

Toxicogenomics in the assessment of immunotoxicity

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Abstract

Microarray analysis is used for simultaneous measurement of expression of thousands of genes in a given sample and as such extends and deepens our understanding of biological processes. Application of the technique in toxicology is referred to as toxicogenomics. The examples of assessment of immunotoxicity by gene expression profiling presented and discussed here, show that microarray analysis is able to detect known and novel effects of a wide range of immunomodulating agents. Besides the elucidation of mechanisms of action, toxicogenomics is also applied to predict consequences of exposing biological systems to toxic agents. Successful attempts to classify compounds using signature gene expression profiles have been reported. These did, however, not specifically focus on immunotoxicity. Databases containing expression profiles can facilitate the applications of toxicogenomics. Platforms and methodologies for gene expression profiling may vary, however, hampering data compiling across different laboratories. Therefore, attention is paid to standardization of the generation, reporting, and management of microarray data. Obtained gene expression profiles should be anchored to pathological and functional endpoints for correct interpretation of results. These issues are also important when using toxicogenomics in risk assessment. The application of toxicogenomics in evaluation of immunotoxicity is thus not yet without challenges. It already contributes to the understanding of immunotoxic processes and the development of *in vitro* screening assays, though, and is therefore expected to be of value for mechanistic insight into immunotoxicity and hazard identification of existing and novel compounds.

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1. Introduction

Microarray technology allows quantitative measurement of transcriptional activity of thousands of genes at the same time [1]. The technique is nowadays widely employed in research on cell biology, pathology, pharmacology, and toxicology and extends the scale and depth of understanding of biological processes. Toxicogenomics is the term used for identification of specific gene expression profiles in biological systems associated with xenobiotic exposure [2]. Assuming that the expression pattern of a gene product and its function are tightly cor-

related, this provides insight into the underlying mechanisms of action of toxicants [3]. Since exposures leading to the same endpoint will presumably share comparable changes in gene expression, gene expression profiling can at the same time aid in characterization of classes of compounds and identification of biomarkers to be used for prediction of specific toxic effects [4–7]. This idea is supported by several proof-of-principle studies with well-characterized chemicals [5,8–16]. Immunotoxicity was, however, not addressed in those studies. Studying gene expression changes associated with immunotoxic processes is a relatively new approach in the field of immunotoxicology. We will highlight and discuss examples of this application of toxicogenomics. Before that, some technical aspects of the microarray analysis procedure,

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the establishment of public databases, and the value of toxicogenomics for risk assessment are discussed.

2. Microarray analysis

The basic principle of microarray technology is the hybridization between nucleic acids, one of which is immobilized on a solid matrix [17]. This is the same for different platforms, platforms meaning types of arrays or manufacturers of arrays. Per gene, a single probe or a few different probes are generated, using either PCR-amplified complementary DNA (cDNA) or synthetic DNA segments (oligonucleotides or oligos) devised on the basis of these cDNA sequences. Oligos are computer-designed in such a way that they have a similar hybridization optimum and lack significant cross-hybridization, these being significant advantages over cDNAs. In addition, oligos require fewer production steps and these steps can easily be automated, making oligos less prone to sequence errors. Hence, oligos are currently the platform of choice especially for large arrays. Some companies manufacture oligos *in situ*, either using photolithography (Affymetrix) or chemical coupling (Agilent). Oligos and cDNAs are spotted onto a glass surface in a regular array, a process requiring dedicated machinery. Several options exist to obtain arrays: (1) ready-made arrays (e.g., Affymetrix, Agilent, Amersham, ABI, and many others), (2) custom-made arrays (e.g., Affymetrix, Agilent), (3) in-house spotting of a PCR-amplified clone collection (e.g., Invitrogen) or an oligo collection (e.g., Operon, Sigma), and (4) in-house spotting of an in-house prepared PCR-amplified clone collection. Several array manufacturers also market their own suited hard- and software.

The number of genes to be analyzed is of interest. Obviously, for mechanistic studies as well as for populating databases aimed to identify patterns of toxicity or toxic compounds, the number of genes should be maximal, nowadays meaning virtually all genes. With statistics aiding in the process of gene selection, signatures of toxicants (such as peroxisome proliferators) or pathology (such as hepatocellular necrosis) may eventually be addressed by interrogating a small number of genes.

Prior to a microarray experiment, RNA or mRNA is isolated from cells or tissues. Since microarray analysis requires very high quality purified RNA, dedicated machinery for RNA quality control has been developed, such as the Bioanalyzer (Agilent). RNA is used for cDNA synthesis and depending on the amount of starting material this cDNA can be used in an amplification step to yield antisense RNA (aRNA or cRNA). For RNA amplification, linear amplification based on the Eberwine T7 transcription method [18] has become most common as it is less sensitive to introducing changes in proportions of different RNA transcripts between samples compared to exponential (PCR-based) amplification. Several linear amplification kits are available from e.g., Ambion. Either during or after synthesis, the cDNA, aRNA, or cRNA is labeled using biotin

(Affymetrix) or a fluorescent label (spotted arrays). Since the amount of starting RNA as well as labeled nucleic acid is limited, quality control must be performed on very small sample sizes. This is enabled by equipment such as the NanoDrop spectrophotometer (NanoDrop technologies).

Labeled nucleic acids are then hybridized to the array. The Affymetrix platform uses a single labeled sample cRNA per hybridization, whereas most spotted array platforms rely on two samples labeled with Cy3 and Cy5. Most often these are a test sample and a common reference used on all arrays. Other experimental designs (e.g., a loop design in which samples are compared to one another chain wise [19]) are not frequently used, mainly because technical failure of one or more slides hampers the accompanying analysis.

After hybridization and washing, the array is read using a scanner (with fitted laser(s)) that measures the fluorescence intensity (intensities). Scanned images are processed using image analysis software (e.g., ArrayVision, GenePix, Imagen) resulting in signal data per spot. Opinion is divided as to whether the background signal should be subtracted from the spot signal data. In our experience it is better not to do so as background subtraction adds noise to the data and results in zero or negative signal values that complicate further data processing such as log transformations.

During and after the experiment a number of controls have to be performed to assure that the results obtained are reliable. Array controls include the shape of the spots and the amount of DNA spotted (e.g., by hybridization of labeled random nonamers). General hybridization controls include a similar average staining intensity over the entire array, and the absence of stains or scratches. Also, the Cy3/Cy5 ratio is plotted against their geometric average intensity in an MA-plot [20]. This ratio should be independent on the intensity for most of the genes interrogated.

In the subsequent normalization step the data are adjusted in such a way that the signal intensities obtained are comparable for most genes across the arrays, as it is generally assumed that only a minority of genes are affected in most experiments. For two-dye arrays various locally weighted linear regression (lowess)-based normalization algorithms are in use to smooth out minor artifacts [21]. For Affymetrix chips many public algorithms have been developed in addition to the vendor's microarray suite v.5 (MAS5) statistical package. Of these public algorithms, robust multi-chip average (RMA) analysis [22] is well-known and currently regarded as most reliable.

If replicate samples are tested, statistics can be performed. In the literature, the criteria on whether a gene can be considered affected by the experimental conditions (i.e. differentially regulated) vary between studies. However, we find that a false discovery rate (FDR) [23] <0.05 to 0.1 as well as a fold ratio (FR) of >1.5 to 2 between expression levels in treatment and control generally answer the aims of a study. Though in many publications merely a list of regulated genes is mentioned, often more information can be

extracted from the obtained data by multivariate statistics such as cluster analysis and/or principal component analysis (PCA) [24]. This results in clusters of similarly and therefore potentially co-regulated genes, indicating possible physical or functional connections between the corresponding gene products. Public algorithms to perform such analyses are available for the R statistical package (<http://www.r-project.org>). Commercial software packages sometimes have the advantage of easier data handling, as a command-based interface may be hard to run for less experienced users.

For a useful interpretation of microarray data, perhaps most important of all is to link the results to biological phenomena. A general method is to look for pathway or function enrichment when a (sub)set of regulated genes is compared to the entire array. Such analyses based on the gene ontology annotation (<http://www.geneontology.org>) can be performed by several web-based tools, e.g., EASE (<http://apps1.niaid.nih.gov/david/>) or GoStat (<http://gostat.wehi.edu.au/>). This type of analysis is obviously restricted by the deficiencies in annotations that exist for many genes. Within a given pathway individual gene expression can be visualized by tools such as GenMAPP (www.genmapp.org), KEGG (<http://www.genome.ad.jp/kegg/>), or MetaCore (www.GeneGO.com). Finally, thorough knowledge of the literature in the field concerned is also indispensable for adequate interpretation.

3. Toxicogenomics databases and standardization

Discrimination of specific classes of toxicants on the basis of signature expression profiles is an important goal of toxicogenomics. In order to reliably classify compounds using gene expression profiles, validation of such signatures on an extensive data set is needed [25]. The potential for microarray data to be informative for chemical classification is therefore dependent on the sharing of data generated in individual gene expression profiling projects [26]. As a result, the research community needs standards for reporting and management of gene expression data.

3.1. Conveying microarray data

To facilitate compiling and comparison of data produced at different laboratories using various methodologies and bioinformatics tools for microarray and data analysis, a document was established that describes which information needs to accompany published microarray results for others to interpret and verify them [27]. These ‘minimum information about a microarray experiment (MIAME)’ guidelines (<http://www.mged.org/Workgroups/MIAME/miame.html>), produced by the microarray gene expression database (MGED) society, have been adopted by publishers and used in public resources in the past years [28]. The set of guidelines is now also extended to MIAME/Tox in order to meet toxicogenomics-specific requirements (<http://www.mged.org/Workgroups/rsbi/rsbi.html>).

For storage and exchange of results of microarray experiments in a standardized format, several public repositories have been created. Examples are the gene expression omnibus (GEO) in the USA (<http://www.ncbi.nlm.nih.gov/geo/>), ArrayExpress in Europe (www.ebi.ac.uk/arrayexpress/), and the Center for Information Biology Gene Expression Database (CIBEX) in Japan (<http://cibex.nig.ac.jp>) [29]. Several journals now oblige authors to deposit their experimental data into such a repository when publishing microarray results [30]. Efforts are underway to link gene expression data stored in the major repositories [31]. In addition, initiatives have been started to build public toxicogenomics databases that besides gene expression data also incorporate associated information on biologic and toxicological endpoints so as to establish a complete knowledge base. This will advance further interpretation of expression patterns and understanding of biological systems. Some examples, for instance chemical effects in biological systems (CEBS) (<http://cebs.niehs.nih.gov/nct/cebs>) and Tox-MIAM-Express (<http://www.ebi.ac.uk/tox-miamexpress/>), have been described by Mattes et al. [32].

The Reporting Structure for Biological Investigations (RSBI) working group represents several communities that aim to contribute towards the development of an international and compatible informatics platform for investigations employing multiple functional genomic technologies (<http://www.mged.org/Workgroups/rsbi/rsbi.html>). The working group intends to coordinate standardization initiatives to come to a unified approach for describing and reporting genomics experiments so that communication can be maximized and duplicated effort minimized [33]. One of the challenges that still exist is harmonization of diverse ontologies and vocabularies. Use of common annotation standards, as addressed by, among others, the Standards and Ontologies for Functional Genomics (SOFG) Conference (<http://www.sofg.org>), will facilitate deposition of data in public databases and data mining [33].

3.2. Standardization of the microarray technology

Now that functional genomics technologies begin to be broadly accepted and used, it is important that the microarray procedures itself are also standardized and that consistency across experiments and platforms is created. Concerns about validity of the technique could hamper uptake in diagnostics and industry. Therefore, variability of microarray data, validation of the technology, and production of standard materials is the focus of many initiatives [33]. The Health and Environmental Science Institute (HESI), for example, initiated an extensive collaborative research program, partly to identify sources of biological and technical variability associated with experimental protocols in toxicogenomics [34]. The results showed that gene expression patterns related to biological pathways are robust enough to allow insight into mechanisms, even across different platforms and laboratories. In a parallel effort, the Toxicogenomics Research Consortium (TRC)

(<http://www.niehs.nih.gov/dert/trc/intro.htm>) evaluated technical variability within science to achieve results (STAR) projects. Here it was concluded that identification of differentially expressed genes correlates between platforms, and that the main source of variability is interlaboratory variation [32]. Other investigations yielded similar findings [35,36]. The Measurements for Biotechnology (MfB) program (<http://www.mfbprog.org.uk>) also determined accuracy of microarray analysis and found the normalization strategy to have the largest impact on data comparability. In the future, standardization of protocols and use of similar platforms as much as possible could reduce (interlaboratory) variation in gene expression measurements, thereby facilitating data exchange [32].

3.3. Use in risk assessment

The construction of databases and attention for standardization are also required for risk assessment to benefit from toxicogenomics research. Microarray analysis might provide a promising avenue for improving risk assessment for several reasons. Firstly, gene expression profiling has been suggested to have the potential of reducing the amount of experimental animals and time needed for toxicological investigation of compounds compared to the established procedures for hazard identification [5,37]. Toxicological effects may be identified at an earlier time point than when traditional toxicity tests are used, and when similarities to classes of known toxicants are found, chemicals can be prioritized for further study [26]. Furthermore, understanding of mechanisms of toxicity could improve insight into adverse effects associated with exposure to compounds and aid in the choice of reliable biomarkers of exposure and effect [26,38]. Since sequence and function of genes are often very similar in various organisms and microarray analysis can be conducted directly in human cells [3], information on molecular responses to compounds generated by toxicogenomics studies can also show if signaling pathways and mechanisms of action are conserved across species. If so, uncertainty in inter-species extrapolations will be reduced. High-to-low dose and short-to-long term extrapolations are argued to benefit from the technique as well, resulting in more scientifically founded safety factors [34,37].

However, despite the progress in development and implementation of toxicogenomics, several obstacles have limited the interpretation of gene expression data and extraction of meaningful and useful information from it [38,39]. For example, mechanisms of action of compounds can depend on dose, timing, and duration of exposure and cell phenotype [40]. Besides, gene expression responses are dynamic and reversible, in contrast to other toxicological endpoints [38]. This hampers establishment of dose-response relationships and extrapolation between model systems. For accurate hazard characterization, insight into the relationship between genomics-based endpoints and known health outcomes is needed. A significant change in

gene expression cannot be concluded to represent an adverse effect (or a small change to represent its absence) until results are placed in an appropriate biological context [34,38,39] and the natural range of physiological variability of gene expression is known [41]. Moreover, toxicogenomics data may not cover all endpoints evaluated by animal studies [37]. For instance, microarray analysis will miss posttranslational modification of proteins by regulatory signals or interactions of compounds with other chemicals or metabolites *in vivo*, whereas both can also cause adverse effects [40,42]. Therefore, confidence in accuracy, sensitivity, and robustness of the method [42] and an extensive amount of interdisciplinary information are needed to advance the application of toxicogenomics in risk assessment [25,39]. Several initiatives have been started to deal with these issues [33,34,37].

4. Applications of microarray analysis in immunotoxicology

Microarray technology has been employed in immunology to study molecular functions associated with immune-related genes in order to better understand immune function and regulation, also referred to as ‘immunomics’ [43]. Gene transcripts that are specifically expressed in immune cell subsets and participate in their maintenance and function have been identified [44,45]. Gene expression profiling is also broadening the understanding of basic and clinical immunological processes by revealing changes in genes that accompany lymphocyte differentiation, activation, and signaling, self-non self recognition, regulation of innate and adaptive immunity, interindividual variations in immune response, inflammation, infection, allergic conditions, autoimmune and other immune-related diseases, and tumor antigen recognition [44,46–57]. Toxicogenomics is now increasingly applied to study alterations in gene expression after immunotoxicant exposure, but published data are still limited. Examples in the literature are discussed here.

4.1. Hexachlorobenzene exposure of Brown Norway rats

Hexachlorobenzene (HCB) is a persistent environmental pollutant with toxic effects in man and rat. Reported adverse effects include hepatic porphyria, toxic effects on the immune and reproductive system, and neurotoxicity. The brown norway (BN) rat strain is very susceptible to HCB-induced immunopathology. Oral exposure to HCB induces, among other things, enlargement of liver, spleen, and lymph nodes, increased serum immunoglobulin (Ig) M, IgG and IgE levels, and inflammatory skin and lung lesions. The mechanisms of HCB-induced immunopathology are not yet fully understood, they are very complex and involve multiple factors.

To gain more insight into the molecular mechanisms of HCB-induced toxicity, gene expression profiling was performed [58]. BN rats were exposed to a diet supplemented with 0, 150, or 450 mg HCB per kg food for 4 weeks. Blood, thymus, kidney, liver, spleen, and mesenteric lymph nodes

(MLN) were collected. An Affymetrix array (rat genome U-34A), containing 8,799 probes identifying primarily annotated genes was used. Principal component analysis showed that effects of HCB on transcript abundance were clearly dose-related. Statistically significant ($p < 0.001$) changes in the spleen and MLN included genes associated with granulocytes, chemokines, cytokines, Ig's, and genes involved in drug metabolism and acute phase responses. Genes of which the expression was significantly changed in the liver included cytochrome P450 genes, genes involved in estrogen and porphyrin metabolism, and also genes involved in immune function. In the blood, genes encoding for instance MHC class II and T cell markers were affected. Alterations in the kidney included genes for cytokines and complement components, but also for cytochrome P450 enzymes. As expected, the thymus was only weakly affected since it is not a target organ of HCB.

Microarray analysis proved to be a suitable tool to reveal changes in gene expression that are consistent with a number of the known (immuno)toxicological effects of HCB in the BN rat and its induction of enzymes involved in metabolism and reproduction. Novel findings included increased gene expression of pro-inflammatory cytokines, chemokines, complement components, cell adhesion molecules, antioxidants, and acute phase proteins. These results are indicative for the involvement of macrophages and granulocytes and the mediators they release in the inflammatory response to HCB, which is accompanied by oxidative stress and an acute phase response. This confirms previous findings [59–61]. The study thus revealed the complexity of cells and mediators that participate in the response to HCB and provided more insight into the mechanisms of HCB toxicity. Measurement of gene expression at more than a single time point could, however, have yielded even more mechanistic information.

4.2. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on gene expression

The xenobiotic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) produces a variety of toxic effects, one of the targets being the immune system. Immunotoxic effects such as induction of thymus atrophy, suppression of cytotoxic T cell activity, and reduction of humoral immunity were often shown to be mediated through the aryl hydrocarbon receptor (AhR) (reviewed by Inadera [62]), which is a transcription factor that can alter the expression of many genes. Zeytun et al. therefore studied effects of TCDD *in vivo* in mice at the transcriptome level [63]. Pathway-specific microarray analysis interrogating 83 genes involved in apoptosis, cytokine production, and angiogenesis revealed upregulation of expression of apoptosis-related genes in the thymus and spleen and to a lesser extent the liver 1 and 3 days after TCDD administration. The results suggested that TCDD-induced apoptosis is mediated by the death receptor pathway in thymocytes. Previous observations showing that the thymus is the most sensitive target of

TCDD and that TCDD can induce apoptosis in a range of cell types were thus confirmed. Besides, differential regulation of expression of genes encoding cytokines was detected in the thymus, also confirming previous observations. Cytokine expression was affected in spleen as well, but the profile differed from that in the thymus. In this study, most genes were regulated at both time points, although the expression levels varied. When additional doses of TCDD were tested, a low dose was found to induce gene expression changes already, and a small subset of genes was regulated dose-dependently [63].

In another study, gene expression changes that underlie suppression of antibody production by TCDD were investigated in lymphocytes of mice immunized with ovalbumin and adjuvant [64]. The Affymetrix Mouse Expression Array 430 A was used to analyze isolated CD4 T cells and B cells. Although immunization alone mainly upregulated gene expression, prior exposure to TCDD particularly resulted in downregulation of gene expression in the T cells 3 and especially 24 hours after immunization, including 3 and 7 genes, respectively, that were upregulated by immunization alone. Inhibition of antibody production by TCDD may thus be mediated by suppression of immunization-induced gene expression. Moreover, many of the suppressed genes coded for proteins that are involved in G protein-linked signaling in CD4 T cells, possibly activating the ERK pathway and thereby inhibiting T cell activation, which is another clue to the mechanism of action of TCDD with respect to reduction of humoral immunity. In B cells, TCDD mainly upregulated gene expression, indicating that TCDD causes cell-type-specific effects [64].

Similar to the HCB study, these two experiments on TCDD-induced immunotoxicity demonstrate that microarray analysis is able to both detect known effects (even with a limited number of specific genes) and expand the knowledge on specific mechanisms of action of compounds.

4.3. Induction of gene expression changes by sensitizers

We have shown that oxazolone (OXA) and toluene diisocyanate (TDI) differ in their induction of IL-4, IL-10, and IFN- γ in the local lymph node assay (LLNA) [65]. He et al. [66] topically applied these two allergens, as well as the non-sensitizing irritant nonanoic acid (NA), on the ears of mice on four consecutive days, and excised the draining (auricular) lymph nodes (LN) on day 5. The resulting expression profiles were compared using Affymetrix arrays comprising 6,500 genes, and 32, 19, and 19 differentially expressed genes were found between TDI and OXA, TDI and NA, and OXA and NA, respectively. These genes included immune response-related genes and genes coding for transcription factors and signal transduction molecules, and may aid in the understanding of molecular mechanisms underlying chemical-induced hypersensitivity. The expression of selected genes was also investigated by RT-PCR. While RT-PCR was able to detect IL-4, IL-10, and interferon (IFN)- γ , the microarray failed to do so. A reason for

this might be the lower detection limit of RT-PCR compared to microarray analysis. In a panel of 5 other genes, only 47% concordance was observed between microarray and RT-PCR results.

Betts et al. [67] measured effects on global gene expression in the draining LN after single topical exposure to the contact allergen dinitrofluorobenzene (DNFB) on the ears of mice. Expression was measured at 18 and 48 hours after application, using an Incyte array interrogating 8,734 genes. Importantly, the authors showed that the technique was both sensitive, since thresholds of detection were similar to the LLNA, and robust, since the kinetics and dose-responses could be confirmed by Northern blot and/or RT-PCR. The authors described 3 genes that were significantly affected: GlyCAM-1 (downregulated), guanylate binding protein 2, an IFN- γ inducible GTPase present in macrophages (upregulated), and onzin (also upregulated). The authors suggested that reduced GlyCAM-1 expression might be linked to increased LN cellularity in the absence of LN cell proliferation. GlyCAM-1 is expressed by high endothelial venules (HEV), its ligand being L-selectin (CD62-L). It regulates migration of circulating lymphocytes into the periphery, and an inverse relation exists between its expression and LN weight. The authors thus hypothesized that reduced GlyCAM-1 expression is involved in recruitment of lymphocytes into the LN.

The upregulation of guanylate-binding protein-2 and onzin expression may be in keeping with induction of T-helper 1 (Th1) responses, which is generally accepted to occur after contact allergen exposure. Guanylate-binding protein-2 is induced by the prototypical Th1 cytokine IFN- γ . Onzin has 98% nucleotide sequence homology and complete amino acid sequence homology with C-15, a cytokine or defensin, that is produced by plasmacytoid dendritic cells (DC) [68]. Plasmacytoid DC enter LN by extravasation across HEV, drive Th1 polarization *in vitro* by producing interleukin (IL)-12 and type I IFN [69], and produce CCL3, thereby recruiting Th1 cells [70]. Plasmacytoid DC were shown to be present in the inflammatory infiltrate in allergic contact dermatitis [71]. Whether DNFB exposure indeed induces plasmacytoid DC influx into the draining LN remains to be established.

Ryan et al. [72] have demonstrated dose-dependent changes in gene expression in blood-derived DC induced by the contact allergen dinitrobenzenesulfonic acid (DNSB) (a water soluble analogue of dinitrochlorobenzene) that are associated with DC maturation, a process that is proposed to occur during DC migration to LN after activation by encountering a chemical allergen [73]. Analysis by means of Affymetrix chips (U95Av2) examining approximately 10,000 genes revealed upregulation of 60 genes and downregulation of 58 genes after exposure to both doses used, representing several cellular processes such as transcription, signal transduction, protein modification and small molecule transport. A number of gene expression changes were consistent with known features of DC maturation, and some of those were recently reported to also occur after

exposure of human DC to the contact allergen nickel sulphate [74]. Ryan et al. obtained findings that were inconsistent with DC maturation as well, maybe due to differences in kinetics of the transcriptional response compared to other methods, or because the observed changes are only a portion of the changes that take place during DC maturation, or because they are chemical-specific. In this study reproducibility (among platforms and DC donors) of the observed changes was also demonstrated, as well as specificity: expression of many genes was altered only by DNSB and not by a structurally similar non-allergen. From the results of this study a list of target genes was derived that could serve to predict skin sensitization by chemicals [75]. These genes were evaluated and further prioritized using real time RT-PCR analysis of PBMCs exposed to an extended set of chemicals including irritants, contact allergens, and non-sensitizers. The 10 genes that were brought forth by this approach showed selectivity, specificity, and a proper dynamic range, and may therefore be used in screening for skin-sensitizing chemicals [75].

In conclusion, clues to mechanisms of sensitization can be inferred from the data obtained. In addition, the sensitivity, specificity, and robustness demonstrated in the different studies support the viewpoint that methods relying on microarray analysis have the potential to—at least in part—replace existing methods to uncover sensitizing effects of compounds. In general, global gene expression profiles after *in vitro* exposure of specific cell types to various model compounds may provide novel (sets of) biomarkers that can predict *in vivo* adverse effects, such as immunotoxicity. It is envisaged that ultimately small sets of biomarkers are sufficient to identify immunotoxicity, enabling high-throughput screening [1,25]. Since various cell types of human origin, most notably DC, can now be obtained (by culture) from human peripheral blood, a readily accessible source, the possibilities to use human cells for immunotoxicity testing have increased, making inter-species comparison superfluous. It is well imaginable, therefore, that transcript changes identified by microarray analyses such as performed by Gildea et al. [75], can serve as new markers for allergenicity which could be used for predictive *in vitro* assays in the future [72].

Besides, Hansen et al. [76] were able to identify 26 differentially expressed ($p < 0.01$) genes by microarray analysis in chromium-stimulated peripheral blood mononuclear cells (PBMCs) from allergic patients compared to healthy controls. Real time RT-PCR showed similar expression changes for three selected genes using a second allergen, nickel, in PBMCs of a nickel allergic patient. Although a larger experimental population and a higher degree of significance would be desirable, this indicates that gene expression profiling also has the potential to identify diagnostic markers for contact sensitivity.

4.4. Other examples

Not only chemical compounds and metals but also mycotoxins can produce immunomodulatory effects. An

example is the mycotoxin deoxynivalenol (DON), which was the subject of an experiment in mice [77]. Although the authors recognized limitations of the study design, such as investigation of a limited number of genes, assessment at a single time point, and the possibility of detecting a nonspecific stress response, microarray analysis in spleens of mice two hours after exposure to DON or vehicle showed alterations in genes involved in immunity, inflammation, and chemotaxis that are likely to contribute to DON's immunological effects. Changes in gene expression were not highly significant ($p < 0.05$) but they were confirmed with real time RT-PCR for a selection of the genes.

Probiotic bacteria, that are claimed to have health promoting effects, are another example of non-chemical immunomodulators. Various strains have been found to induce suppressive as well as stimulating effects on host innate and acquired immune responses, which are often explained by modulation of the Th1/Th2 balance. Microarray analysis using a rat oligo set did, however, not reveal overt effects of *Lactobacillus casei* Shirota on gene expression in liver, spleen, thymus, and MLN of Wistar rats to which we administered the lactic acid bacteria during eight days [78]. The reason for this could be that the immunomodulating effects of the probiotic were only subtle. Nevertheless, the same *Lactobacillus* treatment did stimulate autoimmunity in a Lewis rat model [78]. Therefore, T cell triggering, as occurred in the disease model, may be a prerequisite for induction of effects by this probiotic. Alternatively, probiotics may exert their immunomodulating effects at the protein instead of the gene expression level.

As pointed out in paragraph 4.2 with reference to contact sensitization, microarray analysis is believed to increase the opportunities for *in vitro* (pre-)screening for toxicity. Besides the studies on sensitization, several other experiments on changes in gene expression induced by different compounds illustrate the potency of *in vitro* examination of immunotoxicity. An example is a study on JP-8 jet fuel. Inhalation or dermal exposure to JP-8 jet fuel can, among a range of other effects, impair immune function in mice [79]. JP-8 is known to induce apoptosis in several cell types. In one of these, the Jurkat cell line, gene expression of 439 stress response- and apoptosis-related genes upon exposure to JP-8 was assessed. Gene expression analysis indeed identified 16 up- and 10 downregulated genes ($FR > 2$) associated with termination of cell proliferation or induction of apoptosis. This result may provide clues on the molecular basis of JP-8 induced cytotoxicity in immune cells [80].

In another study, the effects of various concentrations of the environmental contaminant depleted uranium (DU), a byproduct of enrichment of natural uranium, were investigated in murine peritoneal macrophages and splenic CD4⁺ T cells. Damage to immune cells contributes to DU's toxic effect, and DU has been shown to accumulate in macrophages and to subsequently induce apoptosis. A concentration-dependent induction of apoptosis and necrosis was detected by flow cytometry. Also, short term DU exposure

of macrophages was found to influence their interaction with CD4⁺ cells when co-cultured resulting in enhanced T cell proliferation. Gene expression alterations as assessed by a cytokine gene array consisting of 514 cytokine-related cDNAs occurred in both cell types: mostly genes related to signal transduction (for instance the NF- κ B signaling pathway), cytokine production (for instance upregulation of IL-10 in macrophages), chemokines and chemokine receptors, and neurotrophic factors were affected. These effects can be linked to carcinogenesis, development of autoimmune disease and Th2 polarization, which in part may explain observed health effects of DU exposure. As a whole, the results help to understand the mechanisms of DU-induced immune modulation [81].

The effects of a low and a high concentration of the toxic metal cadmium were investigated by Tsangaris et al. [82] in an immature T-cell line at an early and late time point using cDNA microarray slides measuring 1,455 genes. Effects on the gene expression profile were time- and dose-dependent and associated with cell function, cell differentiation, malignant transformation, and cell death. Affected cell viability and apoptosis were demonstrated by other assays as well. Direct and indirect as well as early and late apoptotic effects of cadmium were detected at the transcriptome level and are in line with effects that were demonstrated previously in this and other immune cell lines. The gene expression alterations contribute to the knowledge on toxic and apoptotic effects of cadmium.

Cholera toxin (CT), finally, is not only responsible for the clinical symptoms of cholera but also a powerful mucosal adjuvant. The latter effect is partly caused by enhancement of the production of various cytokines and suppression of IL-12 expression, stimulating the development of Th2 cells. In order to better understand the mechanisms of action of CT, the transcriptional responses in cultured human lymphocytes and monocytes were studied. Using cDNA microarrays expression of 800 selected genes was examined after varying concentrations and time periods. Expression of more than 200 genes changed time- and dose-dependently. Particularly genes associated with immunomodulation, inflammation, and oxidative stress responded to CT. As expected, expression of Th1 markers was downregulated whereas Th2 markers were upregulated. Many genes affected by CT were regulated through the NF- κ B pathway. The gene expression profiles were compared with those induced by an activator and an inhibitor of adenylate cyclase, since this enzyme is activated by CT resulting in intracellular cAMP accumulation. Overlap and differences of gene expression alterations induced by these three compounds yielded insight into the involvement of cAMP in CT toxicity [83]. Furthermore, in a second microarray experiment effects of CT on expression of additional genes involved in immune responses were analyzed. Expression of proinflammatory genes such as IL-8, IL-1 β , IL-6, and VEGF was found to be affected the most, which corresponds to earlier findings. Since the proteins encoded by the regulated genes are known to mediate the recruitment,

migration, activation, proliferation, and development of lymphocytes, they are likely to contribute to the adjuvanticity of CT [84].

5. Discussion

The previous paragraphs describe examples of toxicogenomics in immunotoxicological research. In general, the *in vivo* and *in vitro* experiments illustrate the potential of microarrays to confirm known molecular effects of the compounds studied as well as to be of help in elucidating their mechanisms of action.

Some issues should however be considered. In the HCB exposure study 6 organs were analyzed at three doses including vehicle control, providing a rather complete picture of the exposure effects in a 28-day study. The inclusion of organs other than immunological ones is very informative, since genes expressed outside of the immune system can still have a function in the immune response [44]. In addition, HCB is known to exert toxic effects on systems other than the immune system.

The use of a low and a high dose of HCB was especially valuable since many xenobiotics are studied at levels of significant toxicity, whereas insight into gene expression changes in the absence of pathological or cellular change is likely to be more meaningful. For example, at higher doses cellular influx can occur, as was seen after exposure to the high dose of HCB, causing secondary effects on gene expression. This can obscure the interpretation of microarray experiments since it may by itself cause altered abundance of certain mRNAs. Pathology may identify which cell types have entered a certain organ, while hematology can measure the changes in blood cell numbers. Genes that are expressed only or predominantly by the various blood cell types can also be used to characterize the nature and amount of cellular influx. These genes (e.g., CD markers) may or may not be included in further analysis. Certain genes will not be expressed by blood cells, regulation of them thus suggesting other processes to occur besides cell influx, while for other genes the expression by blood cells may not be known.

To evaluate the possible benefits of microarray analysis for immunotoxicology in general on the basis of the examples mentioned, it is important to stress that several studies are limited in their size. Dose- and time-dependency of gene expression effects were found in all studies including more than a single dose and time point, indicating that this is an important aspect to be considered when designing microarray experiments. An in-depth investigation on the immune response to chemical allergens, for instance, requires several doses, time points, and compounds to be tested. Additional experience with a wider range of allergens and non-allergens is also required to determine selectivity of markers to be used in screening for contact allergy, thereby improving hazard identification. Another variable, the number and timing of allergen applications, adds extra variability, further increasing the number of experiments needed.

Another drawback of some microarray studies is the limited number of genes that are investigated, such as in the studies investigating effects of TCDD, DON, JP-8 jet fuel, and DU. Interrogating a broader range of genes will yield a more general view on effects on gene expression and give insight into involvement of additional cellular pathways.

As pointed out before, for correct interpretation of gene expression data it is important to link the observed changes to other adverse effects, and to confirm results by measuring the same endpoints in different assays. This was for instance done in the studies on DNFB and cadmium. In the CT study another approach was chosen to confirm mechanisms involved, being comparison of the gene expression profile with that of compounds with the same or different mechanisms of action. In the same way, transcriptome changes after DNSB exposure were related to those of a structurally similar non-allergen. In the experiment with *Lactobacillus casei* Shirota we showed that it is equally important to establish correlation of absence of changes in gene expression with functional effects, since effects may only be observable in specific experimental models or at other cellular components than the transcriptome. Results of *in vitro* approaches should most ideally be confirmed with *in vivo* effects, since functional differences may exist between cells in culture or *in vivo*, and *in vitro* designs lack interaction of various biological entities [4]. This is for instance illustrated by a study comparing effects of TCDD on gene expression *in vivo* and *in vitro* [85], that did not detect immunotoxic effects *in vitro* whereas these were found *in vivo* [63,64]. Use of human cells, as was done in the studies concerning DNSB, CT, and nickel and chromium, is useful to gain insight into human relevance.

Not only immunomodulatory effects of chemicals, metals, and sensitizers but also of a bacterial toxin exhibiting adjuvant activity were successfully studied by means of gene expression profiling. This shows the ability of microarray analysis to detect immunomodulatory effects of a wide range of agents. However, from the examples discussed as well as our own experience it can be concluded that examination of compounds besides model compounds at more doses and time points, as well as inclusion of the results in a database, is needed to construct reliable biomarkers for specific immunotoxic processes. By combining transcriptomics with the other 'omics' approaches, proteomics and metabolomics, an even more complete view on effects of toxicants and their relevant metabolites at the gene expression and protein level will be obtained, facilitating evaluation of consequences of human exposure to immunotoxicants [7].

6. Concluding remarks

Toxicogenomics may not, or not yet, be able to replace the current methods for assessment of immunotoxicity, but it is surely a valuable supplement providing additional insights into molecular mechanisms of toxicity. The opportunities that toxicogenomics offers for *in vitro* (pre-)screen-

ing for immunotoxicity are certainly worth further exploitation. When the standardization and validation issues are solved, implementation of transcript, protein, and metabolite profiling (within existing initiatives) in the assessment of immunotoxicity will eventually result in improvement of mechanistic understanding of immunotoxicity and hazard identification of existing and novel compounds.

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