through the DRE site, it follows that hu-hs1,2 could also be a transcriptional target of TCDD, perhaps with greater sensitivity with an increasing number of IS and DRE sites. Therefore, we evaluated the effect of TCDD, as well as B cell activation by LPS, or the combination of both on luciferase reporter plasmids regulated by the V_H promoter and the human α1 hs1,2 enhancer with one, two, or three IS (representing alleles α1A, α1B, or α1C; Fig. 4A). Surprisingly, TCDD had a striking, but divergent, effect on the hu-hs1,2 alleles compared with mo-hs1,2. In the absence of LPS stimulation, TCDD significantly activated hu-hs1,2 in a concentration-dependent manner and at TCDD concentrations as low as 0.1 nM for α1A and α1B and 0.01 nM for α1C (Fig. 4B). LPS stimulation synergistically enhanced TCDD-induced activation of hu-hs1,2 but not of the V_H promoter (Fig. 4B). Because LPS was shown to increase AhR protein expression and to activate transcription factors with binding sites within hu-hs1,2 (i.e., AP-1, NF-κB, Oct) that are also influenced by TCDD and/or AhR (4, 13, 15, 21, 22, 45), we further evaluated the synergy between TCDD and LPS by conducting concentration-response studies with LPS. Synergism was dependent on both the TCDD and LPS concentration and was solely mediated by hu-hs1,2 (no synergistic activation of the V_H promoter) (Figs. 4B, 5A). Additionally, the magnitude of synergism was greater with more ISs. In contrast, the TCDD-induced fold effect, although dependent on the concentration of TCDD and LPS, was not dependent on the number of ISs (Figs. 4C, 5B). In the absence of LPS stimulation, the V_H promoter alone exhibited noticeable variation in TCDD-induced fold activation. This variation may be due to the very low basal activity of the V_H promoter, typically well below 100 RLU compared with ≥200 RLU following LPS stimulation or the addition of the hs1,2 enhancer. Correspondingly, LPS stimulation or the addition of the hs1,2 enhancer exhibited less variation (Figs. 4C, 5B). In any case, hu-hs1,2 induced a markedly greater level of overall reporter activity compared with the V_H promoter alone, although a strict dependence on the number of ISs was less consistent when all of the reporters were analyzed together. Additionally, the synergistic activation of the hs1,2 enhancers by TCDD and LPS, as opposed to the TCDD-induced fold change, was consistently influenced by the number of ISs, particularly with the α1C allele.

The effect of TCDD on hu-hs1,2 was not limited to a mouse cell line; a similar effect was demonstrated in a human B cell line. IM-9. IM-9 cells were shown to express a functional AhR-signaling pathway (41), which we validated by Western blot analysis for AhR and by TCDD-induced CYP1A1 mRNA induction (hallmark end point of a functional AhR-signaling pathway). Notably, IM-9 cells express much lower levels of AhR, which correlated with a weaker TCDD-induced CYP1A1 induction compared with CH12.LX cells (data not shown). Similarly to CH12.LX cells, TCDD treatment of IM-9 cells transiently transfected with the hu-hs1,2 reporters demonstrated increased transcriptional activity; however, the greatest transcriptional activity and fold change were seen most consistently with the α1B, C allele (Fig. 6). Additionally, TCDD induced a lower fold induction of the hu-hs1,2 reporters in

![FIGURE 6. TCDD activates the polymorphic hu-hs1,2 enhancer in the human IM-9 B cell line. IM-9 cells were transiently transfected with V_H, α1A, α1B, or α1C reporter constructs. Transfected cells were cultured for 24 h with media alone (Control), 0.01% DMSO, or 30 nM TCDD. (A) Luciferase enzyme activity (mean ± SEM, n = 3) is represented on the y-axis as RLU normalized to transfection efficiency. **p < 0.01, ***p < 0.001, compared with the corresponding DMSO vehicle control, two-way ANOVA followed by a Bonferroni post hoc test. Significance of poly- morphic hu-hs1,2 reporters compared with the V_H reporter at p < 0.05 for α1A and p < 0.01 for α1A and α1C (not represented on the bar graph). Results are representative of three separate experiments. (B) TCDD-induced fold changes in reporter activity is represented on the y-axis as fold change relative to the respective DMSO vehicle control and was generated from 6–12 separate experiments; each symbol represents the mean (n = 3) from a single experiment. *p < 0.05, **p < 0.01, compared with the V_H reporter, one-way ANOVA followed by a Dunnett post hoc test.](http://www.jimmunol.org/)
IM-9 cells compared with CH12.LX cells, and a higher concentration of TCDD (30 nM versus 10 nM) was required to induce this effect, which correlates with the lower basal AhR expression in IM-9 cells. Interestingly, and in contrast to CH12.LX cells, the VSH promoter was minimally affected by TCDD and exhibited less variability, perhaps as a result of the greater basal transcriptional activity of the reporters in IM-9 cells. In CH12.LX cells, cellular activation by LPS (TLR4 ligand) and cotreatment with TCDD led to an enhanced activation of hu-hs1,2, which may relate to the well-documented increase in AhR protein expression shortly after B cell activation (15, 21, 22). However similar attempts to activate IM-9 cells with a TLR ligand were not successful. LPS, CpG, and R848 (ligands for TLR4, TLR7, and TLR9, respectively) did not induce IM-9 cells to secrete Ig (data not shown). Regardless, our results support that the polymorphic hu-hs1,2 is a significant transcriptional target of TCDD.

ISs contribute to hu-hs1,2 activation, and AhR mediates TCDD-induced activation

The IS contains a DRE, AP-1, NF1, and NF-κB binding site (Fig. 1B, 1C) and appears to influence basal hu-hs1,2 activity, as well as the overall and synergistic activation by TCDD and LPS, but less so for TCDD-induced fold change. Because there are other putative transcription factor binding sites, including AP-1/Ets and Oct, outside of the IS, we used site-directed mutagenesis to delete the IS from our reporter constructs (Table I) to determine the role of the IS in hu-hs1,2 activity. Deletion of an IS from the α1A (α1AΔIS1) or α1B (α1BΔIS1) hu-hs1,2 alleles significantly lowered basal activity, as well as LPS- and/or TCDD-induced activation in CH12.LX cells (Fig. 7A, 7B). However, with TCDD treatment, α1BΔIS1 still exhibited significantly greater activity than did α1A (Fig. 7B). Interestingly, deletion of IS1 in the α1A hu-hs1,2 allele (α1AΔIS1) did not significantly alter the fold induction by TCDD relative to DMSO (Fig. 7C), but there was a noticeable nonsignificant decrease in fold induction by TCDD with deletion of the IS1 in the α1B hu-hs1,2 allele (α1BΔIS1) (Fig. 7D). Collectively, these results show a significant contribution of the IS to overall hs1,2 activity (i.e., naïve and in response to TCDD and/or LPS); however, the transcription factor binding sites, including the DRE-like site within the IS, do not appear to greatly influence fold induction by TCDD.

Therefore, our next objective was to determine the role of AhR in TCDD-induced hu-hs1,2 activity. To this end, we used an AhR

FIGURE 7. Deletion of the IS from the α1A and α1B hu-hs1,2 constructs reduces overall reporter activity. CH12.LX cells were transiently transfected with reporter constructs regulated by the wild-type α1A (Δ/), α1A with the IS deleted (α1AΔIS1: Δ/Δ), wild-type α1B (○/○), or α1B with the 5′ IS deleted (α1BΔIS1; ▼/▼). Transfected cells were cultured for 24 h with either media alone (NA) or with varying concentrations of TCDD (0–10.0 nM) in the absence (open symbols) or presence (filled symbols) of LPS (1.0 μg/ml) stimulation. Luciferase enzyme activity (mean ± SEM; n = 3) of α1A and α1AΔIS1 (A) or of α1A, α1B, and α1BΔIS1 (B) is represented on the γ-axis as fold change relative to transfection efficiency. Results are representative of at least five separate experiments. TCDD-induced activation of α1A and α1AΔIS1 (C) or of α1A, α1B, and α1BΔIS1 (D) is represented on the y-axis as fold change relative to the respective DMSO vehicle control and was generated from five to seven separate experiments; each symbol represents the mean (n = 3) from a single experiment. *p < 0.05, **p < 0.01, compared with the corresponding vehicle control (0.01% DMSO [0 nM TCDD]), one-way ANOVA, followed by a Dunnett post hoc test. Significance between the reporters was determined by a two-way ANOVA, followed by a Bonferroni post hoc test. The vertical line in (A) and (B) represents significant differences (p < 0.001) between two reporters at all treatment conditions. ††p < 0.01, †††p < 0.001, α1B versus α1BΔIS1. The reporters were not significantly different in (C) and (D). C, LPS stimulation alone.
antagonist, CH-223191, that was shown to bind AhR and prevent its translocation to the nucleus (35). In CH12.LX cells, CH-223191 pretreatment inhibited TCDD-induced Cyp1A1 expression (data not shown) and efficiently antagonized the effect of TCDD on hu-hs1,2 (Fig. 8A, 8B). The AhR antagonist inhibited both TCDD-induced overall activation and fold change of α1A, α1B, and α1C hu-hs1,2 reporter activity, with no effect on basal or LPS-induced activity (Fig. 8A, 8B). Similar results were obtained in human IM-9 cells; the AhR antagonist completely reversed TCDD-induced α1A, α1B, and α1C hu-hs1,2 activation (Fig. 8C, data not shown). These results support a role for AhR in mediating TCDD-induced activation of hu-hs1,2.

Pax5 and the species differences in regulation of the hs1,2 enhancer

A major difference between the human and mouse hs1,2 enhancers is the presence of a high- and low-affinity Pax5 (paired box protein 5) binding site within mo-hs1,2 that is not conserved in hu-hs1,2 (34). Pax5, a central regulator of B cell development and differentiation (46, 47), specifically inhibits IgH expression by binding Pax5 motifs within the hs1,2 and hs4 enhancers and recruiting corepressors (48–51). During B cell differentiation, Pax5 expression is progressively decreased, lifting transcriptional repression of the Ig genes (47, 52, 53). Interestingly, TCDD treatment prevents the downregulation of Pax5 expression in CH12.LX cells and splenic mouse B cells stimulated with LPS; this sustained Pax5 expression may contribute to the inhibitory effect of TCDD on Ig expression and Ab secretion (17, 18). The lack of a Pax5 binding site in the hu-hs1,2 may partially explain the species differences seen in this study in hs1,2 enhancer regulation. Therefore, we evaluated the effect of inserting the high-affinity mo-hs1,2 Pax5 binding site within the α1A hu-hs1,2 reporter (α1A+Pax5; Table I). We chose the α1A allele because it is most similar to mo-hs1,2, which does not contain polymorphic tandem repeats. Insertion of the Pax5 binding site significantly suppressed TCDD-induced reporter activity in unstimulated CH12.LX cells, with a minimal effect on the basal reporter activity. In LPS-stimulated CH12.LX cells, insertion of the Pax5 binding site resulted in a marked suppression of LPS-induced reporter activity (Fig. 9A), but TCDD-induced fold change was not significantly altered (Fig. 9B). Similarly to the mouse CH12.LX cell line, insertion of the Pax5 binding site in the α1A reporter did not consistently alter basal reporter activity in the human IM-9 cell line, but suppression of TCDD-induced reporter activity was observed.

**FIGURE 8.** TCDD activates the polymorphic hu-hs1,2 enhancer in an AhR-dependent manner. CH12.LX or IM-9 cells were transiently transfected with VH, α1A, α1B, or α1C reporter constructs. Transfected cells were pretreated with 15 μM AhR antagonist (CH-223191), 0.15% DMSO, or media alone and then cultured for 24 h in the absence or presence of LPS (1.0 μg/ml) stimulation and media alone, 0.01% DMSO, or TCDD (1 nM for CH12.LX; 30 nM for IM-9). The final DMSO concentration was 0.16%. “Control” denotes either unstimulated naive or LPS alone. (A) Luciferase enzyme activity (mean ± SEM, n = 3) in CH12.LX cells is represented on the y-axis as RLU normalized to transfection efficiency. Results are representative of three separate experiments. (B) TCDD-induced fold changes in reporter activity in CH12.LX cells is represented on the y-axis as fold change relative to the respective DMSO vehicle control and was generated from averaging the means of three independent experiments (n = 3 for each treatment group within each experiment). (C) TCDD-induced fold changes in reporter activity in IM-9 cells is represented on the y-axis as fold change (mean ± SEM, n = 3) relative to the respective DMSO vehicle control and is representative of three experiments. Significance was determined by one-way ANOVA, followed by a Dunnett post hoc test (A) or two-way ANOVA, followed by a Bonferroni post hoc test (B, C): *p < 0.05, **p < 0.01, ***p < 0.001, versus corresponding DMSO vehicle control; †p < 0.05, ††p < 0.01, †††p < 0.001, versus corresponding VH reporter, two-way ANOVA with a Bonferroni post hoc test.
A L or IM-9 cells were transiently transfected with either wild-type a

not significantly different, as determined by an unpaired

way ANOVA, followed by Bonferroni post hoc test. Vertical line in (p

represents significant differences (p < 0.001) at all treatment conditions.

FIGURE 9. Insertion of a Pax5 binding site in the α₁₄ h₁₂,1 construct

inhibits reporter activity in mouse CH12.LX and human IM-9 cells. CH12.

LX or IM-9 cells were transiently transfected with either wild-type α₁₄ or

α₁₄ with an inserted high-affinity Pax5 binding site from the mo-h₁₂,1 enhancer (α₁₄+Pax5). (A and B) Transfected CH12.LX cells were cultured

for 24 h with varying concentrations of TCDD (0–10.0 nM) in the absence

(open symbols) or presence (filled symbols) of LPS (1.0 μg/ml) stimulation.

(C) Transfected IM-9 cells were cultured for 24 h with media alone

(Control), 0.01% DMSO, or 30 nM TCDD. (A and C, left panel) Luciferase

enzyme activity (mean ± SEM, n = 3) of α₁₄ and α₁₄+Pax5 is represented

on the y-axis as RLU normalized to transfection efficiency. Results are

representative of five (A) and seven (C, left panel) separate experiments.

(B and C, right panel) TCDD-induced activation of α₁₄ and α₁₄+Pax5 is

represented on the y-axis as fold change relative to the respective DMSO

vehicle control and was generated from five (B) and seven (C, right panel)

separate experiments; each symbol represents the mean (n = 3) from a single experiment. Significance was determined by one-way ANOVA,

followed by a Dunnett post hoc test (A) or two-way ANOVA, followed by a

Bonferroni post hoc test (B, C); t < 0.05, **p < 0.01, ***p < 0.001, compared with the corresponding DMSO vehicle control (0 nM TCDD).

p < 0.01, †p < 0.05, ††p < 0.01, α₁₄ versus α₁₄+Pax5 reporters, two-

way ANOVA, followed by Bonferroni post hoc test. Vertical line in (A)

represents significant differences (p < 0.001) at all treatment conditions.

(C, right panel) TCDD-induced fold changes of α₁₄ and α₁₄+Pax5 were

not significantly different, as determined by an unpaired t test.

There was no consistent effect on TCDD-induced fold change (Fig.

9C). Cellular activation of IM-9 cells would likely exhibit greater effects,

as demonstrated in CH12.LX cells; however, as described above, we were unable to activate IM-9 cells. Taken together, these results

support an inhibitory role for Pax5, but it does not appear to

mediate the inhibitory effect of TCDD on mo-h₁₂,1.

Discussion

Our previous work identified the mouse 3′IghRR as a sensitive

target of various chemicals, including TCDD and other ligands for

AhR. Effects of these chemicals on 3′IghRR reflected their effects

on endogenous H chain mRNA and protein expression (4–7),

underscoring the association of 3′IghRR activity and Ig expression

(3). Given that TCDD-induced inhibition of Ig expression

and secretion is AhR dependent (9, 15, 16), the 3′IghRR may be a

molecular target of AhR. Indeed, recent studies using an AhR

antagonist or short hairpin RNA directed against AhR identified

a reversal in the inhibitory effect of TCDD on 3′IghRR activation

and endogenous Ig expression (19, 20) (M.J. Wourms and C.E.W.

Sulentic; R.L. Salisbury and C.E.W. Sulentic, manuscripts in prepa-

ration). The current results suggest that inhibition of mouse 3′IghRR

activation by TCDD is mediated through the h₁₂,1 enhancer.

Because of sequence homology within the mouse and human 3′IghRR

enhancers (34) and the presence of a fairly well conserved DRE

site within hu-h₁₂,1, we hypothesized that hu-h₁₂,1 is also a target

of TCDD. Indeed, hu-h₁₂,1 activity was modulated by TCDD.

Surprisingly, it was activated, which was opposite from the inhibi-

tory effect of TCDD on mo-h₁₂,1. Furthermore, our inhibitor

studies with an AhR antagonist strongly support a role for AhR

in the effects of TCDD on hu-h₁₂,1 activity. Because several human

diseases, including celiac disease, IgA nephropathy, Burkitt’s

lymphoma, systemic sclerosis, plaque psoriasis, and rheumatoid

arthritis (23–28), have been associated with the human 3′IghRR

and all (with the exception of Burkitt’s lymphoma) have been

associated with the polymorphic hu-h₁₂,1, the occurrence and

severity of these disease states could be altered by AhR ligands.

Although sequence homology was identified between the mouse

and human 3′IghRR enhancers, species differences in the effects

of TCDD on the h₁₂,1 enhancer are in agreement with previous

suggestions of potential differences in regulation due to a lack of

conservation of specific transcription factor binding sites (e.g.,
Pax5) outside of the species-conserved regions (30, 34). Sepulveda

and colleagues (54) demonstrated a difference in regulation of the

human versus mouse hs₄ enhancer; Oct-2 and NF-κB/Rel proteins

cooperatively regulated human hs₄ activity, but not Oct-1 and

Pax5, as seen previously with the mouse hs₄ enhancer (50). A

similar mechanism may occur for hs₁₂,1 enhancer regulation be-

cause insertion of the mo-h₄,1 high-affinity Pax5 binding site into

hu-h₁₂,1 (α₁₄+Pax5) significantly decreased TCDD and LPS-

induced activation of hu-h₁₂,1. However, under the current ex-

perimental conditions, Pax5 does not appear to mediate the species

differences in the effects of TCDD on h₁₂,1 activity (i.e., TCDD-

induced activation of hu-h₁₂,1 versus inhibition of mo-h₁₂,1).

Alternatively, the mo-h₁₂,1 enhancer contains a high-affinity and

a low-affinity Pax5 binding site; it is possible that both are nec-

essary for the inhibitory effect of TCDD.

The effects of TCDD on hu-h₁₂,1 resemble the effects on mo-

hs₄, which was shown to be synergistically activated by cotreat-

ment with TCDD and LPS (5, 6). Activation of mo-hs₄ was ini-

tially difficult to reconcile with the overall inhibitory effect of

TCDD on mouse 3′IghRR activation. However, as shown in this

study, this dichotomy is balanced by the negative effect of TCDD

on mo-h₄,1 activity, which may mediate the net inhibitory effect

of 3′IghRR activation. Interestingly, κB, Pax5, and Oct binding

sites were shown to positively regulate mo-hs₄ and negatively

regulate mo-hs₁₂,1 in mature B cells, but not in plasma cells where

κB and Oct binding sites become positive regulators of mo-hs₁₂,1,

and Pax5 has no influence because its expression is downregulated

in plasma cells (50). Similarly, TCDD increased κB and DRE

binding within mo-hs₄, and mutational analysis identified these

binding sites as positive modulators of the synergistic activation of

the mo-hs₄ activity by TCDD and LPS (5, 6). Because both a κB

and DRE site (but not Pax5) are conserved within hu-h₁₂,1, and

previous studies identified a physical interaction between AhR and
NF-κB proteins (reviewed in Refs. 55, 56), we speculate that these proteins may cooperatively interact to influence hu-hs1,2 activation by TCDD.

Additionally, our AhR antagonist studies strongly support a role for AhR in the effects of TCDD on hu-hs1,2 activity; however, the lack of an influence of the IS on the fold induction by TCDD also suggests the involvement of transcription factors identified outside of the IS (i.e., Oct, AP-1/Ets, Sp1) that are modulated by AhR. In fact, TCDD and the AhR-signaling pathway were shown to alter AP-1 and Sp1 binding to either consensus sites or sites within specific transcriptional promoters (13, 45, 57–60). Additionally, Kel et al. (61) identified, through in silico genomic footprinting, a composite regulatory module in promoters of AhR-regulated genes that contains binding sites for AhR, as well as binding sites for several other transcription factors, including a high occurrence of binding sites for Oct, AP-1/NF-1, and Sp1, suggesting transcriptional regulation that is dependent on an interaction between these transcription factors and AhR. Therefore, TCDD could modulate a number of transcription factors to induce hu-hs1,2 activation. Notably, in contrast to the fold effect by TCDD, the number of IS did reproducibly influence the magnitude of the synergistic effect induced by cotreatment of TCDD and LPS on hu-hs1,2 activation. In addition to LPS and TCDD likely modulating a common set of transcription factors (e.g., NF-κB, AP-1, Oct), another potential reason for this synergism is that cellular stimulation, in general, was shown to upregulate AhR expression in CH12.LX cells (i.e., increased AhR protein expression by 8 h), as well as primary mouse and human B cells (15, 21, 22). Perhaps the synergistic activation of hu-hs1,2 reflects increased AhR levels and, therefore, increased AhR activation and interactions between AhR and other transcription factors inside and outside of the IS. Using site-directed mutagenesis, current efforts are focused on elucidating the contribution of individual transcription factor binding sites within and outside the IS to basal hu-hs1,2 activity and activation induced by TCDD, LPS, or a cotreatment of LPS and TCDD.

The sensitivity of the Ab response to TCDD-induced suppression in animal models suggests that human B cells could be a sensitive target of TCDD; however, very few studies have evaluated the effect of TCDD on human B cell function and Ig expression. Results of these studies appear to support a variation in the magnitude of the response (i.e., altered Ig expression) of human primary tonsillar lymphocytes and primary peripheral blood B cells to TCDD, with the most responsive cells demonstrating a similar sensitivity to TCDD as mouse models (62, 63). Interestingly, the actual effect of TCDD on the Ig response also varied and demonstrated an inhibitory effect in some donor cells versus no effect or an increase in the Ig response in other donor cells, which may relate to differences in AhR function (63). In a different study, TCDD induced an increase in IgE production in B cells from patients with allergic rhinitis, atopic eczema/dermatitis syndrome, or bronchial asthma but not healthy controls (64). These limited studies support a complex effect of TCDD on Ig responses in human cells, which likely involves the influence of many factors, including AhR function, disease states, and perhaps the hu-hs1,2 polymorphism.

The 3′ end of the IgH locus in humans is duplicated, resulting in four extra constant regions and two 3′IghRR, one downstream of each α C region (Coα1 and Coα2); unlike the mouse, the human 3′IghRR contains only three enhancers: hs3, hs1,2, and hs4 (Fig. 1A) (34). Of the mouse and human 3′IghRR enhancers, only hu-hs1,2 contains a polymorphism that is characterized by the presence of one to four 38-bp ISs, with 14–20-bp spacers between the IS repeats (32, 65). The hu-hs1,2 alleles of Coα (i.e., α1A, α1B, α1C, and α1D [also referred to as A1, A2, A3, and A4], representing one, two, three, or four IS, respectively) have been associated with various autoimmune diseases and exist at relatively high frequencies in certain ethnic groups (66). A correlation between particular hs1,2 alleles and overall Ig levels was reported in one study in which a higher frequency of α1A was associated with lower total Ig levels, and a higher frequency of α1B was associated with higher total Ig levels (33). Because the only difference between the hu-hs1,2 alleles is the addition of the ISs containing binding sites for NF-κB, NF1, AP-1, Sp1, and AhR, the IS and proteins binding within it would appear to be the major contributing factor in the association between the hu-hs1,2 alleles and specific disease states and total Ig levels. In the current study, a strict correlation was not consistently seen; however, generally, an increase in IS led to an increase in basal activity and TCDD- and/or LPS-induced activity, which was further supported by deletion of the IS (α1AΔIS1 and α1BΔIS1). Differences in activity of the hu-hs1,2 alleles may have physiological relevance in that the IS-dependent increase in the overall magnitude of hu-hs1,2 activation by TCDD, LPS, and a cotreatment of LPS and TCDD (i.e., synergistic activation) may push an Ig locus over the threshold for activity. Current efforts are focused on identifying human cell lines with a functional AhR-signaling pathway that can be stimulated to express Ig to further elucidate the mechanism by which TCDD and other AhR ligands affect the polymorphic hu-hs1,2 enhancer and Ig expression.

Many factors are likely involved in the overall effect of TCDD on human Ig expression, not all of which are likely mediated through hu-hs1,2. The current study focused on the hs1,2 enhancer because of recent studies that identified an association between human immune-related disorders and hu-hs1,2 polymorphisms (23–28). Our results demonstrate an AhR-dependent modulation of the hs1,2 alleles, and it is becoming increasingly evident that exogenous activators of the AhR-signaling pathway are not limited to TCDD or dioxin-like compounds. A diverse array of chemicals, including those of dietary or therapeutic origin, were shown to have affinity for AhR, and studies also identified indoles, tetrapyroles, and arachidonic acid metabolites as potential endogenous, albeit low-affinity, ligands for AhR (reviewed in Refs. 67, 68). With nondioxin, low-affinity AhR ligands of dietary or therapeutic origin [e.g., indolo(3,2,b)carbazole, omeprazole, primaqine], we showed modulation of 3′IghRR activity and Ig expression (7). Consequently, human exposure to a broad range of AhR ligands, some yet to be discovered, could modulate hu-hs1,2 activity and influence the occurrence and/or severity of human diseases associated with hu-hs1,2.

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Disclosures

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References

10. Swanson, H. I., and C. A. Bradfield. 1993. The AH-receptor: genetics, structure
32. Giambra, V., A. Fruscalzo, M. Giufre', C. Martinez-Labarga, M. Favaro,
26. Frezza, D., V. Giambra, B. Toluso, M. De Santis, S. Bosello, S. Vettori,
24. Ducati, C., D. E. Drouet, E. Pinaud, Y. Denizot, J. C. Aligdir, F. Broidoux,
16.②
15. Denizot, Y., E. Pinaud, C. Aupertit, C. Le Morvan, E. Magnoux, J. C. Aligdir,
14.②
13. Chen, C., and B. K. Biristein. 1997. Virtually identical enhancers containing a segment of homology to murine 3'IgH-E(hs1,2) lie downstream of human Ig C
12.②
11.②
10.②
9.②
8.②
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