Toxicogenomics-Based Identification of Mechanisms for Direct Immunotoxicity

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Compounds with direct immunotoxic properties, including metals, mycotoxins, agricultural pesticides, and industrial chemicals, form potential human health risks due to exposure through food, drinking water, and the environment. Insights into the mechanisms of action are currently lacking for the majority of these direct immunotoxicants. Therefore, the present work aimed to gain insights into the molecular mechanisms underlying direct immunotoxicity. To this end, we assessed in vitro the effects of 31 test compounds on the transcriptome of the human Jurkat T-cell line. These compounds included direct immunotoxicants, immunosuppressive drugs with different mode of actions, and nonimmunotoxic control chemicals. Pathway analysis of the microarray data allowed us to identify canonical pathways and Gene Ontology processes that were transcriptionally regulated in common by immunotoxicants (1) with structural similarities, such as tributyltin chloride and tributyltin oxide that activated the retinoic acid/X receptor signaling pathway and (2) without structural similarities, such as As2O3, dibutyltin chloride, diazinon, MeHg, ochratoxin A (OTA), S9-treated OTA, S9-treated cyclophosphamide, and S9-treated benzo[a]pyrene, which activated unfolded protein response, and FTY720, lindane, and propanil, which activated the cholesterol biosynthesis pathway. In addition, processes uniquely affected by individual immunotoxicants were identified, such as the induction of Notch receptor signaling and the downregulation of acute-phase response genes by OTA. These findings were validated by quantitative real-time PCR analysis of genes involved in these processes. Our study indicated that diverse modes of action are involved in direct immunotoxicity and that a set of pathways or genes, rather than one single gene, can be used to screen compounds for direct immunotoxicity.

Key Words: toxicogenomics; direct immunotoxicity; Jurkat.

Direct immunotoxicity is defined as direct deleterious effects of a xenobiotic on the functioning of the immune system, whereas indirect immunotoxicity is an allergic reaction, which causes tissue damage upon exposure to a xenobiotic. Direct immunotoxicity can be elicited by either suppression or by activation of the immune system, better known as immunosuppression and immune enhancement, respectively (Corsini and Roggen, 2009; Descotes, 2005; Galbiati et al., 2010). Numerous compounds with direct immunotoxic properties form potential human health risks due to exposure through food (Borchers et al., 2010), drinking water, and the environment (Coelho et al., 2012; Corsini et al., 2012a; Veraldi et al., 2006). Direct immunotoxicants include metals (Tchounwou et al., 2003), mycotoxins (Milićević et al., 2010), agricultural pesticides (Corsini et al., 2012b), industrial chemicals (Veraldi et al., 2006), and certain drugs (Descotes, 2005). Currently, insights into the molecular mechanisms of direct immunotoxicity are lacking for the majority of these compounds.

During the registration and approval of chemicals and drugs, the in vivo identification of direct immunotoxicity is based on gross pathology and hematological parameters in rodent-based animal models (Food and Drug Administration, 2006; Institoris et al., 1998). After the identification of direct immunotoxicity, mechanistic information of direct immunotoxicity at the cellular level is mostly obtained by applying classical assays for immune cell functionality. These assays mainly determine the effects of the xenobiotics on the proliferation, cytokine release, or antibody production of immune cells in vitro upon antigenic, mitogenic, or viral stimulation (Lankveld et al., 2010). However, experiments to characterize the effects at the molecular level are generally lacking. In the last decade, (immuno)toxicogenomics, the application of genomics techniques in (immuno)toxicology, has rapidly become a new promising approach, which can be employed to get insight into the molecular mechanisms of (immuno)toxic compounds (Luebke et al., 2006).

Recently, the application of genomics techniques has led to improved mechanistic understanding for a limited set of direct
immunotoxic compounds. The rodent in vivo studies by Baken et al. (2008) and Frawley et al. (2011) showed that different direct immunotoxicants could have overlapping mechanisms of action at the level of gene expression: cell cycle arrest was elicited by the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP), the immunosuppressive drug cyclosporine A (CsA; calcineurin inhibitor), and the industrial chemical tributyltin oxide (TBTO; organotin) in murine spleens (Baken et al., 2008), whereas T-cell receptor and CD28 signaling were altered in murine thymocytes by the immunosuppressive drugs cyclophosphamide (CP; alkylating agent), diethylstilbestrol (estrogen), and dexamethasone (glucocorticoid) (Frawley et al., 2011). The application of immunotoxicogenomics in in vitro studies has led to improved insights into the modes of action (MOAs) of direct immunotoxic compounds. For instance, transcriptome analyses of human lymphocytes exposed in vitro to immunotoxicants have led to the identification of calcium-mediated induction of endoplasmic reticulum (ER) and oxidative stress by the organotin compound TBTO (Katika et al., 2011, 2012a) and the trichothecene mycotoxin deoxynivalenol (DON) (Katika et al., 2012b).

Currently, a wide survey study on the potential MOAs of direct immunotoxicants is lacking. Therefore, the present study aims to fill this knowledge gap by assessing the effects of a large variety of compounds on the transcriptome of the human T cell in vitro. These compounds include chemicals that are known to cause direct immunotoxicity, chemicals structurally related to the direct immunotoxicants, nonimmunotoxic control chemicals, and immunosuppressive drugs with well-characterized MOAs. We have chosen to use the Jurkat T-cell line for this in vitro transcriptome profiling study because Jurkat cells are of human origin and are easy to culture. In the present study, Jurkat T cells were exposed to subcytotoxic concentrations of the test compounds in order to identify primary MOAs of the immunotoxicants rather than less specific cell death–related gene expression signatures. The exposure was done for a relatively short time period of 6h in order to identify the initial pathways involved in direct immunotoxicity. Subsequently, the exposed cells were subjected to transcriptome analysis. This approach enabled us to identify several common MOAs for direct immunotoxicity and to assign MOAs to several immunotoxicants for which mechanistic insights were previously limited or lacking.

MATERIALS AND METHODS

Cell culture. The human lymphoblastic T-cell line (Jurkat) was obtained from the American Type Culture Collection. The Jurkat cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 2mM glutamine, 1mM sodium pyruvate, 1mM nonessential amino acids, 100U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured at 37°C with 5% CO₂ in a humidified atmosphere. The medium was refreshed every 2 days.

Compound selection. The direct immunotoxicants were selected on the basis of their severity of toxicity and environmental abundance (Table 1 and Supplementary table 1). The total number of compounds included in the present study is 31. Besides environmental contaminants, we also incorporated a group of immunosuppressive drugs on the basis of their well-defined molecular targets (Table 1). The MOAs of these immunosuppressive drugs include DNA alkylation, glucocorticoid receptor activation, inhibition of purine synthesis, calcineurin, mammalian target of rapamycin, and the sphingosine-1-phosphate receptor 1. All chemicals tested were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands), except mono-2-ethylhexyl phthalate (MEHP; Wako chemicals, Neuss, Germany) and fengolinol (FTY720; Selleck Chemicals, via Bio-Connect Diagnostics, Huissen, The Netherlands). Stock solutions of all chemicals were made by dissolving the substances in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany).

Metabolic activation system. BaP, ochratoxin A (OTA), and CP were subjected to in vitro metabolic activation using a human liver S9 fraction (BD Bioscience, Breda, The Netherlands). The S9 reaction mixtures, having a total volume of 1 ml, consisted of 570 µl H₂O (MQ), 200 µl 0.5M potassium phosphate buffer (pH 7.4), 100 µl NADPH regeneration system solution A (BD Bioscience), 20 µl NADPH regeneration system solution B (BD Bioscience), 10 µl compound stock in DMSO, and 100 µl S9 mix (BD Bioscience). After incubating for 1, 6, and 24 h, the S9 chemical reaction mixtures were heated inactivated (5 min at 56°C) and pooled at equal volumes.

Viability and Cytotoxicity Assays

WST-1 assay. WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, Roche Diagnostic Ned BV, Almere, The Netherlands) is a water soluble tetrazolium salt, which is converted by mitochondrial dehydrogenases to a colored formazan complex. The amount of formazan correlates to the viability of the cells. Twenty hours before exposure, Jurkat cells were plated in advance in 96-well plates resulting in about 20,000 cells per well at the starting point of exposure. Exposure was done in triplicate in 100 µl medium for 24 h to increasing concentrations of the compounds or to the vehicle controls. In the last 2h of exposure, 10 µl of WST-1 reagent was added. Absorbance was measured at 450nm in a microplate reader (BioTek, Winooski, VT).

ATPlite assay. ATPlite assay (Perkin Elmer, Oosterhout, The Netherlands) is based on the production of light caused by the reaction of ATP with luciferase and D-luciferin. The emitted light is proportional to the amount of ATP, which is a marker for cell viability. Jurkat cells were plated as described above for the WST-1 assay. Exposure was done in triplicate in 100 µl medium in 96-well plates for 24h to increasing concentrations of compounds or to the vehicle controls. After exposure, the assay was performed according to the manufacturer’s protocol.

ToxiLight assay. ToxiLight assay (Lonza, Breda, The Netherlands) is luminescent cytotoxicity assay based on the release of adeny late kinase (AK) from damaged cells. Jurkat cells were plated as described above for the WST-1 assay. Exposure was done in triplicate in 100 µl medium in 96-well plates for 24h to increasing concentrations of compounds or to the vehicle controls. After exposure, the assay was performed according to the manufacturer’s protocol.

Chemical exposures. Jurkat cells (passage number 16–20) were seeded in 2.7 ml medium per well in 6-well plates (750,000 cells/well). After growing the cells for 20h, exposure was initiated by adding 0.3 ml medium containing noncytotoxic concentration of the compounds or vehicle controls. Subsequently, cells were exposed to the compounds for 6h. The final DMSO concentration in the medium was 0.1% (vol/vol) for all the samples. Each exposure was performed in quadruplicate at four different days.

RNA isolation and quality control. After exposure, the culture medium was removed after the centrifugation of the cell suspension (5 min at 300 g, 4°C). The resulting cell pellet was homogenized in 600 µl red cell lysis buffer (Qiagen, Venlo, The Netherlands) supplemented with 10% β-mercaptoethanol and stored at −80°C until further processing. RNA was isolated with the Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. RNA yield was assessed spectrophotometrically (NanoDrop...
<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>CAS</th>
<th>Known MOA</th>
<th>Concentration</th>
<th>Source/application</th>
<th>Compound class</th>
<th>Structurally related control compound</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic trioxide</td>
<td>As$_2$O$_3$</td>
<td>1327-53-3</td>
<td>Oxidative stress induction</td>
<td>3µM</td>
<td>Environmental, heavy metal, contamination of food chain</td>
<td>ITOX</td>
<td></td>
<td>Gómez et al. (2005) and Qian et al. (2007)</td>
</tr>
<tr>
<td>Benzo[a]pyrene S9</td>
<td>BaP S9*</td>
<td>50-32-8</td>
<td>Aryl hydrocarbon receptor activation; DNA adduct formation</td>
<td>5µM</td>
<td>Environmental, polycyclic aromatic hydrocarbon, contamination of food chain</td>
<td>ITOX</td>
<td>BaP</td>
<td>Shimizu et al. (2000) and Suh et al. (1995)</td>
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<tr>
<td>Cobalt(II) chloride</td>
<td>CoCl$_2$</td>
<td>7646-79-9</td>
<td>Chemical hypoxia induction</td>
<td>80µM</td>
<td>Environmental, heavy metal, contamination of food chain</td>
<td>ITOX</td>
<td></td>
<td>Stenger et al. (2011)</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>DON</td>
<td>51481-10-8</td>
<td>Regulation of metabolic pathways; protein synthesis; signaling transduction</td>
<td>250nM</td>
<td>Environmental, mycotoxin, contamination of food chain</td>
<td>ITOX</td>
<td></td>
<td>Pestka (2010)</td>
</tr>
<tr>
<td>O,O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate (diazinon)</td>
<td>DZN</td>
<td>333-41-5</td>
<td>Modulation of cytokine production; oxidative stress induction</td>
<td>200µM</td>
<td>Environmental, organophosphate insecticide, contamination of food chain</td>
<td>ITOX</td>
<td></td>
<td>Alhwaimi and Hussein (2007) and Slotkin and Seidler (2008)</td>
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<tr>
<td>Gamma-hexachlorocyclohexane (lindane)</td>
<td>LIN</td>
<td>58-89-9</td>
<td>Modulation of cytokine production; apoptosis induction</td>
<td>130µM</td>
<td>Environmental, organochlorine insecticide, contamination of food chain</td>
<td>ITOX</td>
<td></td>
<td>Battaglia et al. (2010b) and Dar et al. (2012)</td>
</tr>
<tr>
<td>Methylmercury</td>
<td>MeHg</td>
<td>22967-92-6</td>
<td>Oxidative stress induction</td>
<td>1µM</td>
<td>Environmental, heavy metal, contamination of food chain</td>
<td>ITOX</td>
<td></td>
<td>Shenker et al. (2004)</td>
</tr>
<tr>
<td>Mono-2-ethylhexyl phthalate</td>
<td>MEHP</td>
<td>4376-20-9</td>
<td>Oxidative stress induction; caspase 3 and 7 activation</td>
<td>300µM</td>
<td>Environmental, metabolite plasticizer DEHP</td>
<td>ITOX</td>
<td>DEHP</td>
<td>Rosado-Berrios et al. (2011)</td>
</tr>
<tr>
<td>Ochratoxin A S9</td>
<td>OTA S9*</td>
<td>303-47-9</td>
<td>Oxidative stress induction; protein synthesis inhibition</td>
<td>10µM</td>
<td>Environmental, mycotoxin, contamination of food chain</td>
<td>ITOX</td>
<td>OTA</td>
<td>Al-Anati and Petzinger (2006) and Liu et al. (2012)</td>
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<tr>
<td>N-(3,4-Dichlorophenyl)propanamide (propamid)</td>
<td>PROP</td>
<td>709-98-8</td>
<td>Inhibition of NF-kB activity; modulation of Ca(2+) signaling</td>
<td>100µM</td>
<td>Environmental, herbicide</td>
<td>ITOX</td>
<td></td>
<td>Salazar et al. (2008)</td>
</tr>
<tr>
<td>Tributyltin chloride</td>
<td>TBTC</td>
<td>1462-22-9</td>
<td>Apoptosis induction via Fas pathway; activation of LXRα/RXRα pathway induction of ER stress and mitochondrial stress</td>
<td>100nM</td>
<td>Environmental, organotin, biocide</td>
<td>ITOX</td>
<td>DBTC</td>
<td>Chen et al. (2011) and Cui et al. (2011)</td>
</tr>
<tr>
<td>Bis(trichlorobutyl) oxide</td>
<td>TBTO</td>
<td>56-35-9</td>
<td>Induction of ER stress and mitochondrial stress</td>
<td>100nM</td>
<td>Environmental, organotin, biocide</td>
<td>ITOX</td>
<td>DBTC</td>
<td>Katika et al. (2011)</td>
</tr>
<tr>
<td>Cyclophosphamide S9</td>
<td>CP S9*</td>
<td>50-18-0</td>
<td>Nitrogen mustard alkylation</td>
<td>3mM</td>
<td>Immunosuppressive drug</td>
<td>ISD</td>
<td>CP</td>
<td>Macaubbin et al. (1991)</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>CsA</td>
<td>59865-13-3</td>
<td>Calcineurin inhibition</td>
<td>8µM</td>
<td>Immunosuppressive drug</td>
<td>ISD</td>
<td></td>
<td>Fakata et al. (1998) and Chun and Hartung (2010)</td>
</tr>
<tr>
<td>Fingolimod</td>
<td>FTY720</td>
<td>162359-55-9</td>
<td>S1P-receptor antagonism</td>
<td>4µM</td>
<td>Immunosuppressive drug</td>
<td>ISD</td>
<td></td>
<td>Griesmacher et al. (1997)</td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>MPA</td>
<td>24280-93-1</td>
<td>IMP dehydrogenase inhibition</td>
<td>10µM</td>
<td>Immunosuppressive drug</td>
<td>ISD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Putative Mechanisms for Direct Immunotoxicity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Source/application</th>
<th>Concentration</th>
<th>CAS</th>
<th>Compound class</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stilbene (resveratrol)</td>
<td>RAPA</td>
<td>mTOR inhibition</td>
<td>5177-18-8</td>
<td>5177-18-8</td>
<td>NON</td>
<td>Ballou and Lin (2008)</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>AgNO</td>
<td>Heavy metal</td>
<td>1.35mM</td>
<td>10035-24-8</td>
<td>NON</td>
<td>Moore et al. (1979)</td>
</tr>
<tr>
<td>Amphotericin</td>
<td>AHP</td>
<td>Antibacterial</td>
<td>853mM</td>
<td>9003-13-5</td>
<td>NON</td>
<td>Briscoe et al. (2008)</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>AZA</td>
<td>Drug (6-mercaptopurine)</td>
<td>50mM</td>
<td>5179-16-5</td>
<td>NON</td>
<td>Takahashi et al. (2006)</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>FLX</td>
<td>Natrium salt</td>
<td>446-46-6</td>
<td>137-72-6</td>
<td>NON</td>
<td>(2000)</td>
</tr>
<tr>
<td>Furosemide</td>
<td>FURO</td>
<td>Loop diuretic drug</td>
<td>50mM</td>
<td>7761-88-8</td>
<td>NON</td>
<td>(2000)</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>SSRI</td>
<td>ASAR syndrome</td>
<td>30mM</td>
<td>9051-80-3</td>
<td>NON</td>
<td>(2000)</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>MA</td>
<td>Acetate sweeter</td>
<td>100mM</td>
<td>60-55-1</td>
<td>NON</td>
<td>(2000)</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>NF</td>
<td>Salt</td>
<td>20mM</td>
<td>168-03-5</td>
<td>NON</td>
<td>(2000)</td>
</tr>
<tr>
<td>Carbamyl monomer</td>
<td>UC</td>
<td>Plastic monomer</td>
<td>20mM</td>
<td>50-24-8</td>
<td>NON</td>
<td>(2000)</td>
</tr>
</tbody>
</table>

Microarray hybridization and data normalization. Gene expression profiling was performed using Affymetrix U133 Plus 2.0 Arrays (Santa Clara, CA). For each sample, complementary DNA (cDNA) was synthesized from 5 μg of total RNA. cDNA synthesis and subsequent synthesis of biotin-labeled cRNA was performed using GeneChip One-Color Target Labeling and Control Reagents including the One-Color cDNA Synthesis Kit, Poly-A RNA Control Kit, Sample Cleanup Module, IET Labeling Kit, and Hybridization Control Kit (all from Affymetrix) according to the manufacturer’s protocol. Yields of cRNA were quantified spectrophotometrically (Nanodrop). cRNA integrity was assessed using the Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Upon fragmentation, 12 μg of cRNAs were hybridized to the arrays for 16 h at 45°C (GeneChip Hybridization Oven 640; Affymetrix). After washing and staining (GeneChip hybridization station, Fluidics Station 450, Affymetrix), the chips were scanned (Affymetrix GeneChip Scanner 3000 7G). Raw data were extracted using GeneChip Operating Software (Affymetrix).

In order to filter out probes with suboptimal specificity for the encoding genes, custom CDF files were generated from the raw data files, by using the R package available at http://brainarray.mbi.uc.edu/Braainarray/Database/CustomCDF/14.0.1/entrez.csp. Then robust multichip average normalization was applied to the complete data set (Bioconductor). Bioconductor packages were used for the quality control of the microarray data (www.arrayanalysis.org). Boxplots of log-intensity distribution before and after normalization were constructed for between-array comparison (Supplementary fig. 1).

Microarray Data Analyses

Hierarchical clustering. Unsupervised hierarchical clustering was performed with the publicly available programs Cluster (uncentered correlation; average linkage clustering) and Treeview (Eisen et al., 1998).

Metacore. Functional interpretation of differentially expressed genes for each chemical was done using Metacore software (GeneGo, St Joseph, MI). Metacore uses hypergeometric distribution to assess significance for over-representation of affected genes in signaling and metabolic pathways (Ekins et al., 2006). For each compound, the affected genes were analyzed with Metacore, separately for the up- and downregulated genes. Pathways with a p value < 0.01 were considered as significantly regulated. Pathways that were determined by less than four genes or affected by less than two compounds were left out for further analysis (see Supplementary table 4 for the resulting pathway list). Then, pathways irrelevant for the present study (for instance, regross of virus within host cell, response to biotic stimulus, etc.) or irrelevant for the immune system (for instance, brain development, nervous system development, etc.) were manually removed. The p values were then converted into Z values to enable clustering. Pathways obtained a positive or negative Z value when up- or downregulated, respectively. Hierarchical clustering was done as described above.

Gene set enrichment analysis. Gene set enrichment analysis (GSEA) was used to detect the differential expression of biologically relevant gene sets and to provide insight into the affected molecular mechanisms. GSEA makes use of predefined gene sets that are based on previous experimental results and literature. It ranks all genes on the basis of their expression ratios and then calculates whether a particular gene set is significantly enriched at the top or the bottom of the ranking list (Subramanian et al., 2005). Therefore, GSEA has the advantage over other statistical tools that no initial filtering is applied to the data set to select significantly differentially expressed genes. We used the gene sets downloaded from the Gene Ontology (GO) consortium (http://www.geneontology.org/) including biological process, cellular component, and molecular function. Gene sets with a p value < 0.01 and an false discovery rate value < 0.25 were considered as significantly regulated. Up- and downregulation of significant gene sets were visualized with heat maps. The p values were converted into Z values to enable clustering. Gene sets obtained a positive or negative Z values
when up- or downregulated, respectively. Hierarchical clustering was done as described above.

**Quantitative real-time PCR verification.** Verification of the microarray outcome was performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the 96.96 BioMark Dynamic Array for real-time PCR (Fluidigm Corporation, San Francisco, CA), according to the manufacturer’s instructions. cDNA samples were synthesized from the same RNA samples as used for the microarray experiment using miScript Reverse Transcription kit according to the manufacturer’s protocol (Qiagen). Before use on the BioMark array, the cDNA was first subjected to 14 cycles of Specific Target Amplification using a 0.2x mixture of all TaqMan Gene Expression assays in combination with the TaqMan PreAmp Master Mix (Applied Biosystems), followed by fivefold dilution. Thermal cycling and real-time imaging of the BioMark array was done on the BioMark instrument, and Ct values were extracted using the BioMark real-time PCR analysis software, and ΔΔCT values were used to determine compound effects on mRNA expression levels.

**RESULTS**

**Selection of Test Compounds**

We selected 31 test compounds for assessing their effects on the transcriptome of the human Jurkat T-cell line in vitro. These test compounds comprise direct immunotoxic chemicals, immunosuppressive drugs with known MOAs, compounds structurally related to the immunomodulating compounds, and nonimmunotoxic control compounds (Table 1). BaP, OTA, and CP were bioactivated using human liver S9 fraction. For these three compounds, both the parent compounds and the bioactivated samples were included in the study.

**Determination of Exposure Concentrations**

The effects of the test compounds on the Jurkat cell transcriptome were assessed upon 6h of exposure to subcytotoxic concentrations (Table 1). Subcytotoxic concentrations were defined as more than 80% viability (CV80) and less than 20% induction when up- or downregulated, respectively. Hierarchical clustering was done as described above.

Then, for each of the test compounds, individual GO enrichment analyses were performed on these 1445 genes using Metacore. These analyses led to the identification of 62 GO processes that were enriched by at least two immunomodulating compounds (p value < 0.01). Thereafter, hierarchical clustering was performed on the Z scores of these enriched GO processes, which resulted in the identification of 13 different clusters of GO processes (Fig. 1). Table 2 summarizes these coclustered GO processes and the compounds involved in each cluster, as well as the individual genes responsible for these enriched processes.

**Metacore Analysis—Common Putative Mechanisms of Direct Immunotoxicity**

Two main GO processes were identified as being most commonly activated by the immunomodulating compounds, cellular stress responses (cluster I) and antiapoptosis (cluster VI) (Fig. 1 and Table 2). These clusters were jointly activated by the heavy metal As$_2$O$_3$, the mycotoxin OTA (+ S9 and −S9), and the organochlorine insecticide lindane, indicating that these immunotoxicants activate both cellular stress responses and cytoprotective antiapoptotic processes. In contrast, the heavy metal MeHg, the organophosphate insecticide diazinon, the organotin compounds (dibutyltin chloride [DBTC], tributyltin chloride [TBTC], TBTO), the alkylating agent CP (+ S9), and the poly acyclic hydrocarbon BaP (+ S9 and −S9) specifically activated cellular stress responses (cluster I) without activating antiapoptotic responses (cluster VI).

Various other GO processes were regulated by three or four immunomodulating compounds and were found among clusters II, III, IV, V, VIII, XI, and XII (Fig. 1 and Table 2). Cluster II includes the regulation of cell proliferation, gene transcription, and protein translation. These processes were mainly activated by lindane and inhibited by OTA (+ S9 and −S9). Heat shock responses (within cluster III) were activated by BaP (+ S9), CP (+ S9), diazinon, DBTC, and OTA (+ S9). Cluster IV includes negative regulation of biological processes and molecular functions. These processes were mainly found to be activated by As$_2$O$_3$, BaP (+ S9), prednisolone, and rapamycin. Cholesterol and lipid metabolism (cluster V) were activated by the S1P-receptor antagonist FTY720 and inhibited by lindane and propanil. Immunomodulation (cluster VIII) was activated by lindane and OTA (+ S9) and inhibited by CP (+ S9). Responses to organic substances (within cluster XI) were inhibited by diazinon, BaP (+ S9), and CP (+ S9). This process was also affected in cluster I, but by another subset of genes (Table 2). Starvation responses (cluster XII) were activated by diazinon and inhibited by As$_2$O$_3$ and lindane.

**Metacore Analysis—Other Mechanisms of Direct Immunotoxicity**

We also identified GO processes that were specifically affected by one or two of the immunomodulating compounds.
CoCl₂ specifically induced the expression levels of genes involved in hypoxia (Fig. 1, cluster XI). OTA (+ S9 and −S9) altered the expression levels of genes involved in cytoprotection, lipid metabolism, and type I interferon production (cluster VII). In addition, the genes of this cluster are involved in Notch and NF-κB signaling. Lindane and BaP (+ S9) altered the expression levels of genes involved in signal transduction related to immunity and antiapoptosis (cluster IX). As₂O₃ and BaP (+ S9) altered the expression levels of genes involved in cellular stress responses related with metabolism and apoptosis (cluster X). Tributyltin compounds (TBTC and TBTO) induced the expression levels of genes involved in response to retinoic acid, and metabolism and transport of lipids (cluster XIII). These effects were highly specific for tributyltins because other compounds, including DBTC, had no effects on the expression levels of these genes.

**Gene Set Enrichment Analysis**

In order to confirm the results of Metacore, we applied GSEA on the full transcriptome data of each individual compound using gene set collections from the GO consortium. The gene sets that were enriched by at least two immunomodulating compounds ($p$ value < 0.01) are shown in Supplementary figure 3. In general, the results from GSEA analyses confirmed most of the biological processes and molecular functions we identified with Metacore. Cell cycle control and regulation of transcription and translation were identified as the most commonly affected biological processes by immunomodulating compounds (Supplementary fig. 3A). Almost half of the compounds (15/31) affected at least one gene set that is related with cell cycle, transcription, or translation. This number of affected compounds is much higher than the results from Metacore analyses, which is expected as the statistical model of GSEA is more sensitive than Metacore. Furthermore, cellular stress responses (ER stress affected by S9-treated OTA and CP, DON, CsA, and DBTC; and hypoxia affected by lindane and CoCl₂) and transport of lipid (affected by TBTO and TBTC) and amino acids (affected by lindane, As₂O₃, S9-treated CP and BaP) were also detected by GSEA (Supplementary figs. 3A and 3C). In addition, GSEA analyses using the gene sets from the cellular
Overview of Core Genes Belonging to Enriched GO Processes and Pathways That Were Coregulated by Different Groups of Immunotoxicants

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Immunomodulating test compounds ≥ 1/2 subclusters</th>
<th>Total (N)</th>
<th>Enriched GO processes</th>
<th>Total (N)</th>
<th>Enriched core genes (symbols)</th>
<th>Total (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Up by AsO₃, BaP S9, CP S9, DBTC, DZN, MeHg, OTA, OTA S9, TBTC, TBTO</td>
<td>10</td>
<td>Cellular stress responses</td>
<td>4</td>
<td>ABCG1, ABL1, ASNS, BCL6, BLNK, CEBPB, CHAC1, CHST2, CHST5, CXCR1, DNAJB1, DNAJB4, DPYS, DUSP10, ERRF1, FANCA, GPR18, GTF2H4, HERPUD1, HMGCS1, HSPA1A, HSPA1B, HSPA6, HSPH1, IL10RB, MAP2K6, MT1X, NQO1, ORM2, PMAIP1, POLI, PP1R15A, SGK1, SLCA7A11, SRXN1, STC2, TM6SF1, TOR1B, VEGFA, VLDLR</td>
<td>41</td>
</tr>
<tr>
<td>VI</td>
<td>Up by AsO₃, LIN, OTA, OTA S9; down by DZN, RAPA</td>
<td>6</td>
<td>Antiapoptosis</td>
<td>2</td>
<td>BIRC3, CASP8, HSPA5, RPS27A</td>
<td>4</td>
</tr>
<tr>
<td>IV</td>
<td>Up by AsO₃, BaP S9, FRD, RAPA</td>
<td>4</td>
<td>Negative regulation of biological processes and molecular functions</td>
<td>3</td>
<td>ACVR1, ASNS, BCL6, BIRC3, CRIM1, DTX1, E2F1, FAS, GRNHI, HSPA1B, ID1, ID2, ID3, IER3, KLFI0, MAP2K6, NFKB1I1, NME5, PDCD4, PP1R15A, RBBP4, SMARCC2, SRGN, TNFAIP3, TRIB3, VEGFA</td>
<td>26</td>
</tr>
<tr>
<td>II</td>
<td>Down by OTA, OTA S9; up by LIN</td>
<td>3</td>
<td>Cell cycle control, regulation of transcription and translation</td>
<td>9</td>
<td>AARS, ABL1, ACVR1, ASPR01, BCL6, BCR, BLZF1, CAPN3, CASP8, CDC88C, CCNG2, CDK12, CHD2, CSTA, CTSG, CUZD1, DDIT3, DNAJB6, DUSP2, DUS6, DUSP10, E2F1, EEF1A1, EE2K, EGR1, Fbx16, FBXO32, FUT4, GCM1, GSG2, HBS1L, ID1, ID2, ID3, KDM4A, KIAA1804, KLFI2, KLKB1, MAP2K6, PDCD4, PIF1, POLI, PTPRH, PXK, PYGM, SARS, SCG5, SGK1, SGSH, SMARCC2, SREBF1, SRGN, TAF9, TLR3, TSC22D3, UBE2L3, WNK4, ZNF263</td>
<td>58</td>
</tr>
<tr>
<td>III</td>
<td>Down by RAPA; up by BaP S9, CP S9</td>
<td>3</td>
<td>Inhibition cysteine-type endopeptidase activity involved in apoptosis, heat shock response and biosynthesis of cholesterol and lipids</td>
<td>5</td>
<td>ATP3, DDIT3, DNAJB4, DNAJB6, DUSP2, DUS6, DUSP10, E2F1, EEF1A1, EE2K, EGR1, Fbx16, FBXO32, FUT4, GCM1, GSG2, HBS1L, ID1, ID2, ID3, KDM4A, KIAA1804, KLFI2, KLKB1, MAP2K6, PDCD4, PIF1, POLI, PTTRH, PXK, PYGM, SARS, SCG5, SGK1, SGSH, SMARCC2, SREBF1, SRGN, TAF9, TLR3, TSC22D3, UBE2L3, WNK4, ZNF263</td>
<td>12</td>
</tr>
<tr>
<td>V</td>
<td>Up by FTY720; down by LIN, PROP</td>
<td>3</td>
<td>Metabolism and biosynthesis of cholesterol and lipids</td>
<td>5</td>
<td>ABCG1, ALDH8A1, BAAT, CEL, DHR3, HDLBP, HMGCS1, HMGR, HSD3B7, ID1, IL2A, G4C, SGMS1, SQLE, SREBF1, VLDLR</td>
<td>15</td>
</tr>
<tr>
<td>XI</td>
<td>Down by BaP S9, CP S9; up by CoCl (hypoxia)</td>
<td>5</td>
<td>Cell activation and responses to organic substance and hypoxia</td>
<td>5</td>
<td>AK4, AP, LAT2, NFKB1A, WNT10A</td>
<td>5</td>
</tr>
<tr>
<td>XII</td>
<td>Up by DZN, MPA, RAPA</td>
<td>3</td>
<td>Cellular responses to starvation or hypoxia: negative regulation of gene expression, immune responses, and metabolism</td>
<td>6</td>
<td>ASNS, BCL6, DUSP6, E2F1, FLI1, ID1, ID2, ID3, KLFI0, PDCD4, SLC3A2, SMARCC2, SREBF1, STC2</td>
<td>14</td>
</tr>
<tr>
<td>VIII</td>
<td>Down by CP S9; up by LIN, OTA S9</td>
<td>3</td>
<td>Immunomodulation</td>
<td>2</td>
<td>CD69, TLR3</td>
<td>2</td>
</tr>
<tr>
<td>VII</td>
<td>Up by OTA, OTA S9</td>
<td>2</td>
<td>Notch receptor–mediated signaling, NF-xB-mediated TF activity, cytoprotection, lipid metabolism, and type I interferon production</td>
<td>9</td>
<td>B2M, CTS5, GPR17, MAML1, MESDC2, RBBP4, RPS27A, SLC3A2, SLC16A6, SLC43A1</td>
<td>11</td>
</tr>
<tr>
<td>IX</td>
<td>Up by LIN; down by BaP S9</td>
<td>2</td>
<td>Signal transduction involved in cellular differentiation and immunity</td>
<td>4</td>
<td>ABL1, ACVR1, ASNS, CASP8, CD38, CDC7, DNAJ2A, DTX1, EGR1, FLI1, GRK4, LMT2, PMAIP1, RGS2, RGS5, RGS6, SMARCC2, TAF8, TBC1D8, TLR3, TNFAIP3, TNFSF13B, TRAF3IP2, VEGFA</td>
<td>24</td>
</tr>
<tr>
<td>X</td>
<td>Up by AsO₃; down by BaP S9</td>
<td>2</td>
<td>Cellular stress responses regulating apoptosis and metabolism</td>
<td>5</td>
<td>BCL6, BIRC3, CCDC88C, DDIT3, DNAJB6, FAS, MT1E, MT1F, MT1G, MT1H, MT1X, MT2A, PMAIP1, TLR3, VEGFA</td>
<td>14</td>
</tr>
<tr>
<td>XIII</td>
<td>Up by TBTC, TBTO</td>
<td>2</td>
<td>Retinoic acid–mediated responses and regulation of lipid metabolism and transport</td>
<td>3</td>
<td>ABCA1, ABCG1, ACACA, KLFI2, SREBF1, STC2, VPREB1</td>
<td>6</td>
</tr>
</tbody>
</table>

Note. The roman cluster numbers correspond to the 13 clusters of enriched GO processes depicted in Figure 1. The immunotoxicants presented for each cluster are the compounds that regulated at least half of the GO processes in the cluster (except for CoCl, which only regulated response to hypoxia in cluster XI). The clusters are ranked according to the total number of compounds that enriched a GO process in Metacore. Gene symbols depicted in bold have been verified by Q-RT-PCR using the BioMark platform (Fluidigm).
component database of the GO consortium also revealed that a number of compounds, lindane, TBTO, fluoxetine, prednisolone, diazinon, rapamycin, As$_3$O$_4$, and propanil, downregulated the genes sets related with ribonucleoprotein complex, nucleus, and mitochondria (Supplementary fig. 3B).

Data Comparison Using the Outcome of Other Toxicogenomics Studies

In order to determine the degree of overlap between our data and the data from other toxicogenomics studies, we selected the genes that were up- or downregulated, after considering a fold change cutoff of $>2$log 0.7 or $<2$log 0.7 (numerical $>1.62$ up or down) in at least three of the four biological replicates, for three model compounds, As$_3$O$_4$, TBTO, and CsA. The data of As$_3$O$_4$ were compared with the data of As$_3$O$_4$-treated human myeloma cells (Matulis et al., 2009) and human liver cells HepG2 (Kawata et al., 2007) (Supplementary fig. 4). The data of TBTO were compared with the data of TBTO-treated mouse thymocytes (van Kol et al., 2012) and HepG2 cells (Magkoufopoulou et al., 2012) (Supplementary fig. 5). The data of CsA were compared with the data of peripheral blood mononuclear cells (PBMCs) that were isolated from CsA-treated patients (Brouard et al., 2010) and also to the data of CsA-treated HepG2 cells (Magkoufopoulou et al., 2012) (Supplementary fig. 6).

For As$_3$O$_4$, part of the genes that were up- or downregulated in Jurkat cells were also shown to be affected to the same direction in myeloma cells and HepG2 cells (Supplementary fig. 4). However, the degree of overlap is clearly higher in myeloma cells (Supplementary fig. 4A) than in HepG2 cells (Supplementary fig. 4B).

For TBTO, most of the genes that were upregulated in Jurkat cells were also shown to be upregulated after 3 and 6 h of exposure to 0.5µM of TBTO in mouse thymocytes (Supplementary fig. 5A). We have also identified some overlap in the upregulated genes between our data set and that of TBTO-treated HepG2 cells (Supplementary fig. 5B). Only four genes were downregulated by TBTO in Jurkat cells after considering the fold change cutoff ($>2$log 0.7 or $<2$log 0.7), and none of them can be found in the data of TBTO-treated mouse thymocytes or HepG2 cells.

For CsA, most of the genes that were upregulated in Jurkat cells were also shown to be upregulated in 13 out of 14 CsA-treated patients (Supplementary fig. 6A, genes upregulated in Jurkat cells). Only four genes were downregulated by CsA in Jurkat cells after considering the fold change cutoff ($>2$log 0.7 or $<2$log 0.7), and the expression levels of these found genes were quite random among the patients (Supplementary fig. 6A, genes downregulated in Jurkat cells). Not much similarity can be found between our data and the data of CsA-treated HepG2 cells (Supplementary fig. 6B).

Verification of the Microarray Gene Expression Patterns by Quantitative Real-Time PCR

We proceeded with verifying the microarray mRNA expression patterns and relevant GO processes that were associated with exposure to direct immunotoxicants. For this purpose, we employed quantitative real-time PCR (Q-RT-PCR) on three out of four independent experiments that were also used for the microarray experiments. Out of the 1445 genes that were transcriptionally regulated by one or more compounds, we selected 28 signature genes by using the following criteria: we first identified 93 genes, consisting of 80 genes that were modulated by at least three compounds, and 13 genes that were specifically regulated by one single compound or compound class. Then, we selected 28 genes out of these 93 genes, based on the largest up- or downregulation in response to the immunotoxicants.

As shown in Tables 2 and 3, these 28 genes can be linked to GO processes that are potentially relevant for immunotoxicity. From these 28 genes, 10 genes are from cluster I, CHAC1, GPR18, HMGCS1, HSPA1B, HSPA5, NQO1, SLC7A11, SRXN1, TM6SF1, and VLDLR, and are associated with cellular stress responses (cluster I, Table 2). CHAC1, HSPA1B, and HSPA5 are well-known markers for ER stress and the unfolded protein response (UPR). NQO1 and SRXN1 are specifically related with response to oxidative stress. HSPA1B and HSPA5 are also part of cluster III and are involved in heat shock responses. HSPA5 is also one of the cluster VI genes and is involved in antiapoptosis. The cluster II genes CCNG2, ID1, and ID2 are involved in cell cycle control and regulation of gene transcription and protein translation.

HMGCS1, VLDLR, and ABCG1 are from cluster V and associated with metabolism, biosynthesis, and transport of cholesterol and lipids. ABCG1, together with ABCA1, KLF2, and VPREB1, is part of cluster XIII genes and is associated with response to retinoic acid. The cluster XI gene AK4 and cluster VII gene GPR17 are specifically related with response to hypoxia and G protein–coupled cell signaling, respectively.

For 27 of the 28 genes, the Q-RT-PCR-based expression patterns generally correlated well with the microarray data (Pearson correlation, $R \geq 0.69$) (Table 3). The only exception here was NANOS1 ($R = 0.14$) that was excluded from further comparisons. Furthermore, for three genes and two compounds, the mRNA expression patterns showed opposite effects between the Q-RT-PCR and the microarray data, ID2 for lindane, and GPR18 and HSPA5 for OTA. For all other test compounds, Q-RT-PCR gave similar results for GPR18, HSPA5, and ID2 as the microarray (Pearson correlation, $R \geq 0.69$). Examples of the comparison between the results from microarray and the Q-RT-PCR are given in Figure 2. In general, the effects found in Q-RT-PCR analysis were larger than those shown by microarray analysis. This suggests that Q-RT-PCR was a more sensitive method to detect chemically induced gene expression changes than microarray mRNA expression profiling.

**DISCUSSION**

The present study was aimed at the identification of mechanisms for chemical-induced direct immunotoxicity. To that end,
### TABLE 3

Major GO Processes and Molecular Functions in Which the 28 Genes Are Involved, Pearson Correlation Results Between the Microarray and Q-RT-PCR Data, and the Putative Molecular Targets of the 28 Genes

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>mRNA accession</th>
<th>Affymetrix ID</th>
<th>TaqMan assay</th>
<th>Pearson correlation (R)</th>
<th>GO biological process/molecular function (GO, Genecards)</th>
<th>Putative molecular targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNG2</td>
<td>NM_004354</td>
<td>901_at</td>
<td>Hs00171119_m1</td>
<td>0.86</td>
<td>Regulation of cell cycle</td>
<td>FOXO (Chen et al., 2006)</td>
</tr>
<tr>
<td>HMGC5</td>
<td>NM_001098272</td>
<td>3157_at</td>
<td>Hs00940429_m1</td>
<td>0.84</td>
<td>Cholesterol biosynthesis SREBP</td>
<td>SREBP (Bensinger et al., 2008)</td>
</tr>
<tr>
<td>ALDHA1</td>
<td>NM_001193480</td>
<td>64577_at</td>
<td>Hs00988965_m1</td>
<td>0.83</td>
<td>Metabolism and biosynthesis of retinoic acid</td>
<td></td>
</tr>
<tr>
<td>BZRAP1</td>
<td>NM_004758</td>
<td>9256_at</td>
<td>Hs00720490_m1</td>
<td>0.94</td>
<td>Benzodiazepine receptor binding</td>
<td></td>
</tr>
<tr>
<td>NANOS1</td>
<td>NM_199461</td>
<td>340719_at</td>
<td>H00996068_s1</td>
<td>0.14</td>
<td>Inhibition of translation, zinc ion binding</td>
<td></td>
</tr>
<tr>
<td>FBXO32</td>
<td>NM_001242463</td>
<td>114907_at</td>
<td>Hs01041408_m1</td>
<td>0.92</td>
<td>Protein ubiquitination</td>
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<tr>
<td>ARRD3</td>
<td>NM_0028081</td>
<td>57561_at</td>
<td>Hs00385845_m1</td>
<td>0.84</td>
<td>Protein binding</td>
<td></td>
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<tr>
<td>CRM1</td>
<td>NM_0016441</td>
<td>51232_at</td>
<td>Hs00212750_m1</td>
<td>0.82</td>
<td>Insulin-like growth factor receptor activity, serine-type endopeptidase inhibitor activity, regulation of cell growth</td>
<td></td>
</tr>
<tr>
<td>GPR17</td>
<td>NM_001161415</td>
<td>2840_at</td>
<td>Hs00171137_m1</td>
<td>0.92</td>
<td>GPCR signaling, dual-specificity receptor for uracil nucleotides and cysteinyl leukotrienes (Genecards)</td>
<td></td>
</tr>
<tr>
<td>GPR18</td>
<td>NM_001098200</td>
<td>2841_at</td>
<td>Hs00245542_m1</td>
<td>0.73</td>
<td>GPCR signaling, N-arachidonyl glycine receptor</td>
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<tr>
<td>KLHL24</td>
<td>NM_0017644</td>
<td>54800_at</td>
<td>Hs00214210_m1</td>
<td>0.81</td>
<td>Protein binding</td>
<td></td>
</tr>
<tr>
<td>ABCA1</td>
<td>NM_005502</td>
<td>19_at</td>
<td>Hs01059118_m1</td>
<td>0.92</td>
<td>Response to retinoic acid, ATP-dependent transmembrane transport of sterols</td>
<td></td>
</tr>
<tr>
<td>ABCG1</td>
<td>NM_004915</td>
<td>9619_at</td>
<td>Hs00245154_m1</td>
<td>0.87</td>
<td>Response to retinoic acid, ATP-dependent transmembrane transport of purine nucleotides, L-tryptophan, and sterols</td>
<td></td>
</tr>
<tr>
<td>KLF2</td>
<td>NM_0016270</td>
<td>10365_at</td>
<td>Hs00360439_g1</td>
<td>0.91</td>
<td>Response to stress, transcription factor activity, regulates T-cell trafficking by promoting expression of the lipid-binding receptor S1P1 and the selectin CD62L (Weinreich et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>VPREB1</td>
<td>NM_007128</td>
<td>7441_at</td>
<td>Hs00356766_g1</td>
<td>0.72</td>
<td>Immune response, antigen binding</td>
<td></td>
</tr>
<tr>
<td>MT1F</td>
<td>NM_005949</td>
<td>4494_at</td>
<td>Hs01582977_gH</td>
<td>0.95</td>
<td>Response to metal ion and oxidative stress</td>
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</tr>
<tr>
<td>CHAC1</td>
<td>NM_001142776</td>
<td>79094_at</td>
<td>Hs00225520_m1</td>
<td>0.84</td>
<td>Possibly proapoptotic component of UPR by mediating the ATF4-ATF3-DDIT3/CHOP cascade (Genecards), negative regulation Notch signaling (Mungrue et al., 2009)</td>
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<tr>
<td>HSPA1B</td>
<td>NM_005346</td>
<td>3304_at</td>
<td>Hs01040501-sH</td>
<td>0.81</td>
<td>Response to unfolded protein, antiapoptosis HSF1</td>
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<tr>
<td>HSPA5</td>
<td>NM_005347</td>
<td>3309_at</td>
<td>Hs00946084_g1</td>
<td>0.69</td>
<td>Response to unfolded protein, starvation response, involved in the folding and assembly of proteins in the ER (Genecards) ATF6 and XBP-1</td>
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<tr>
<td>NQO1</td>
<td>NM_000903</td>
<td>1728_at</td>
<td>Hs01045995_m1</td>
<td>0.91</td>
<td>Response to toxin, NAD(P)H dehydrogenase (quinone) activity</td>
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<tr>
<td>SRXN1</td>
<td>NM_008725</td>
<td>140809_at</td>
<td>Hs00607800_m1</td>
<td>0.69</td>
<td>Response to oxidative stress, antioxidant activity</td>
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<tr>
<td>ID1</td>
<td>NM_002165</td>
<td>3397_at</td>
<td>Hs00357821_g1</td>
<td>0.79</td>
<td>Regulation of transcription from RNA polymerase II promoter, regulation of apoptosis</td>
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<tr>
<td>ID2</td>
<td>NM_002166</td>
<td>3398_at</td>
<td>Hs00747379_m1</td>
<td>0.73</td>
<td>Immune cell differentiation, inhibition of gene transcription</td>
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<tr>
<td>AK4</td>
<td>NM_001003535</td>
<td>205_at</td>
<td>Hs03405743_g1</td>
<td>0.79</td>
<td>Amino acid biosynthesis, AK activity</td>
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<td>SLC7A11</td>
<td>NM_014331</td>
<td>23657_at</td>
<td>Hs00921938_m1</td>
<td>0.88</td>
<td>Response to oxidative stress, response to toxin, cystine-glutamate antipporter activity</td>
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<tr>
<td>VLDLR</td>
<td>NM_001018056</td>
<td>7436_at</td>
<td>Hs01047538_m1</td>
<td>0.81</td>
<td>ER stress response (Dombroski et al., 2010), lipoprotein receptor activity</td>
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<tr>
<td>CEBPB</td>
<td>NM_005194</td>
<td>1051_at</td>
<td>Hs00270923_s1</td>
<td>0.81</td>
<td>Transcriptional activation of immune and inflammatory response genes (Genecards)</td>
<td></td>
</tr>
<tr>
<td>TM6SF1</td>
<td>NM_001144903</td>
<td>53346_at</td>
<td>Hs00224823_m1</td>
<td>0.92</td>
<td>ER stress response (Taylor et al., 2011)</td>
<td></td>
</tr>
</tbody>
</table>
we assessed the *in vitro* effects of a large variety of immunotoxic and nonimmunotoxic test compounds on the transcriptome of the human Jurkat T-cell line.

For three compounds also the effect of biotransformation on gene expression was examined by *in vitro* treatment of the compounds with human liver S9. BaP, OTA, and CP were chosen...
because it was shown by others that these compounds need to be bioactivated before exerting their immunomodulatory effects (Carlson et al., 2004; Ekhart et al., 2009; Manderville, 2005). In addition, this issue was addressed by including immunotoxic metabolites of TBTC and bis(2-ethylhexyl) phthalate (DEHP), being DBTC and MEHP, respectively. For TBTC, it is known from literature that both the parent compound and its metabolite DBTC are immunotoxic because they can both cause atrophy in rat thymus (Snoeij et al., 1988). For DEHP, it is known to be rapidly hydrolyzed into MEHP in vivo (Koch et al., 2006), and MEHP is immunotoxic to immune cells in vitro (Rosado-Berrios et al., 2011; Schlezinger et al., 2004).

The biological interpretation of the genes that were up- or downregulated by the exposures enabled the identification of
the processes affected by immunotoxicants. Some cellular pathways, processes, and functions were commonly modulated by three or more immunotoxic compounds, whereas other mechanisms were specifically affected by one or two immunotoxicants. The processes most commonly affected by direct immunotoxicants included ER stress, oxidative stress, antiapoptotic responses, negative regulation of biological process and molecular functions, cell cycle control, regulation of transcription and translation, and metabolism of cholesterol and lipids (Fig. 1 and Table 2). The involvement of these mechanisms in the transcriptional responses of Jurkat T cells to direct immunotoxicants was substantiated by Q-RT-PCR analysis.

On the basis of the enriched core genes involved in cellular stress responses (cluster I, Table 2), we could identify two types of cellular stress responses, ER stress/UPR (CHAC1, HSPA1B, HSPA5, SLC7A11) and oxidative stress (NQO1, SRXN1, SLC7A11). The induction of ER stress can be triggered by continued accumulation of incorrectly folded proteins in the ER (Kaufman et al., 2002) or by compounds that directly affect the function of ER, such as thapsigargin (Treiman et al., 1998). ER stress leads to activation of several transcription factors including ATF3, ATF4, ATF6, and DDIT3 (Galluzzi et al., 2012). Prolonged ER stress can initiate terminal programs in the cells, such as autophagy and apoptosis (Chakrabarti et al., 2011). In T cells, ER stress leads to increased intracellular calcium levels and eventually to T-cell activation (Katika et al., 2011, 2012a). A number of compounds inducing the ER stress response in our study also have been shown to induce this response in other studies. For instance, both As2O3 and MeHg have been found to induce ER stress in myoblasts in vitro (Usuki et al., 2008; Yen et al., 2012). HSPA5 (also upregulated by S9-treated BaP in our experiment, see Supplementary fig. 1), which is a well-known marker gene for ER stress (Galluzzi et al., 2012), has been identified recently as a biomarker for BaP-induced immunotoxicity in Xenopus laevis (Martini et al., 2012). ER stress has also been demonstrated as one of the overrepresented pathways for immunotoxicity in a previous toxicogenomics study (Hochstenbach et al., 2012). Our results further supported ER stress as being one of the major mechanisms for immunotoxicity.

The induction of oxidative stress has also been implied in cluster I as two well-known oxidative stress marker genes, NQO1 and SRXN1, are among the enriched core genes of this cluster (cluster I, Table 2). SLC7A11 in this cluster encodes the transporter of cysteine/glutamate (see the expression pattern of SLC7A11 in Fig. 2). This transporter plays an important role in maintaining cellular glutathione level, which is crucial in protecting cells against oxidative stress (Sakakura et al., 2007). Oxidative stress has been shown to be involved in in vivo and in vitro immunotoxicity induced by a wide range of chemicals, such as fungal toxins, heavy metals, and organic substances (Ashry et al., 2010; Hannam et al., 2010; Liu et al., 2012; Mishra et al., 2008). NQO1 and SRXN1 are also known as the downstream target genes of NRF2 pathway (Schäfer et al., 2010). The induction of oxidative stress, mainly through activation of the NRF2 pathway, has also been identified as the key mechanism to distinguish skin sensitizers from irritants (Vandebriel et al., 2010), thus emphasizing the relevance of this pathway for immunotoxicity in a broad sense.

Besides these two types of stress responses, the response to hypoxia was also identified as being a potential relevant process for immunotoxicity (cluster XI, Fig. 1 and Table 2). This response was specially induced by CoCl2, which is a well-known hypoxia-mimetic agent (Stenger et al., 2011) and can activate gene expression in a HIF1α-dependent fashion (Gao et al., 2012). Cobalt chromium has also been shown to induce hypoxia in fibroblasts in vitro (Madathil et al., 2010). The CoCl2-mediated induction of hypoxia could be confirmed by Q-RT-PCR, as AK4, a well-known hypoxia-inducible gene (Greijer et al., 2005), was highly induced by CoCl2 (Fig. 2).

Hypoxia increases the production of reactive oxygen species in the mitochondria, which eventually leads to oxidative stress and cell death (Duranteau et al., 1998; Guillemin and Krasnow, 1997; Wang et al., 2000). Some cohort studies suggested that patients with metal-on-metal implants are at a higher risk of developing lymphopenia and metal hypersensitivity because of the cobalt and chromium nanoparticles released from the bearing surface of these devices (Gill et al., 2012). One possible cause for this could be the induction of hypoxia.

Another stress response, response to metals, was found to be highly induced by the heavy metal As2O3, which is related to the induction of several metallothioneins (MTs) (cluster X, Table 2, also see Supplementary fig. 1). We selected one MT, MT1F, for Q-RT-PCR analysis and could verify the increased expression of this gene as initially observed in the microarray experiment (Fig. 2). MTs can bind to heavy metals, especially to cadmium, and control cellular stresses such as hypoxia and oxidative stress (Otsuka et al., 2007; Wang et al., 2004). MT-null mice are more susceptible to the hematotoxic and immunotoxic effects of cadmium exposure, indicating the involvement of MTs in the detoxification of heavy metals and cytoprotection (Liu et al., 1999). MTs have also been suggested to act as chemoattractants to support the movement of leukocytes to the site of inflammation, which further supported their important roles in the immunomodulation against toxicant exposure (Yin et al., 2005).

Microarray data analysis at the level of GO processes also led to the observation that genes involved in the inhibition of apoptosis were commonly activated by direct immunotoxicants, including As2O3, lindane, and OTA (+S9 and −S9) (cluster VI, Fig. 1 and Table 2). This observation indicates that transcriptional activation of antiapoptotic genes is possibly a common cytoprotective mechanism upon exposure to direct immunotoxicants. We used relatively low doses that were subcytotoxic for all the compounds in this study. It could be that at higher concentrations, this putative compensatory mechanism is not sufficient anymore to prevent apoptosis. As2O3, lindane,
and OTA have also been found to induce apoptosis in various human and murine cell lines (Battaglia et al., 2010a; Olgun et al., 2004; Qian et al., 2007; Zhou et al., 2005).

The other cluster of processes, which was affected by more than three immunotoxicants (including As$_2$O$_3$, BaP [+ S9], prednisolone, and rapamycin), is negative regulation of biological process and molecular functions (cluster IV, Fig. 1 and Table 2). ID1 and ID2, which encode two important helix-loop-helix transcription factors that participate in cell differentiation and proliferation, are among the enriched core genes of this cluster (see the expression patterns of ID1 and ID2 in Fig. 2). The expression of ID1 has been linked with attenuated cytotoxicity induced by TiO$_2$ in lung cells in vitro (Lee et al., 2009). The expression level of ID2 has been found to be suppressed by DEHP in mouse liver in vivo (Wong and Gill, 2002). In T cells, both ID1 and ID2 have been shown to inhibit T-cell lineage commitment (Jones-Mason et al., 2012; Wang et al., 2009). ID2 has also been shown to inhibit lymphoid tissue development and hematopoietic stem cell differentiation (Cherrier et al., 2012). Therefore, the compounds that affect the mRNA levels of these genes can potentially alter the differentiation status of lymphoid organs and T cells.

Both ID1 and ID2 are also among the enriched core genes of cluster II, which comprises the processes related with cell cycle control and regulation of gene transcription and protein translation. This cluster was mainly upregulated by OTA (+ S9 and −S9) and downregulated by lindane (cluster II, Fig. 1 and Table 2). Regulation of transcription and translation is likely associated with cell cycle control (DiPaola, 2002). Due to the high proliferation rate of immune cells, the cell cycle controlling pathways in immune cells might be more sensitive to toxicants than other cell types. Our observations that OTA and lindane both affected the mRNA levels of genes involved in cell cycle regulation are in agreement with previous studies. Induction of cell cycle arrest by OTA has been reported in human PBMCs (Liu et al., 2012). Lindane has also been demonstrated to disrupt cell cycle in various cell lines (Kalantzi et al., 2004). In addition, our observation that OTA affected the genes involved in regulation of transcription and translation is in line with previous studies, in which inhibition of RNA (Dirheimer and Creppy, 1991) and DNA synthesis (Stormer and Lea, 1995) have been linked with OTA-induced toxicity, both in vivo and in vitro.

Metabolism and biosynthesis of cholesterol and lipids were found to be activated by the S1P-receptor antagonist FTY720 and inhibited by the organochlorine insecticide lindane and the organophosphate herbicide propanil (cluster V, Fig. 1 and Table 2). Recent murine studies have shown that (1) enrichment of plasma membrane cholesterol drives T cells toward a Th1 phenotype, which indicates a predisposition toward autoimmune and inflammatory diseases (Surls et al., 2012), and (2) deficiency of LXR$\beta$, a key nuclear receptor for cellular cholesterol efflux, results in lymphoid hyperplasia and enhances responses to antigenic challenge (Bensinger et al., 2008).

These studies underline the relevance of cholesterol metabolism in maintaining immune system homeostasis. Our results are in line with previous in vivo studies as FTY720 has been shown to induce hypercholesterolemia in mice (Klingenberg et al., 2007) and propanil exposure has been found to decrease serum cholesterol level in rats (Santillo et al., 1995).

Furthermore, we observed that the mRNA levels of two G protein–coupled receptors (GPCRs), GPR17 (cluster VII) and GPR18 (cluster I) (see the expression patterns of GPR17 and GPR18 in Fig. 2), were altered by various immunotoxicants. These GPCRs are known to be expressed by T cells and play important roles in mediating cellular immune responses. For instance, GPR17 is known to aggravate Th2 immune responses mediated by cysteinyl leukotrienes (Laidlaw and Boyce, 2012), and GPR18 expression correlates with the Th1 phenotype of human T cells (Herrera et al., 2006). Further research is needed to establish whether these GPCRs could also mediate chemically induced immunotoxicity.

Besides the processes that were commonly affected by immunotoxicants, we also identified processes that were affected by one or two of the immunomodulating compounds. Notch and NF-κB signaling pathways were found to be activated by OTA (+ S9 and −S9) (cluster VII, Fig. 1 and Table 2). Notch signaling has been well characterized for its role in promoting T lineage commitment and maturation (Yuan et al., 2010). It also contributes to the peripheral T-cell response as the inhibition of endogenous Notch activation decreases the proliferation of activated T cells via enhancing a positive feedback loop involving IL-2 and its high-affinity receptor CD25 (Adler et al., 2003). The importance of NF-κB signaling in the immune system has been well described. In general, NF-κB family members are transcription factors that regulate various aspects of both innate and adaptive immune responses by controlling the transcription of cytokines and other soluble factors that regulate cellular proliferation, differentiation, and survival (Hayden et al., 2006; Vallabhapurapu and Karin, 2009). The NF-κB signaling pathway can be activated by ER stress, increased calcium levels, and oxidative stress (Fardoun et al., 2007; Pahl and Baueuerle, 1996). This pathway is also known to play a role in T-cell activation (Katika et al., 2011). NF-κB signaling pathway has already been associated with OTA-induced toxicity both in vivo and in vitro (Ferrante et al., 2008; Kumar et al., 2013; Schwerdt et al., 2007).

Response to retinoic acid was found to be induced by TBTC and TBTO (cluster XIII, Fig. 1 and Table 2). Retinoic acid is known to be a ligand of retinoic acid receptors (RARs) and retinoic X receptors (RXRs) (Pfahl and Chytil, 1996). Three enriched core genes of cluster XIII, ABCA1, ABCG1, and KLF2, are known target genes of the nuclear receptor LXR (Zelcer and Tontonoz, 2006), RAR (Berry et al., 2012), and RXR (Costet et al., 2003). The upregulation of ABCA1, ABCG1, and KLF2 by TBTC and TBTO indicated the activation of these receptors (see the expression patterns of ABCA1 and ABCG1 in Fig. 2). Our results are in line with the recently reported findings of
Cui et al. (2011) that TBTC exposure increased ABCA1 mRNA expression by activating LXRα/RXR signaling and thereby modulating cellular efflux of cholesterol in mouse macrophages. Effects of TBTO on lipid metabolism have also been suggested in previous toxicogenomics studies with rodent thymocytes (Baken et al., 2007; van Kol et al., 2012). LXR, RXR, and RAR signaling have been shown to be a key pathway in controlling immune tolerance and adaptive immune responses (Hall et al., 2011; Kidani and Bensinger, 2012). Another study with TBTO-treated Jurkat cells has demonstrated not only the induction of ER stress and oxidative stress but also the activation of NF-κB and nuclear factor of activated T cells pathways and the induction of apoptosis (Katika et al., 2011). However, the concentrations of TBTO used in the latter study (200 and 500nM) were higher than the one applied in the present study (100nM) and might represent the onset of cytotoxicity.

Furthermore, we observed that the mycotoxin OTA (+ S9 and −S9) and the organochlorine insecticide lindane both induced ALDH8A1, a gene encoding an enzyme involved in the biosynthesis of retinoic acid (cluster V. Table 1 and Fig. 2). ALDH8A1 converts 9-cis-retinal into RXR ligand 9-cis-retinoic acid. Presently, it is not known whether T lymphocytes can produce retinoic acid, although retinoic acid that is produced by dendritic cells or epithelial cells can regulate the differentiation and functionality of T lymphocytes (Hall et al., 2011). Therefore, further research is required to verify whether the transcriptional activation of ALDH8A1 by OTA or lindane could result in increased cellular retinoic acid biosynthesis in T lymphocytes, or in other immune cells, and could thereby potentially be involved in OTA- or lindane-mediated immunotoxicity.

Most of the biological processes and molecular functions that we identified with Metacore were confirmed by GSEA. Compared with Metacore, GSEA is a more sensitive statistical tool because it uses the full transcriptome data as the input without enforcing a statistical cutoff beforehand. In this way, GSEA allows the detection of subtle effects on the mRNA levels of large groups of genes. GSEA analyses showed that the gene sets related with ribonucleoprotein complex, nucleolus, and mitochondria were commonly affected by direct immunotoxicants (Supplementary fig. 3B). The effects on the ribonucleoprotein complex and the nucleolus are likely related with regulation of transcription and translation, which are among the affected biological processes that were identified by Metacore analyses. Mitochondrial dysfunction has been linked to direct immunotoxicity of a wide range of chemicals, including metals, organotin compounds, dioxins, and immunosuppressive drugs (Baken et al., 2007; Bustamante et al., 2004; Kobayashi et al., 2009; Mitra et al., 2013). Mitochondrial dysfunction is also known to be associated with oxidative stress and apoptosis, both in vivo and in vitro (Gerlach et al., 2007; Yuzefovych et al., 2013). These latter processes were also among the biological processes that were implied by our data.

Some of the pathways and processes that we identified overlap with those described in previous immunotoxicogenomics studies that also aimed at identifying common pathways/genes involved in immunotoxicity, including the processes related with cellular stress responses (response to chemical stimulus, response to unfolded proteins, and response to oxidative stress) and regulation of NF-κB signaling (Hochstenbach et al., 2010, 2012; Vandebriel et al., 2010). Response to oxidative stress was also found in other toxicogenomics studies, for instance, for OTA in rat kidney cells in vivo (Lube et al., 2003) and human liver cells in vitro (Hundhausen et al., 2008), and for MeHg in fish liver in vivo (Yadetie et al., 2013). Furthermore, our study revealed new processes and pathways that are potentially associated with immunotoxicity, such as modulation of lipid metabolism, retinoic acid signaling, and Notch signaling. Further research is required to validate the relevance of these potential mechanisms.

When comparing our data with the As2O3, TBTO, and CsA data from other toxicogenomics studies, the degree of overlap between Jurkat and other immune cells was higher than the overlap between Jurkat cells and the liver cell line HepG2, which suggested a cell type–dependent effect of the compounds on gene expression (Supplementary figs. 4–6).

In order to estimate the potential relevance of these effects for immunomodulation in humans in vivo, we compared the exposure concentrations that we applied in vitro with circulating concentrations in humans in vivo (Supplementary table 3). This approach showed that for some crucial test compounds, being the immunomodulators As2O3, DON, TBTC, TBTO, FTY720, and mycophenolic acid and the nonimmunotoxic control AgNO3, furosemide, and azathioprine, the exposure concentrations that we applied in vitro were within the same range as humans in vivo (0.33–3×). For the other test compounds, the concentrations that we used were different from the in vivo situation, as a consequence of the selection criteria for the concentrations (± CV80 at 24h).

Furthermore, some of the genes we identified overlap with human in vivo data. For instance, we have found an induction of the gene FBXO32 by As2O3 (Supplementary fig. 1). A similar effect on FBXO32 has been found in lymphocytes of human individuals chronically exposed to As2O3-contaminated drinking water (Andrew et al., 2008). This observation underlines the relevance of the effects on mRNA expression levels that we observed in the present in vitro study for human direct immunotoxicity in vivo.

In conclusion, by applying an in vitro toxicogenomics approach, we have identified cellular pathways and processes that are transcriptionally modulated upon exposure to direct immunotoxicants. The present study has demonstrated the value of the Jurkat T-cell line as a sensitive system for identifying mechanisms underlying direct immunotoxicity. Because apparently diverse MOAs are involved, it is envisaged that an assay based on a set of pathways or genes, rather than one single gene, will allow to screen compounds for direct immunotoxicity. Finally, it should be noted that the Jurkat cell model may not be useful to test compounds that specifically target immune cells.
other than T cells. Therefore, the assay that can be developed based on the results of the present study should not be considered as a universal screening assay but as a promising assay to complement animal-free immunotoxicity testing approaches.

**SUPPLEMENTARY DATA**

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

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**REFERENCES**


