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REVIEW ARTICLE

Present and future of *in vitro* immunotoxicology in drug development

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Abstract

The realization, that the immune system can be the target of many chemicals including environmental contaminants and drugs with potentially adverse effects on the host's health, has raised serious concerns within the public and the regulatory agencies. At present, assessment of immunotoxic effects relies on different animal models and several assays have been proposed to characterize immunosuppression and sensitization. The use of whole animals, however, presents many secondary issues, such as expense, ethical concerns, and eventual relevance to risk assessment for humans. Furthermore, due to the new policy on chemicals (REACH), in the European Union, *in vitro* methods will play a major role in the near future. In addition, there is still a lack of human cell-based immunotoxicity assays for predicting the toxicity of xenobiotics toward the immune system in a simple, fast, economical, and reliable way. Hypersensitivity and immunosuppression, for which animal models have been developed and validated, are considered the primary focus for developing *in vitro* methods in immunotoxicology. Nevertheless, *in vitro* assays, as well as *in vivo* models, to detect immunostimulation and autoimmunity are also needed. Even if no validated alternative *in vitro* tests to assess immunotoxicity exist, in the last decade, much progress has been made toward these assays. Such models can be, at least, used for the pre-screening and hazard identification of unintended immunosuppression and contact hypersensitivity of direct immunotoxicants. Following a brief introduction to immunotoxicology and to *in vivo* models use to assess immunotoxicity, this manuscript will review the state-of-the-art in the field of *in vitro* immunotoxicity.

Keywords: *In vitro immunotoxicology; immunosuppression; allergy; autoimmunity; human; cell lines*

Introduction

Immunotoxicology is important aspect of the safety evaluation of drugs and chemicals. Toxicity to the immune system encompasses a variety of adverse effects (Figure 1), including:

1. Decreased immunocompetence (immunosuppression): A decreased capacity to neutralize external organisms, which may result in repeated, more severe, or prolonged infections. A decreased immunosurveillance may result in the development of cancer. Infectious complications have often been described in patients treated with corticosteroids (Klein et al., 2001), radiation or immunosuppressive drugs in the post-transplantation period (Sia and Paya, 1998; Sleijffers et al., 2002). Immunocompromised patients are at a greater risk of developing virus-related malignancies. Organ transplant patients treated with long-term immunosuppressive regimens also develop more frequent malignancies (Vial and Descotes, 2003).
2. Immunoenhancement: An exaggerated expression of the immune response, which, as an adverse effect, may lead to immune-mediated diseases such as hypersensitivity reactions or autoimmune diseases. Hyperthermic reactions (> 38.0–38.5°C) with chills, arthralgias, and malaise have been described in patients treated with immunostimulatory medicinal products or following the administration of vaccines and recombinant cytokines. Hypersensitivity reactions are (in industrialized countries) by far the most frequently reported immunotoxic effects of drugs and other chemicals in human beings (Esser and Jux, 2009). Hypersensitivity reactions are

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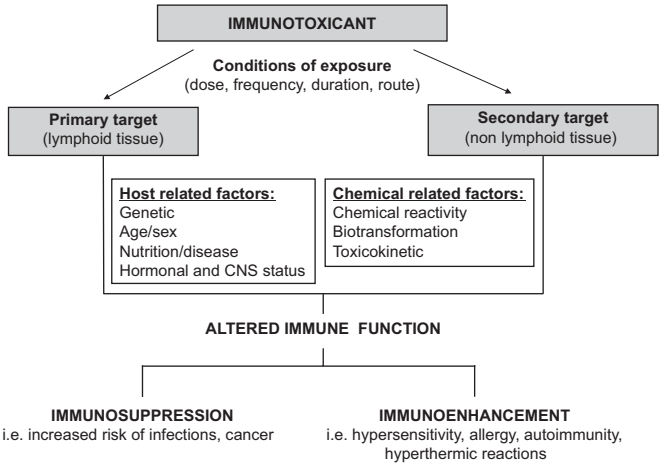

Figure 1. Schematic view of chemical-induced immunotoxicity. CNS, central nervous system.

also relevant drug adverse reactions, as approximately one-third of all drug-induced adverse reactions consists of either immunoallergic or pseudoallergic hypersensitivity reactions (Demoly and Bousquet, 2001). One of the most frequent immune-mediated adverse effects of some, but not all, recombinant cytokines is a marked increase in the incidence of autoimmune diseases (Miossec, 1997). A number of treated patients developed extremely varied autoimmune diseases, with autoimmune thyroiditis being by far the most common finding. Although post-marketing surveillance data are scarce, exacerbation of asthma, eczema and rhinitis was reported shortly after initiation of treatment with immunostimulatory drugs (Bini and Weinshel, 1999).

It has been hypothesized that altered immune functions may be an early indicator of immunotoxicity, eventually having an effect on immunologically based diseases such as cancer, hypersensitivity, and autoimmunity (Luster and Rosenthal, 1993). Industrialised countries have been facing a significant increase in some of these conditions over the past few decades (Sly, 1999), although the rate of increase has recently slowed (Fleming et al., 2000; van Schayck and Smit, 2005). Even if there have been no satisfactory explanations for this observation, regulatory interventions aimed: to reduce exposures; to promote the use of appropriate work practices; to enhance the training and education of workers; and, to provide overall a better survey and classification of symptoms, may all have contributed to such trend. The epidemiology of metal allergy has recently changed in Europe as nickel allergy has decreased following regulatory intervention concerning nickel release from consumer products (Thyssen and Menné, 2010).

Although the levels of evidence differ, these diseases can potentially be attributed to an environmentally-related alteration of the immune system's function. The observed changes in prevalence have been too rapid to be accounted for by modification of the gene pool and there is a general consensus that alterations in lifestyle (i.e., changes in diet, reduced exposure during early life to pathogens, vaccination programs), combined with changing environmental factors (i.e., both outdoor and indoor air quality), are responsible for the observed increase in prevalence (ECETOC, 2005).

Xenobiotics, including drugs, may play an important role in genetically susceptible individuals, where they may initiate, facilitate or exacerbate pathological immune processes, resulting in autoimmunity, asthma and cancer. Theoretically, they can do so by the induction of mutations or changes in regulation of genes coding for immunoregulatory factors, modification of immune tolerance and regulation, resulting in immunostimulation and immunosuppression.

Current in vivo models to assess immunotoxicity

Guidelines from the European Medicines Agency (EMEA, 2000) emphasize the need to assess immunotoxic effects during the preclinical phase of drug development, and recommend specific tests to measure immunotoxicity. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), which brings together the regulatory authorities of Europe, Japan, and the United States and experts from the pharmaceutical industry to discuss scientific and technical aspects of product registration, have recently agreed to adhere to the so-called S8 guideline for immunotoxicity evaluation of pharmaceuticals (ICH Safety Number 8: Immunotoxicity studies for human pharmaceuticals. EMEA/CHMP/167235/2004). This guideline states that immunotoxicity studies should be conducted on a case-by-case base, when alerting data are found in repeated dose toxicity studies. The preclinical assessment of immunotoxicity is, at present, however, restricted to animal models and assays to predict unexpected immunosuppression and contact hypersensitivity.

The ICH S8 guideline advocates a weight-of-evidence approach, which should be interpreted to indicate that specific immunotoxicity testing would be conducted based on identified cause(s) for concern in routine toxicology studies rather than as a routine screening method. In Table 1, the signs of concern of immunotoxic potential in standard

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toxicology studies are reported. The ICH S8 guideline provides:

1. Recommendations on non-clinical testing approaches to identify compounds that have the potential to be immunotoxic; and,
2. Guidance on a weight-of-evidence decision making approach for immunotoxicity testing.

If a cause for concern is identified, additional immunotoxicity studies should be performed to verify the immunotoxic potential of the compound. These studies can also help determine the cell type affected, reversibility, and the mechanism of action. Where a specific target is not identified, an immune function study such as a T-lymphocyte-dependent antibody response is recommended. In addition, immunophenotyping of leukocyte populations, a non-functional assay, together with natural killer (NK) cells activity, a functional assay, can be conducted to identify the specific cell populations affected and immune functionality, which might also provide useful clinical biomarkers.

Despite the important health and economic impact of autoimmunogenicity or systemic allergenicity by pharmaceuticals, drug-induced systemic hypersensitivity and autoimmunity are excluded from the guideline as well as the assessment of immunogenicity of biologicals, which will be discussed later in this manuscript. No standard approaches for human drugs are currently available for testing for respiratory or systemic allergenicity (antigenicity) or drug-specific autoimmunity; testing for these endpoints is not currently required. The most important reason for this is the related complex interplay of multiple factors, and, moreover, the clinical effects are quite diverse, and involve both organ-specific and systemic effects, including a diversity of skin diseases. For the assessment of systemic hypersensitivity and autoimmunity, readers are encouraged to see recent reviews by Pieters (i.e., Pieters et al., 2002 and Pieters, 2007). Among the different models, the reporter antigen-popliteal lymph node assay (RA-PLNA) holds a strong promise to distinguish sensitizing and/or neoantigen-forming capacity of low-molecular-weight pharmaceuticals.

The evaluation of contact sensitization is usually done using the local lymph node assay (LLNA) as described in the Organization for Economic Cooperation and Development (OECD) guideline 429 (Gerberick et al., 2007a). The LLNA is a murine model developed to evaluate the skin sensitization potential of chemicals (Kimber and Weisenberger, 1989). The LLNA is an alternative approach to traditional guinea pig methods and, in comparison, provides important animal welfare benefits. The assay relies on measures of events during the induction phase of skin sensitization—specifically lymphocyte proliferation in the draining lymph nodes—as a hallmark of a skin sensitization response. In addition to providing a robust method for skin sensitization hazard identification, the LLNA has proven very useful in assessing the skin sensitizing potency of test chemicals. The primary method to make comparisons of the relative potency of chemical sensitizers is based on the estimation of the concentration of chemical required to induce a stimulation index of three relative to concurrent vehicle-treated controls (i.e., the EC_3).

To summarize, assessment of immunotoxic effects relies on different animal models and several assays have been proposed to characterize immunosuppression and sensitization. Current available animal models and assays are not valid to assess the potential for systemic hypersensitivity and, at this time, autoimmunity is 'not predictable' at all, with the RA-PLNA holding a strong promise.

Table 1. Signs of immunotoxic potential in standard toxicology studies.

1. Hematological changes such as leukocytopenia/leukocytosis, granulocytopenia/granulocytosis, or lymphopenia/lymphocytosis;
2. Alterations in immune system organ weights and/or histology;
3. Changes in serum immunoglobulins;
4. Increased incidence of infections;
5. Increased occurrence of tumors, in the absence of genotoxicity, hormonal effects, or liver enzyme induction.

To assess drug-induced immunotoxicity, a generally-accepted study design in rodents is the 28-day repeated dose toxicity testing study.

In vitro assessment of immunotoxicity

Although evaluation of immune function following *in vivo* exposure to a test material is arguably the most relevant situation, it is increasingly desirable to limit the use of animals whenever possible. In Table 2, the general recommendations from the ECVAM workshop on "The use of *in vitro* systems for evaluating Immunotoxicity" are reported. Many of the techniques that serve as the *in vitro* portion of *ex vivo* immunotoxicology testing could serve as stand-alone assessments in a totally *in vitro* test system (House, 2000). Hypersensitivity and immunosuppression are considered the primary focus for developing *in vitro* methods in immunotoxicology, as validated animal models exist, with the clear notion that *in vitro* assays to detect developmental immunotoxicity, immunostimulation and autoimmunity are also highly needed.

In the last decade, much progress has been achieved in the development of alternative *in vitro* testing to assess

Table 2. General recommendations from the ECVAM workshop on "The Use of *In Vitro* Systems for Evaluating Immunotoxicity."

- Hypersensitivity and immunosuppression are considered the primary focus for developing *in vitro* methods in immunotoxicology.
- It is recommended to use a flow chart/decision tree approach to evaluate whether or not a compound is immunotoxic (initial screening).
- To maximize human relevance, and due to the lack of species limitations for these assays, it is recommended that human cells be used for all *in vitro* test systems.
- Although the use of primary human cells will be of the highest clinical relevance, consideration may eventually be given to the use of sufficiently well-characterized and validated cell lines (human or animal) for certain aspects of the test systems.
- The validation of an *in vitro* method to detect immunotoxicity must depend on high quality *in vivo* data. It is essential that a sufficiently large number of positive and negative reference compounds including both drugs and chemicals, be tested. To this aim, the establishment of a human database is strongly recommended.

See Gennari et al. (2005) for details.

immunotoxicity, mainly immunosuppression and contact hypersensitivity. These advances are such that these models should be, at least, used for the pre-screening and hazard identification of direct immunotoxicants where direct immunotoxicity is caused by the effects of chemicals on immune cells. Following a brief description of possibilities to identify immunosuppressive agents, possibilities to identify contact and respiratory allergens are reported.

General strategy to identify in vitro immunosuppressive agents

Before starting with *in vitro* tests, bioavailability should be considered. If the compound does not have appreciable bioavailability, immunotoxicity is unlikely to occur. As a general strategy, *in vitro* testing for direct immunotoxicity can be done in a tiered approach, the first tier measuring myelotoxicity. Compounds that are capable of damaging or destroying the bone marrow will often have a profound immunotoxic effect, since the effectors of the immune system itself will no longer be available. Therefore, if a compound is myelotoxic the material will be a *de facto* immunotoxicant. The methodology for evaluating myelotoxicity *in vitro* using bone marrow culture systems is well-characterized (Pessina et al., 2001). The assay (validated by an International study supported by the EC) can be applied to test drug toxicity to myeloid progenitors "*in vitro*" by using as source human umbilical cord blood and mouse bone marrow cells. *In vitro* clonogenic assays can be used to assess proliferation and the differentiation both of pluripotent haemopoietic stem cells and of the different progenitors of blood cell lineages: megakaryocytes (colony forming unit [CFU]-Mk) granulocyte-macrophage (CFU-GM), and erythrocytes (blast-forming unit [BFU]-E/CFU-E). The clonogenic assay is very useful to investigate the pathogenic mechanisms of drug-induced blood disorders and also for screening compound during preclinical safety study.

Compounds that are not overtly myelotoxic may still selectively damage or destroy lymphocytes, which are the primary effectors and regulators of acquired immunity. Compounds are therefore tested for lymphotoxicity (second tier). This toxicity may result from the destruction of rapidly dividing cells by necrosis or apoptosis; alternatively, chemicals may interfere with cell activation affecting signal transduction pathways. A variety of methods are available for assessing cell viability (e.g., colorimetric, flow-cytometric assays). After myelotoxicity and overt cytotoxicity are excluded as endpoints, basic immune cells functionality should then be assessed by performing specific functional assays, i.e., proliferative responses, cytokine production, NK cell activity, etc. (third tier), using non-cytotoxic concentrations of the tested chemicals (viability > 80%).

Functional assays

In the following paragraphs, a brief description of *in vitro* methods to assess both innate and acquired immune functions, namely NK cell activity, lymphocyte activation, and cytokine production, is reported. These assays can, in many instances, be performed using both murine and human cells. However, to avoid interspecies extrapolation and the considerable species differences in the response of the host immune system as well as the species specificity of some agents to be tested, *in vitro* assays should preferably use human cells.

It is also important to stress that any dysregulation(s) of immune cell homeostasis can result in severe adverse changes in immune functions, increasing the susceptibility to infections and cancer, as well as favoring the development of autoimmune diseases. Therefore, any significant change in the functionality of immune cells must be consider as a hazard, which effective risk for human beings should be carefully evaluated during the risk assessment phase. A normal immune system is comprised of complementary and compensatory mechanisms. Failure to identify alterations in host resistance in the face of significant changes in functional ability does not necessarily mean the absence of risk to man. Due to genetic polymorphisms, the response to immunotoxicants in humans is variable. Thus, alterations in immune functions, which may be tolerated well in normal individuals, could have more serious consequences for those who are chronically ill, malnourished, or whose immune system has yet to develop or is in decline. Therefore, the detection of potential immunotoxicity of chemicals (hazard), including drugs, is an important aspect of safety evaluation. The available data suggest that if a large enough population is exposed and that the challenge dose or virulence of pathogenic organisms or tumor cells is sufficient, small changes in immune surveillance could increase the background incidence and burden of disease in the human population (Germolec, 2004).

Due to the high predictivity of human immunotoxicants provided by the *in vivo* antibody induction assay (Luster et al., 1992), the development of this *in vitro* system is highly desirable. Many other *in vitro* functional tests can, however, be performed, including, NK cell activity, proliferative responses, cytokine production, etc. (Gennari et al., 2005).

Innate immunity: NK cell

NK cells or large granular lymphocytes are involved in non-specific immunity. NK cells are involved in defense against viruses and some tumors. $CD3^{-}CD16^{+}CD56^{+}$ cells account for 7–41% of the lymphocytes in human peripheral blood (Pasqualetti et al., 2003). They have for a long time been recognized as exquisitely sensitive to toxicants. The inclusion of NK cell activity plus distribution of lymphocyte subsets have been also suggested as an alternative to the primary antibody response to a T-lymphocyte-dependent antigen in *in vivo* immunotoxicology studies (Li, 2010). NK cells are enumerated based on surface markers (mainly CD56) and their cytotoxic function is usually assayed *in vitro* using $[^{51}Cr]$-labeled target cells, classically K562 erythroleukemia cells.

Recently, a flow-cytometric cytotoxicity assay has been developed (Marcusson-Ståhl and Cederbrant, 2003). Intracellular levels of perforin, granzymes, and granulysin can also be analyzed during the evaluation of NK cell function. The proportion or absolute numbers of NK cells are modified after *in vivo* exposure to a consistent number of the substances and physical agents tested (Burns et al., 1994;

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Ross et al., 1996; Marcusson-Ståhl and Cederbrant, 2003). The predictive potential of such assays after *in vitro* exposure has been the subject of intensive investigation (Lebrec et al., 1995; Condevaux et al., 2001). The main limitation of the use of NK cell activity for *in vitro* studies is the rapidly loss of cell viability and of cytolytic function once cells are isolated (Hébert and Pruett, 2001). As a possible alternative, the use of NK cell lines, such as NK-92 or IMC-1, should be explored. Due to the *in vivo* value of this assay, efforts should be devoted to further explore the possibility to use NK cells activity as an alternative *in vitro* test to assess immunotoxicants.

In vitro measurement of lymphocytes activation

In animals, production of T-dependent antibodies is considered to be the "gold standard." However, there are currently no good systems for *in vitro* antibody production using human cells, and there is also some concern whether a primary immune response can actually be induced in human peripheral blood leukocytes (PBL). One potential starting point could be the development of an *in vitro* immunization culture system based on the Mishell–Dutton assay (Mishell and Dutton, 1967). This assay is, at present, not considered optimal for this use due to significant variability in results—and often a complete lack of success—between laboratories. The method for the detection of single cell antibody production by human tonsillar lymphocytes after stimulation with either sheep red blood cells (SRBCs) or polyclonal B-lymphocyte activators was first described in 1976 (Fauci and Pratt, 1976). The culture system was a modified Mishell–Dutton technique with certain critical factors identified. Authors described the assay as a sensitive and reproducible hemolysis-in-gel system, they also identified critical factors essential for optimal responses, i.e., the lots of human AB serum supplements must be extensively absorbed with SRBC to remove a blocking factor present in most human serum, which suppresses the B-lymphocyte response to SRBC targets after stimulation with either SRBC or several polyclonal B-lymphocyte activators. Due to the high predictivity of human immunotoxicants provided by the *in vivo* antibody induction assay, despite all problems mentioned above, the development of this *in vitro* system is highly desirable. Recently, Koeper and Vohr (2009) reported that using a modification of the Mishell–Dutton assay with female NMRI mice splenocytes, all other six immunosuppressive compounds tested (with the exception of cyclophosphamide) and all four non-immunotoxic compounds were correctly identified. Further exploitations of this model are, therefore, recommended.

In alternative, effort should be devoted to the optimization of the *in vitro* T-lymphocyte priming assay. Methods to prime human $CD4^+$ T-lymphocytes *in vitro* would be indeed of significant value for the preclinical evaluation of xenobiotics, including drugs, vaccine candidates and other immunotherapeutics.

The activation of specific immune responses involves the proliferation of lymphocytes. For T-lymphocytes, the stimulatory agent can be a combination of anti-CD3 and anti-CD28 or mitogens such concanavalin A (ConA) and phytohemagglutinin (PHA). For B-lymphocytes, it can be a combination of anti-immunoglobulin and cytokine.

The mitogen-stimulated proliferative response that is widely used in immunotoxicology and in clinical immunology is an *in vitro* correlate of activation and proliferation of lymphocytes specifically sensitized by antigen *in vivo*. *In vitro* stimulation of T-lymphocyte proliferation is an easy assay and, as a stand-alone assay, its predictivity of immunotoxicity is 67% ($P < 0.0003$, Luster et al., 1992). Furthermore, *in vitro* lymphocyte stimulation or transformation can also be performed using the whole blood assay. *In vitro* antigen-specific and mitogen non-specific activation of lymphocytes results in myriad biochemical events, including calcium influx, protein kinase C activation, and phospholipid synthesis, culminating in DNA synthesis and cell division (Bauer and Baier, 2002; Flavell et al., 2002). Thus, xenobiotics interfering with signal transduction pathways are likely to alter mitogen-induced lymphocyte proliferation, making this assay a possible candidate for an *in vitro* test to identify direct immunotoxicants. The lymphocyte proliferation assay, as described below, is currently in a pre-validation phase. The most obvious disadvantage of the lymphocyte proliferation assay is the requirement of [^{3}H]-thymidine. Efforts, therefore, should also be devoted to find alternative readouts for cell proliferation.

Measurement of cytokine production

The activation of any immune response is dependent upon the production and release of cytokines. Cytokines are small molecular weight proteins secreted by many cell types, including immune cells, that regulate the duration and intensity of the immune response. For example, Type 1 cytokines (i.e., interferon [IFN-γ], interleukin [IL-12]) mediate the removal of malignant cells and virally-infected cells, whereas Type 2 cytokines (i.e., IL-4, IL-5, IL-13) mediate the removal of soluble bacterial antigens. Clearly, cytokines play key roles in all immune responses and molecular immunotoxicology has indeed focused on analyses of cytokine levels. Cytokines offer important new avenues to explore, both in terms of mechanistic understanding of immunotoxicity and in terms of developing new assays, the immunotoxic potential of novel compounds.

Cytokines are released as one of the first steps of immune response and quantitative alterations can be used as a measure of immunomodulation. Due to the highly pleiotropic and redundant nature of cytokines, in which a single function may be affected by multiple cytokines simultaneously, it is advisable to include the broadest panel of cytokines possible in any *in vitro* system using such endpoint. A plethora of assay systems are available for measuring cytokines and their receptors such as ELISA, flow cytometry, and molecular biology techniques such as polymerase chain reaction (House, 2001; Corsini and House, 2010). Cytokine production measurements offer outstanding promise and may eventually substitute for other more laborious procedures. The particular profile of cytokine production may also provide important information regarding the nature of any immunotoxic responses. A new *in vitro* system, named "fluorescent cell chip," based

on a number of cell lines derived from T-lymphocytes, mast cells, monocytes, each transfected with various cytokine reporter cell constructs for measuring cytokine expression has been developed (Ringerike et al., 2005). Although further refinement of this system by the expansion with other cell type and cytokines is required, this assay holds promises for *in vitro* screening of chemicals for their immunotoxicity. Cytokine production together with lymphocyte proliferation are currently in a pre-validation phase.

The experience of ECVAM expert group to assess in vitro immunosuppression

As a follow-up to the ECVAM workshop, two consecutive interlaboratory studies involving five European laboratories were performed to evaluate *in vitro* models to assess immunosuppressive potential of compounds (i.e., drugs and chemicals). The first study (Carfi et al., 2007) compared the effects of a number of compounds, e.g., urethane, furosemide, tributyltin chloride (TBTC), verapamil, cyclosporin A, and benzo(a)pyrene on the clonogenic test, mitogen- and/or anti-CD3/CD28-induced proliferative and cytokine responses of rat- or mouse-derived spleen cells and of human peripheral blood lymphocytes. Whereas it was clear that the immune system comprises different mechanisms based upon different cell types, these tests indicate the functionality of lymphocytes as a critical factor for the proper function of the immune system. All the tests confirmed the strong immunotoxic effect of TBTC as well as they confirmed the negative controls. For one chemical, verapamil, the IC_{50} was similar through the different tests. The IC_{50} values obtained with the other chemicals depend on the endpoints and on the animal species. The clonogenic test (CFU-GM) and the mitogen responsiveness showed similar IC_{50} values between human and rodent cells, except for cyclosporin and TBTC. Ranking them in term of potency, TBTC was the most potent followed by cyclosporin A, benzo[α]pyrene, verapamil, and furosemide, that was positive only in the human IFN-γ release.

This study provided a selection of methods to detect lymphocyte stimulation that was then used to test a new selection of compounds in a follow-up pre-validation study. The EVCAM-sponsored project was coordinated by Prof. Henk van Loveren (RIVM, Bilthoven, the Netherlands). Human PBL, murine, or rat splenocytes were used. For each of the three species, basal cytotoxicity was assessed before starting the tests, using the lactate dehydrogenase-release assay. Human PBL were stimulated with anti-CD3/anti-CD28; murine splenocytes with anti-CD3/anti-CD28, ConA, lipopolysaccharide (LPS), and anti-CD40/IL-4; rat splenocytes with ConA and LPS. Cell proliferation and production of IFN-γ and tumor necrosis factor-α (TNF-α) were taken as endpoints. Seven compounds were selected: five clear immunosuppressants, e.g., dexametha-sone, benzo[α] pyrene, rapamycin, cyclophosphamide, methotrexate, 1 weak immunosuppressant (urethane), and one non-immunosuppressant (D-mannitol) were evaluated. Five out of the six positive compounds could be identified as immunosuppressive, whereas the non-immunotoxic compound came out as negative. Urethane was identified as immunosuppressive in only 7% of the experiments; this is within the range of positive classification that can occur by chance, and it was, therefore, classified as negative. Despite the high human donor variation the results of the two studies, the human T-lymphocyte activation assay identified all compounds correctly and is therefore considered to be a very promising assay to predict a compound's immunosuppressive potential. Based on the promising results of these two studies, our laboratories are currently validating this human T-lymphocyte activation test with a balanced set of immunosuppressive and non-immunosuppressive compounds. The central aim of this project is to validate the human lymphocyte activation assay. At the end of the study, the predictive value of this approach will then be evaluated. Currently, the number of chemicals so far tested is too limited to establish the predictive value.

The whole blood assay

The human whole blood cell culture, introduced more then 20 years ago, may also be useful in studying the biological effects of potential allergenic and/or antigenic substances or drugs based on immune cell activation and cytokine secretion. Various clinical uses of whole blood stimulation assays have been suggested, including the assessment of autoimmune diseases, the monitoring of drug and vaccine efficacy, and immunotoxicity (Langezaal et al., 2001; Thurm and Halsey, 2005).

Whole blood assays can be a very useful test due to the easy access of samples from healthy donors and the minimal processing of the sample required. Because the assay mimics the natural environment, whole blood culture may be the best milieu in which to study cell activation and cytokine production *in vitro*. Both plant lectins (e.g., PHA, ConA, pokeweed mitogen, etc.) as well as LPS, purified protein derivative of tuberculin (PPD), anti-CD3 and/or anti-CD28 antibodies, etc. can be used to stimulate T- or B-lymphocyte proliferation in whole blood. Stimulation for 24 hr with LPS leads to the release of IL-1β, IL-6, IL-8, and TNF-α; by prolonging the incubation period from 48 to 72 hr, the whole blood model can be extended to determine also the release of various other cytokines (Hermann et al., 2003), such as IL-2, IL-4, IL-13, and IFN-γ. The potential comparisons to be made between expressions of T-helper lymphocyte type 1 (T_H1) vs. T_H2 cytokines (i.e., IFN-γ vs. IL-4) will be invaluable in ultimately determining if a test agent can induce cytokine dysregulation.

Langezaal et al. (2002) have adapted the whole blood assay for immunotoxicity testing, to permit the potency testing of immunostimulants and immunosuppressants. These Authors proposed the use of LPS-induced IL-1β and staphylococcal enterotoxin B-induced IL-4 release to test the immunotoxic potential of chemicals. This *in vitro* method is capable of determining immunosuppression and immunostimulation, favoring IL-1β release for stimulation and IL-4 release for suppression of cytokines. Thirty-one pharmaceutical compounds, with known effects on the immune system,

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have been used to optimize and standardize the method, by analyzing their effects on cytokine release. The *in vitro* results were expressed as IC_{50} values for immunosuppression, and SC(4) (4-fold increase) values for immunostimulation. The *in vitro* results correlated well with the *in vivo* data, so the test appears to reflect immunomodulation. A sensitivity of 67% and a specificity of 100% for the combined endpoints in the test were calculated. Results were reproducible, and the method could be transferred to another laboratory, suggesting the potential use of the test in immunotoxicity testing strategies. As a general strategy, the Langezaal group also proposed that when unknown compounds are tested for their potency to modulate the immune response, conclusions might be drawn from compounds that are showing clear immunosuppression or immunostimulation. Compounds found to be non-immunotoxic will have to be tested for metabolic activation and for effects on additional endpoints such as antibody formation, lymphocyte proliferation, and sensitization, before it can be concluded whether the compound is immunotoxic or not.

The human whole blood assay is also suitable for intracellular cytokine staining, which may be useful to detect low frequency antigen-specific cell responses with respect to clinical significance in assessing immune status in a variety of clinical conditions and determining efficacy or immunotoxicity of drugs and vaccine antigens (Maino and Picker, 1998). Intracellular staining allows one to identify antigen-specific T- or B-lymphocytes at the single cell level with high sensitivity, providing new insights into antigen-specific immune responses of extremely low frequency (events). Overall, these results suggest that *in vitro* assays are able to detect immunosuppression, holding promise for testing these assays using a wide range of chemicals.

In vitro assessment of contact sensitizers

Skin sensitization potential is an endpoint that needs to be assessed within the framework of existing and forthcoming legislation. Allergic contact dermatitis (ACD) is to a considerable extent a preventable disease. A reduction of ACD can be achieved by: correct detection of skin sensitizers; characterization of potency; understanding of human skin exposure; and, the application of adequate risk assessment and management strategies. A range of *in vivo* methods exist that have been proven to be very accurate in terms of the predictive identification of chemicals that possess skin sensitizing properties, i.e., the LLNA. However, the challenge now to be faced is how to obtain the same quality of information on the potency of skin sensitizing chemicals using *in silico* and *in vitro* methods. With the forthcoming elimination of *in vivo* tests, the opportunities being exploited for *in vitro* test development focus on key elements of the sensitization process, such as peptide binding and dendritic cell (DC) activation (Basketter and Maxwell, 2007). In Table 3, the key passages of contact sensitization and *in vitro* opportunities are described.

In silico methods and the peptide-binding assay

One unifying characteristic of chemical allergens is the requirement that they react with proteins for the effective induction of skin sensitization. The majority of chemical allergens are electrophilic and react with nucleophilic amino acids. One potential alternative approach to skin sensitization hazard identification is the use of (Quantitative) structure-activity relationships ((Q)SARs) coupled with appropriate documentation and performance characteristics. This represents a major challenge. Current thinking is that (Q)SARs might best be employed as part of a battery of approaches that collectively provide information on skin sensitization hazard. A number of (Q)SARs and expert systems have been developed and are described in the literature, i.e., TOPKAT, Derek for Windows and TOPS-MODE, none performed appears to perform sufficiently well to act as a stand-alone tool for hazard identification.

More recently, based on the requirement of chemical reactivity in the induction of skin sensitization, 82 chemicals comprising allergens of different potencies and non-allergenic chemicals were evaluated for their ability to react with reduced glutathione (GSH) or with two synthetic peptides containing either a single cysteine or lysine (peptide-binding test). Following a 15-min reaction time with GSH or a 24-hr reaction time with the two synthetic peptides, the samples were analyzed by high-performance liquid chromatography. The peptide reactivity data were compared with existing LLNA data. Generally, non-allergens and weak allergens demonstrated minimal to low peptide reactivity, whereas moderate to extremely potent allergens displayed moderate to high peptide reactivity. Classifying minimal reactivity as non-sensitizers and low, moderate, and high reactivity as sensitizers, it was determined that a model based on cysteine and

Table 3. Key passages in chemical(s)-induced skin sensitization and *in vitro* opportunities.

Key passage	*In vitro* opportunities	References
Skin absorption	Reconstituted skin epidermis	Howes et al., 1996; Basketter et al., 2007
Binding to macromolecules (i.e., proteins)	QSAR Peptide-binding assay	Netzeva et al., 2005; Patlewicz et al., 2007; Basketter, 2007.Gerberick et al., 2007b, 2008
Antigen processing	DC-like upregulation of class II antigens	Reviewed in Casati et al., 2005.
Langerhans cell maturation and migration	DC-like upregulation of co-stimulatory molecules KC production of specific cytokines required for LC-maturation and migration	Reviewed in Casati et al., 2005.Corsini et al., 1998, 1999; van de Sandt et al., 2002; van Och et al., 2005
Antigen presentation to T_H cells and memory T-cell generation	*In vitro* T-cell activation	Grabbe and Schwarz, 1996; Dai and Streilein, 1998; Rustemeyer et al., 1999; Gorbachev and Fairchild, 2001

lysine gave a prediction accuracy of 89%. The results of these investigations revealed that measurement of peptide reactivity has considerable potential utility as a screening approach for skin sensitization testing, and thereby for reducing reliance on animal-based test methods (Gerberick et al., 2007b, 2008). The peptide-binding assay is currently under formal pre-validation at ECVAM.

Use of KC to identify contact allergens

Besides its barrier function, the skin has been recognised as an immunologically active tissue. Keratinocytes (KC) may convert non-specific exogenous stimuli into the production of cytokines, adhesion molecules and chemotactic factors (Barker et al., 1991). After KC, Langerhans cells (LC) comprise the second most prominent cell type in the skin (2–5% of the epidermal population). These are the principal antigen-presenting cells (APC) in the skin (Katz et al., 1979). Due to their anatomical location and their significant role in the development of ACD, the use of both of these cell types to evaluate sensitizing potency *in vitro* is amply justifiable.

In principle, a test system comprised of KC alone may not be useful in establishing allergenic potency as these cells lack antigen-presenting capacity. However, in addition to chemical processing LC activation requires the binding of cytokines produced by KC as a result of initial chemical exposure. The irritant capacity of allergens might present an additional risk factor so that irritant allergens may be stronger allergens than non-irritant ones (Grabbe et al., 1996). In that case, the potency of chemicals to induce cutaneous sensitization may be assessed as a function of KC cytokine expression.

Starting from the *in vivo* observation that in mouse IL-1α expression by KC was selectively increased after *in vivo* application of contact sensitizers but not tolerogen or irritant (Enk and Katz, 1992), similar results were reproduced *in vitro* using the murine KC cell line HEL30 (Corsini et al., 1998). van Och et al. (2005) obtained similar results and, furthermore, the Authors observed that the ranking of potency was similar to the ranking established using the LLNA. Similarly, using human KC it has been demonstrated that allergens but not irritants or tolerogens induced IL-12 (Müller et al., 1994; Corsini et al., 1999). Trinitrobenzene sulphonic acid induced the expression of CD40 on KC, whereas sodium lauryl sulfate did not (Coutant et al., 1999). Taken together, these studies indicate the possibility to identify contact sensitizers using murine or human KC. Among the cytokines produced by KC, IL-18 has been demonstrated to favor T_H1 type immune responses by enhanc-ing the secretion of pro-inflammatory mediators such as TNF-α, IL-8, and IFN-γ, and to play a key proximal role in the induction of ACD. The increase in intracellular IL-18 content was used to discriminate contact allergens from low-molecular-weight respiratory allergens and irritants (van Och et al., 2005). These initial observations were further expanded using the human KC line NCTC 2544. At not cytotoxic concentrations (cell viability > 80% as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay), all contact sensitizers tested induced a dose-dependent increase in IL-18, whereas both irritants and respiratory allergens failed, indicating that cell-associated IL-18 may provide an *in vitro* tool for identification and discrimination of contact vs. respiratory allergens and/or irritants (Corsini et al., 2009).

Recently, the performance of another KC assay (KeratinoSens), based on a luciferase reporter gene under the control of an antioxidant response element of the human AKR1C2 gene stably inserted into HaCaT KC, has been reported (Emter et al., 2010). Results indicate a good predictive value of this assay for hazard identification, with sensitivity levels of > 88%, a specificity of > 79% and an accuracy of > 85%; these outcomes make this assay a good alternative method.

Use of DCs to identify contact allergens

In contrast, DCs form a sentinel network able to detect, capture, and process antigens such as invading bacteria, viruses, products of tissue damage and haptens (Aiba et al., 1997; Häcker et al., 1998; Cella et al., 1999). Upon antigen capture, the DC undergo a maturation process leading to the upregulation of co-stimulatory molecules (CD86, CD80, and CD40), MHC Class II molecules and the CD83 protein (Caux et al., 1994). Thereafter, these cells migrate to the T-lymphocyte regions of lymphoid organs, where they lose antigen-processing activity and become potent immunostimulatory cells. These maturing DC acquire the ability to migrate through expression of chemokines and chemokine receptors and downregulation of molecules such as E-cadherin.

Knowledge of DC physiology has progressed considerably because of the discovery of culture techniques, in the early 1990s, which support the *in vitro* generation of large numbers of DC from hematopoietic progenitors (Caux et al., 1992). Two main protocols to generate DC, from either monocytes or $CD34^+$ hematopoietic cell precursors (HPC), have been described. Generating DC from murine bone marrow $CD34^+$ HPC has been used as an alternative, but this procedure is time-consuming and requires a significant number of animals. The establishment of human *in vitro* models of DC had offered the possibility to demonstrate that haptens were able to directly activate cultured DC derived from peripheral blood monocytes or from $CD34^+$ HPC (Aiba et al., 1997; Degwert et al., 1997; Rougier et al., 2000; De Smedt et al., 2001; Weigt et al., 2004). Several studies confirmed these observations showing the upregulation of maturation markers (CD83, CD80, CD86, CD40) on human DC (Coutant et al., 1999; Aiba et al., 2000; Tuschl et al., 2000; Arrighi et al., 2001). Cytokine production such as IL-12p40, TNF-α, and IL-1β has also been reported upon hapten stimulation (Aiba et al., 1997, 2003; De Smedt et al., 2001). However, significant differences exist between experimental systems and authors concerning cytokine production.

A recent ECVAM workshop has reviewed the state-of-the-art of the use of DC and human myeloid cell lines for the predictive identification of skin sensitization hazard (Casati et al., 2005). At present, only a limited number of cell lines such as THP-1, U937, KG-1, or MUTZ-3 have shown promising results. Among these, THP-1 (a commercially-available

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human monocytic leukemia cell line) has been proposed by Ashikaga et al. (2002) to identify sensitizers. Recently, Yoshida et al. (2003) reported that naive THP-1 could respond to sensitizers, specifically through augmented expression of the co-stimulatory molecules CD54 and CD86, and considered this as a possible tool to be used as an *in vitro* sensitization test [Human Cell Line Activation Test (h-CLAT)]. The sensitivity (%), specificity (%), positive predictivity (%), negative predictivity (%), and accuracy (%) for the combinations of both CD86 and CD54 (RFI for CD54 ≥ 200 and RFI for CD86 ≥ 150) were as follow: sensitivity 90.5%, specificity 100%, positive predictivity 100%, negative predictivity 80%, and accuracy 93.1% (Sakaguchi et al., 2009).

Among the several endpoints investigated in different experimental models, CD86, IL-8, and p38 MAP kinase appear to be the most promising and robust biomarkers described to date in DC-based assays. Therefore, it is possible that the combined analysis of these or other biomarkers rather than analysis of a single biomarker will give even more satisfactory results. It is anticipated that the combination of different *in vitro* assays will increase the accuracy, i.e., in the KeratinoSens assay, the inclusion of the peptide reactivity data will increase accuracy from 85.1% to 89.6% (Emter et al., 2010); in the h-CLAT system, CD86 ≥ 150 alone has an accuracy of 75.9%, in combination with CD54 ≥ 200, this value increases to 93.1% (Sakaguchi et al., 2009).

Only limited studies address the potency of the sensitizer i.e., whether it is an extreme, strong, moderate, or weak sensitizer (Azam et al., 2006; Mitjans et al., 2008; Nukada et al., 2008). In these studies, not all chemicals tested gave results that were in accordance with LLNA data, indicating that DC-based assays might not be a sufficient stand-alone assay to determine sensitizer potency. In our work (Mitjans et al., 2008), the calculation of concentration of allergen that induced a release of IL-8 of 100 pg/mL by linear regression analysis of data gave the following ranking order: DNCB 0.9 μg/mL, PPD 2.4 μg/mL, TMTD 3.4 μg/mL, HClPt 6.8 μg/mL, cinnamaldehyde 6.9 μg/mL, Penicillin G 13.4 μg/mL, and $NiSO_4$ 22.6 μg/mL. If compared with the available *in vivo* LLNA EC_3 values (DNCB 0.05%, TMTD 5.2%, cinnamaldehyde 3.0%, penicillin G 13.4%, and PPD 0.16%), a significant correlation ($R = 0.924$, $P = 0.0248$) was obtained. There is a general trend for IL-8 release at lower concentration for strong sensitizers, whereas higher concentrations are needed in the case of weak sensitizers. However, investigations are still needed to better address the question of potency.

At present, however, no single cell-based assay nor single marker is yet able to distinguish all sensitizers from non-sensitizers in a test panel of chemicals, nor is it possible to rank the sensitizing potential of the test chemicals. However, many promising methods are in various stages of development and use. It is expected that a predictive method to totally replace animal testing will be a test battery composed of molecular, cell-based, and/or computational methods. At this regards, it is important to mention the 5-years project named SENS-IT-IV, (coordinated by Dr Erwin L. Roggen, Novozyme, Bagsvaerd, Denmark) recently sponsored by the EU (2005–2009). The SENS-IT-IV Consortium comprises 28 research groups overall, of which seven are from industry, 16 groups are universities or research institutes, and four represent organizations. The project is aimed to develop "*in vitro*" alternatives to animal tests currently used for the risk assessment of potential skin or lung sensitizers.

Finally, in the area of skin sensitization, it should also be mentioned that three *in vitro* assays just entered into a formal ECVAM pre-validation activity. These are the:

- h-CLAT, which uses the THP-1 cell line;
- Myeloid U937/CD86 Skin Sensitization Test (MUSST); and, the
- Direct Peptide Reactivity assay.

The first two (h-CLAT and MUSST) assays aim to predict DC activation events, while the third (Direct Peptide Reactivity assay) seeks to model the modification of skin proteins by chemical binding. The next challenge will be to analyze how data from these three *in vitro* approaches, also in combination with other *in vitro* or *in silico* methods can be integrated in safety risk assessment decisions.

Since induction of skin sensitization is a threshold phenomenon, the use of the principles of Quantitative Risk Assessment are as applicable to skin sensitization as any other threshold-based toxicity endpoint. These *in vitro* assays have been developed to identify the hazard of a chemical to induce sensitization, mainly for labeling classification (R43). They were not designed for risk assessment. It is, however, important to note that they may also be used for potency classification. They may be, therefore, useful to establish doses below which the risk for induction of sensitization is unlikely to occur. It is desirable in the future to be able to move focus from hazard identification toward risk assessment—enabling better health protection. A first indication of potency may come, for example, from the concentration required to induce a threshold of positive response (CD86 ≥ 150) in the h-CLAT system. A good correlation ($R = 0.839$, $P < 0.01$) was indeed found between the h-CLAT thresholds and LLNA EC_3 values (Sakaguchi et al., 2009). The quantitative dose-response data should be then integrated into a testing strategy along with the peptide reactivity data, bioavailability data, and some informed rating of structural alerts in order to establish an acceptable exposure level.

In vitro assessment of respiratory sensitizers

There are currently no validated or widely accepted methods/models for the identification and characterization of chemicals that have the potential to induce allergic sensitization of the respiratory tract. Research indicates that respiratory sensitizers may be identified through contact sensitization assays such as the LLNA, as all low-molecular-weight respiratory sensitizers tested so far were also positive in this assay (Arts et al., 2008; Boverhof et al., 2008). The unique defining characteristic of chemical respiratory

allergens, which in most instances distinguishes them from contact allergens, is the ability to provoke the preferential development of T_H2-type immune responses (Kimber and Dearman, 2005). Discrimination between contact and respiratory sensitizers can be eventually achieved *in vivo* by the assessment of cytokine profiles (Dearman et al., 2002; de Jong et al., 2009).

Due to the increasing health concerns associated with occupational asthma and the impending directives on the regulation of respiratory sensitizers and allergens, an approach which can identify these compounds and distinguish them from contact sensitizers is required. New tests should be based on mechanistic understanding and should be preferentially restricted to *in vitro* assays. Various methods have been used to develop (Q)SARs models for prediction of low-molecular-weight organic chemical respiratory sensitizers (Seed et al., 2008). Such methods, however, still require further corroboration from animal or human data. Some of the *in vitro* DC-based tests, previously described, have also been tested and found positive for respiratory sensitizers (Mitjans et al., 2008). The selective upregulation of intracellular IL-18 by contact allergens in KC may also represent a method to discriminate contact allergens from respiratory sensitizers. Hence, a possible strategy for identifying chemical respiratory sensitizers may incorporate one such DC-based model based assay to detect sensitizing activity, followed by the KC-based test to exclude respiratory sensitization potential (Corsini et al., 2009).

Another model relies on using precision-cut lung slices (Wohlsen et al., 2003; Henjakovic et al., 2008) as a potential means of identifying respiratory allergens, based according to the cytokine production profile seen. The production of IL-2 and IL-5 may provide the possibility to identify respiratory allergens. Finally, Verstraelen et al. (2009) have investigated the genetic response, evaluated using the Agilent Whole Human Genome array, of human THP-1 in response to contact ($n=1$) and respiratory sensitizers ($n=3$), to identify genes that are able to discriminate between both groups. Among the 20 most discriminating genes which were categorized into molecular and biological Gene Ontology terms, EIF4E, PDGFRB, SEMA7A, and ZFP36L2 could be associated with respiratory sensitization (Verstraelen et al., 2009).

In conclusion, a number of *in vitro* and *in silico* models show promise and should be further explored for their ability to identify and differentiate contact and respiratory sensitizers.

Immunogenicity of biologicals

Biological/biotechnology-derived proteins are increasingly used as therapeutic agents. The characterization of the immunogenicity of therapeutic proteins is of vital importance in the development and marketing of biotech drugs. Failure to predict and monitor levels of immunogenicity can have serious results for patients and for companies. The consequences of such immune reactions to a therapeutic protein range from transient appearance of antibodies without any clinical significance to severe life-threatening conditions. The predictive value of animal models for evaluation of immunogenicity is low due to inevitable immunogenicity of human proteins in animals. It is, therefore, essential to adopt an appropriate strategy for the development of adequate screening and confirmatory assays to measure an immune response against a therapeutic protein. In April 2008, the EMEA adopted a new guidance on immunogenicity assessment of biologicals.

Many factors may influence the immunogenicity of therapeutic proteins, including patient-related factors that might predispose to an immune response, i.e., underlying disease, genetic background, immune status, and product-related factors, e.g., intensity of treatment (route of administration, source of protein), manufacturing process (impurity profile, contaminants), the formulation and stability characteristics (degradation products, aggregates) of a given protein and dose, dosing interval, and duration of treatment. It is important also to stress that, as stated in the ICH S6 guideline, immunogenicity of a biologic product in an animal model is not predictive of immunogenicity in humans.

It has been recognized that biologicals may induce both humoral and cellular immune responses. It will be, therefore, desirable to develop and standardize *in vitro* methods able to evaluate primary activation of specific immune T-lymphocytes (Grabbe and Schwarz, 1996; Gorbachev and Fairchild, 2001). A serious obstacle is of course the low frequency of allergen-specific T-lymphocytes (Langenkamp et al., 2002). An effective system could be to expand the absolute number of antigen-specific T-lymphocytes from the naïve T-lymphocyte pool of peripheral blood samples. The system has successfully applied to determine T-lymphocyte frequencies for several allergens in human blood samples and for activation of naive hapten-specific T-lymphocytes to study the earliest T-lymphocyte and APC events in the induction of human contact hypersensitivity (Dai and Streilein, 1998; Rustemeyer et al., 1999).

Recently, a fully-human modular immune *in vitro* construct (MIMIC™) was developed to serve as a preliminary screen for efficacy testing of potential vaccine formulations. This autologous method utilizes antigen-triggered monocyte-derived DC to generate immune constructs that can be tailored to generate various cell-mediated and humoral responses depend-ing on culture composition. Consistent results were obtained only for the induction of memory B-lymphocyte responses, reflecting a failure of the system—to a large extent—to induce primary responses (Byers et al., 2009). Indeed, the MIMIC™ system did not generate a measurable tetanus toxoid-specific IgG response following *in vitro* stimulation in 6 out of 13 volunteers. Thus, because it was evident that no primary immune response could be obtained from most donors, the value of this method for screening new vaccines or biologicals remains, as yet, unclear (Byers et al., 2009; Moser et al., 2010).

Further research is clearly needed, but the development of such models will be extremely relevant to confirm *in vitro* the immunogenicity of new chemicals and drugs, including

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biologicals. At present, no such assays described here have been validated for immunotoxicity assessment. The characterization of the immunogenicity of biologicals represents an intensive area of research and it is desirable that the applicability of each test is described and fully exploited.

Limitation and perspectives of *in vitro* methods

Any dysregulation of immune cell homeostasis can result in severe adverse changes in immune functions, increasing the susceptibility to infections and cancer, as well as favoring the development of autoimmune diseases.

Even if no validated alternative *in vitro* test to assess immunotoxicity exist, *in vitro* methods to assess unintended immunosuppression and contact hypersensitivity are available, and for these reasons such methods can be, at least, used for the pre-screening and hazard identification of direct immunotoxicants.

There are several limitations associated with the use of *in vitro* methods that be considered. Materials that require biotransformation would require special culture systems (e.g., culture in the presence of S9). An additional limitation of *in vitro* methods would be the physiochemical characteristics of the test material, which may interfere with the *in vitro* system. Such characteristics may include the need for serum, effects of vehicle on cells (such as DMSO), and chemical binding to cells.

Furthermore, considering the complexity of the immune system, *in vitro* exposure is most straightforward for direct immunotoxicants. Indeed important limitation, particularly relevant for the immune system, is that *in vitro* systems do not take into account the interactions of the different components and it is difficult to reproduce *in vitro* the integrity of the immune system. As *in vitro* systems do not account for potential neuroimmunoendocrine interactions. Therefore, an assessment of *in vitro* immunotoxicity will be valuable only in the cases of a direct immunotoxicant. It is hard to estimate how many immunotoxicants will act directly. It is tempting to speculate that, due to the exquisite sensitivity of the immune system, many of the immunotoxicants will act directly on immune cells. Based on available lists of immunotoxic compounds (i.e., NIEHS or OECD databases), an extensive review of the literature data would be needed to proper answer this question.

Methods such the whole blood assay, the lymphocyte proliferation and cytokine production can be used for the hazard identification of immunosuppressive potential of chemicals, whereas several *in vitro* methods are already available to identify allergens and, possibly, to discriminate contact form respiratory allergens.

In vitro testing has several advantages over *in vivo* testing, such as detailed mechanistic understanding, species extrapolation, reduction, refinement, and replacement of animal experiments. As mentioned above, several *in vitro* assays for lymphotoxicity exist, each comprising specific functions of the immune system (i.e., B- and T-lymphocyte proliferation, cytokine production, NK cell function, and DC function, etc.). Among these, only the human whole blood cytokine release assay, has undergone formal pre-validation, and another one, the lymphocyte proliferation and cytokine production assay, is progressing toward that phase, whereas two DCs maturation assays, namely the h-CLAT and the MUSST, and the direct peptide-binding assay to assess contact allergens are currently under formal pre-validation at ECVAM.

Despite all these efforts, there is, however, still a clear need for continued investment in the development of methods and approaches that will allow the correct identification *in vitro* of potential immunotoxic compounds, including immunogenicity and autoimmunity. Intensive international and interlaboratory cooperation and coordination will be necessary to reach this goal.

Declaration of interest

The Authors declare of not having any financial, personal, or association with any of individuals or organizations that have could inappropriately influence the submitted work. The Authors are alone responsible for the content and writing of the paper. The Authors are not involved in any ECVAM-sponsored validation studies. They are, however, involved in a study sponsored by the EU (SENS-IT-IV) and in two studies sponsored by the Dutch government aimed to develop and refine *in vitro* tests to assess the immunotoxic potential, i.e., both immunosuppression and hypersensitivity, of chemicals.

References

Aiba, S., Manome, H., Nakagawa, S., Mollah, Z. U., Mizuashi, M., Ohtani, T., Yoshino, Y. and Tagami, H. 2003. p38 Mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, $NiCl_2$ and 2,4-dinitrochlorobenzene. *J. Invest. Dermatol*. 120:390–399.

Aiba, S., Manome, H., Yoshino, Y. and Tagami, H. 2000. *In vitro* treatment of human transforming growth factor-beta1-treated monocyte-derived dendritic cells with haptens can induce the phenotypic and functional changes similar to epidermal Langerhans cells in the initiation phase of allergic contact sensitivity reaction. *Immunology* 101:68–75.

Aiba, S., Terunuma, A., Manome, H. and Tagami, H. 1997. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol*. 27:3031–3038.

Arrighi, J. F., Rebsamen, M., Rousset, F., Kindler, V. and Hauser, C. 2001. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. *J. Immunol*. 166:3837–3845.

Arts, J. H., de Jong, W. H., van Triel, J. J., Schijf, M. A., de Klerk, A., van Loveren, H. and Kuper, C. F. 2008. The respiratory local lymph node assay as a tool to study respiratory sensitizers. *Toxicol. Sci*. 106:423–434.

Ashikaga, T., Hoya, M., Itagaki, H., Katsumura, Y. and Aiba, S. 2002. Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive endpoints for contact sensitizers. *Toxicol. In Vitro* 16:711–716.

Azam, P., Peiffer, J. L., Chamousset, D., Tissier, M. H., Bonnet, P. A., Vian, L., Fabre, I. and Ourlin, J. C. 2006. The cytokine-dependent MUTZ-3 cell line as an *in vitro* model for the screening of contact sensitizers. *Toxicol. Appl. Pharmacol*. 212:14–23.

Barker, J. N., Mitra, R. S., Griffiths, C. E., Dixit, V. M. and Nickoloff, B. J. 1991. Keratinocytes as initiators of inflammation. *Lancet* 337:211–214.

Basketter, D. A. 2007. An evaluation of selected global (Q)SARs/expert systems for the prediction of skin sensitisation potential. *SAR QSAR Environ. Res*. 18:515–541.

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Basketter, D., Pease, C., Kasting, G., Kimber, I., Casati, S., Cronin, M., Diembeck, W., Gerberick, F., Hadgraft, J., Hartung, T., Marty, J. P., Nikolaidis, E., Patlewicz, G., Roberts, D., Roggen, E., Rovida, C. and van de Sandt, J. 2007. Skin sensitisation and epidermal disposition: The relevance of epidermal disposition for sensitisation hazard identification and risk assessment. The report and recommendations of ECVAM workshop 59. *Altern. Lab. Anim*. 35:137–154.

Basketter, D. and Maxwell, G. 2007. *In vitro* approaches to the identification and characterization of skin sensitizers. *Cutan. Ocul. Toxicol*. 26:359–373.

Bauer, B. and Baier, G. 2002. Protein kinase C and AKT/protein kinase B in $CD4^+$ T-lymphocytes: New partners in TCR/CD28 signal integration. *Mol. Immunol*. 38:1087–1099.

Bini, E. J. and Weinshel, E. H. 1999. Severe exacerbation of asthma: A new side effect of interferon-alpha in patients with asthma and chronic hepatitis C. *Mayo Clin. Proc*. 74:367–370.

Boverhof, D. R., Billington, R., Gollapudi, B. B., Hotchkiss, J. A., Krieger, S. M., Poole, A., Wiescinski, C. M. and Woolhiser, M. R. 2008. Respiratory sensitization and allergy: Current research approaches and needs. *Toxicol. Appl. Pharmacol*. 226:1–13.

Burns, L. A., White, K. L. Jr, McCay, J. A., Fuchs, B. A., Stern, M., Brown, R. D., Musgrove, D. L., Holsapple, M. P., Luster, M. I. and Bradley, S. G. 1994. Immunotoxicity of mono-nitrotoluenes in female $B_6C_3F_1$ mice: II. Meta-nitrotoluene. *Drug Chem. Toxicol*. 17:359–399.

Byers, A. M., Tapia, T. M., Sassano, E. R. and Wittman, V. 2009. *In vitro* antibody response to tetanus in the MIMIC system is a representative measure of vaccine immunogenicity. *Biologicals* 37:148–151.

Carfi', M., Gennari, A., Malerba, I., Corsini, E., Pallardy, M., Pieters, R., Van Loveren, H., Vohr, H. W., Hartung, T. and Gribaldo, L. 2007. *In vitro* tests to evaluate immunotoxicity: A preliminary study. *Toxicology* 229:11–22.

Casati, S., Aeby, P., Basketter, D. A., Cavani, A., Gennari, A., Gerberick, G. F., Griem, P., Hartung, T., Kimber, I., Lepoittevin, J. P., Meade, B. J., Pallardy, M., Rougier, N., Rousset, F., Rubinstenn, G., Sallusto, F., Verheyen, G. R. and Zuang, V.; Report and Recommendations of ECVAM Workshop 51. 2005. Dendritic cells as a tool for the predictive identification of skin sensitisation hazard. *Altern. Lab. Anim*. 33:47–62.

Caux, C., Dezutter-Dambuyant, C., Schmitt, D. and Banchereau, J. 1992. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* 360:258–261.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I. and Banchereau, J. 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med*. 180:1263–1272.

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I. and Lanzavecchia, A. 1999. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J. Exp. Med*. 189:821–829.

Condevaux, F., Guichard, J., Forichon, A., Aujoulat, M. and Descotes, J. 2001. Compared effects of morphine and nickel chloride on NK cell activity *in vitro* in rats and monkeys. *J. Appl. Toxicol*. 21:431–434.

Corsini, E., Limiroli, E., Marinovich, M., Cohen, C., Roguet, R. and Galli, C. L. 1999. Selective induction of IL-12 by chemical allergens in reconstituted human epidermis. *Altern. Lab. Anim*. 27:261–269.

Corsini, E., Mitjans, M., Galbiati, V., Lucchi, L., Galli, C. L. and Marinovich, M. 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicol. In Vitro* 23:789–796.

Corsini, E., Primavera, A., Marinovich, M. and Galli, C. L. 1998. Selective induction of cell-associated interleukin-1alpha in murine keratinocytes by chemical allergens. *Toxicology* 129:193–200.

Corsini, E. and House, R. V. 2010. Evaluating cytokines in immunotoxicity testing. *Methods Mol. Biol*. 598:283–302.

Coutant, K.D., de Fraissinette, A.B., Cordier, A., Ulrich, P. 1999. Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. *Toxicol Sci*. 52:189–98.

Dai, R. and Streilein, J. W. 1998. Naïve, hapten-specific human T-lymphocytes are primed *in vitro* with derivatized blood mononuclear cells. *J. Invest. Dermatol*. 110:29–33.

de Jong, W. H., Arts, J. H., De Klerk, A., Schijf, M. A., Ezendam, J., Kuper, C. F. and Van Loveren, H. 2009. Contact and respiratory sensitizers can be identified by cytokine profiles following inhalation exposure. *Toxicology* 261:103–111.

De Smedt, A. C., Van Den Heuvel, R. L., Zwi Berneman, N. and Schoeters, G. E. 2001. Modulation of phenotype, cytokine production and stimulatory function of $CD34^+$-derived DC by NiCl(2) and SDS. *Toxicol. In Vitro* 15:319–325.

Dearman, R. J., Warbrick, E. V., Skinner, R. and Kimber, I. 2002. Cytokine fingerprinting of chemical allergens: Species comparisons and statistical analyses. *Food Chem. Toxicol*. 40:1881–1892.

Degwert, J. S., Hoppe, U., and Kligman, L. H. 1997. *In vitro* model for contact sensitization: I. Stimulatory capacities of human blood-derived dendritic cells and their phenotypical alterations in the presence of contact sensitizers. *Toxicol. In Vitro* 11:613–618.

Demoly, P. and Bousquet, J. 2001. Epidemiology of drug allergy. *Curr. Opin. Allergy Clin. Immunol*. 1:305–310.

ECETOC (European Center for Ecotoxicology and Toxicology of Chemicals). 2005. *Workshop on Chemical Pollution, Respiratory Allergy, and Asthma*. Workshop report No. 6.

Emter, R., Ellis, G. and Natsch, A. 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. *Toxicol. Appl. Pharmacol*. 245:281–290.

Enk, A. H. and Katz, S. I. 1992. Early molecular events in the induction phase of contact sensitivity. *Proc. Natl. Acad. Sci. U.S.A*. 89:1398–1402.

Esser, C. and Jux, B. 2009. Small chemicals, bioactivation, and the immune system–a fragile balance of i-tox and benefits? *Chem. Biodivers*. 6:2138–2143.

Fauci, A. S. and Pratt, K. R. 1976. Activation of human B-lymphocytes. I. Direct plaque-forming cell assay for the measurement of polyclonal activation and antigenic stimulation of human B lymphocytes. *J. Exp. Med*. 144:674–684.

Flavell, R. A., Dong, C. and Davis, R. J. 2002. Signaling and cell death in lymphocytes. *Inflamm. Res*. 51:80–82.

Fleming, D. M., Sunderland, R., Cross, K. W. and Ross, A. M. 2000. Declining incidence of episodes of asthma: A study of trends in new episodes presenting to general practitioners in the period 1989-98. *Thorax* 55:657–661.

Gennari, A., Ban, M., Braun, A., Casati, S., Corsini, E., Dastych, J., Descotes, J., Hartung, T., Hooghe-Peters, R., House, R., Pallardy, M., Pieters, R., Reid, L., Tryphonas, H., Tschirhart, E., Tuschl, H., Vandebriel, R. and Gribaldo, L. 2005. The Use of *In Vitro* Systems for Evaluating Immunotoxicity: The Report and Recommendations of an ECVAM Workshop. *J. Immunotoxicol*. 2:61–83.

Gerberick, F., Aleksic, M., Basketter, D., Casati, S., Karlberg, A. T., Kern, P., Kimber, I., Lepoittevin, J. P., Natsch, A., Ovigne, J. M., Rovida, C., Sakaguchi, H. and Schultz, T. 2008. Chemical reactivity measurement and the predicitve identification of skin sensitisers. The report and recommendations of ECVAM Workshop 64. *Altern. Lab. Anim*. 36:215–242.

Gerberick, G. F., Ryan, C. A., Dearman, R. J. and Kimber, I. 2007a. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods* 41:54–60.

Gerberick, G. F., Vassallo, J. D., Foertsch, L. M., Price, B. B., Chaney, J. G. and Lepoittevin, J. P. 2007b. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. *Toxicol. Sci*. 97:417–427.

Germolec, D. E. 2004. Sensitivity and predictivity in immunotoxicity testing: Immune endpoints and disease resistance. *Toxicol. Lett*. 149:109–114.

Gorbachev, A. V. and Fairchild, R. L. 2001. Induction and regulation of T-cell priming for contact hypersensitivity. *Crit. Rev. Immunol*. 21:451–472.

Grabbe, S. and Schwarz, T. 1996. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Am. J. Contact Dermatitis* 7:238–246.

Grabbe, S., Steinert, M., Mahnke, K., Schwartz, A., Luger, T. A. and Schwarz, T. 1996. Dissection of antigenic and irritative effects of epicutaneously applied haptens in mice. Evidence that not the antigenic component but non-specific pro-inflammatory effects of haptens determine the concentration-dependent elicitation of allergic contact dermatitis. *J. Clin. Invest*. 98:1158–1164.

Häcker, H., Mischak, H., Miethke, T., Liptay, S., Schmid, R., Sparwasser, T., Heeg, K., Lipford, G. B. and Wagner, H. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J. 17*:6230–6240.

Hébert, P. and Pruett, S. B. 2001. Selective loss of viability of mouse NK cells in culture is associated with decreased NK cell lytic function. *In Vitro Mol. Toxicol*. 14:71–82.

Henjakovic, M., Martin, C., Hoymann, H. G., Sewald, K., Ressmeyer, A. R., Dassow, C., Pohlmann, G., Krug, N., Uhlig, S. and Braun, A. 2008. *Ex vivo* lung function measurements in precision-cut lung slices (PCLS) from chemical allergen-sensitized mice represent a suitable alternative to *in vivo* studies. *Toxicol. Sci*. 106:444–453.

Hermann, C., von Aulock, S., Graf, K. and Hartung, T. 2003. A model of human whole blood lymphokine release for *in vitro* and *ex vivo* use. *J. Immunol. Methods*. 275:69–79.

House, R. V. 2000. An overview of *in vitro/ex vivo* assays for preclinical evaluation of immunomodulation. *Hum. Exp. Toxicol*. 19:246–250.

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House, R. V. 2001. Cytokine measurement techniques for assessing hypersensitivity. *Toxicology* 158:51–58.

Howes, D., Guy, R., Hadgraft, J., Heylings, J.R., Hoeck, U., Kemper, F., Maibach, H., Marty, J.-P., Merk, H., Parra, J., Rekkas, D., Rondelli, I., Shaeffer, H., Tauber, U. and Verbiese, N. 1996. Methods for Assessing Percutaneous Absorption. ECVAM Workshop Report 13. *Altern. Lab. Anim*. 24:81–106.

Katz, S. I., Tamaki, K. and Sachs, D. H. 1979. Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature* 282:324–326.

Kimber, I. and Dearman, R. J. 2005. What makes a chemical a respiratory sensitizer? *Curr. Opin. Allergy Clin. Immunol*. 5:119–124.

Kimber, I. and Weisenberger, C. 1989. A murine local lymph node assay for the identification of contact allergens. Assay development and results of an initial validation study. *Arch. Toxicol*. 63:274–282.

Klein, N. C., Go, C. H. and Cunha, B. A. 2001. Infections associated with steroid use. *Infect. Dis. Clin. North Am*. 15:423–32, viii.

Koeper, L. M. and Vohr, H. W. 2009. Functional assays are mandatory for a correct prediction of immunotoxic properties of compounds *in vitro*. *Food Chem. Toxicol*. 47:110–118.

Langenkamp, A., Casorati, G., Garavaglia, C., Dellabona, P., Lanzavecchia, A. and Sallusto, F. 2002. T cell priming by dendritic cells: Thresholds for proliferation, differentiation and death and intraclonal functional diversification. *Eur. J. Immunol*. 32:2046–2054.

Langezaal, I., Coecke, S. and Hartung, T. 2001. Whole blood cytokine response as a measure of immunotoxicity. *Toxicol. In Vitro* 15:313–318.

Langezaal, I., Hoffmann, S., Hartung, T. and Coecke, S. 2002. Evaluation and prevalidation of an immunotoxicity test based on human whole-blood cytokine release. *Altern. Lab. Anim*. 30:581–595.

Lebrec, H., Roger, R., Blot, C., Burleson, G. R., Bohuon, C. and Pallardy, M. 1995. Immunotoxicological investigation using pharmaceutical drugs. *In vitro* evaluation of immune effects using rodent or human immune cells. *Toxicology* 96:147–156.

Li, Q. 2010. NK cell assays in immunotoxicity testing. *Methods Mol. Biol*. 598:207–219.

Luster, M. I., Portier, C., Pait, D. G., White, K. L. Jr, Gennings, C., Munson, A. E. and Rosenthal, G. J. 1992. Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. *Fundam. Appl. Toxicol*. 18:200–210.

Luster, M. I. and Rosenthal, G. J. 1993. Chemical agents and the immune response. *Environ. Health Perspect*. 100:219–226.

Maino, V. C. and Picker, L. J. 1998. Identification of functional subsets by flow cytometry: Intracellular detection of cytokine expression. *Cytometry* 34:207–215.

Marcusson-Ståhl, M. and Cederbrant, K. 2003. A flow-cytometric NK cytotoxicity assay adapted for use in rat repeated dose toxicity studies. *Toxicology* 193:269–279.

Miossec, P. 1997. Cytokine-induced autoimmune disorders. *Drug Saf*. 17:93–104.

Mishell, R. I. and Dutton, R. W. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med*. 126:423–442.

Mitjans, M., Viviani, B., Lucchi, L., Galli, C. L., Marinovich, M. and Corsini, E. 2008. Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naive THP-1 cells. *Toxicol. In Vitro*. 22:386–395.

Moser, J. M., Sassano, E. R., Leistritz, d. e. l. C., Eatrides, J. M., Phogat, S., Koff, W. and Drake, D. R. 3rd. 2010. Optimization of a dendritic cell-based assay for the *in vitro* priming of naïve human $CD4^+$ T-cells. *J. Immunol. Methods* 353:8–19.

Müller, G., Saloga, J., Germann, T., Bellinghausen, I., Mohamadzadeh, M., Knop, J. and Enk, A. H. 1994. Identification and induction of human keratinocyte-derived IL-12. *J. Clin. Invest*. 94:1799–1805.

Netzeva, T. I., Worth, A., Aldenberg, T., Benigni, R., Cronin, M. T., Gramatica, P., Jaworska, J. S., Kahn, S., Klopman, G., Marchant, C. A., Myatt, G., Nikolova-Jeliazkova, N., Patlewicz, G. Y., Perkins, R., Roberts, D., Schultz, T., Stanton, D. W., van de Sandt, J. J., Tong, W., Veith, G. and Yang, C. 2005. Current status of methods for defining the applicability domain of (quantitative) structure-activity relationships. The report and recommendations of ECVAM Workshop 52. *Altern. Lab. Anim*. 33:155–173.

Nukada, Y., Miyazawa, M., Kosaka, N., Ito, Y., Sakaguchi, H. and Nishiyama, N. 2008. Production of IL-8 in THP-1 cells following contact allergen stimulation via mitogen-activated protein kinase activation or tumor necrosis factor-alpha production. *J. Toxicol. Sci*. 33:175–185.

Pasqualetti, D., Ghirardini, A., Cafolla, A., Biffoni, M., Coluzzi, S., Vaglio, S. and Girelli, G. 2003. Lymphocyte T subsets and natural killer cells in Italian and Philippino blood donors. *Vox Sang*. 84:68–72.

Patlewicz, G., Aptula, A. O., Uriarte, E., Roberts, D. W., Kern, P. S., Gerberick, G. F., Kimber, I., Dearman, R. J., Ryan, C. A. and Basketter, D. A. 2007. An evaluation of selected global (Q)SARs/expert systems for the prediction of skin sensitisation potential. *SAR QSAR Environ. Res*. 18:515–541.

Pessina, A., Albella, B., Bueren, J., Brantom, P., Casati, S., Gribaldo, L., Croera, C., Gagliardi, G., Foti, P., Parchment, R., Parent-Massin, D., Sibiril, Y. and Van Den Heuvel, R. 2001. Prevalidation of a model for predicting acute neutropenia by colony forming unit granulocyte/macrophage (CFU-GM) assay. *Toxicol. In Vitro* 15:729–740.

Pieters, R. 2007. Detection of autoimmunity by pharmaceuticals. *Methods* 41:112–117.

Pieters, R., Ezendam, J., Bleumink, R., Bol, M. and Nierkens, S. 2002. Predictive testing for autoimmunity. *Toxicol. Lett*. 127:83–91.

Ringerike, T., Ullerås, E., Völker, R., Verlaan, B., Eikeset, A., Trzaska, D., Adamczewska, V., Olszewski, M., Walczak-Drzewiecka, A., Arkusz, J., van Loveren, H., Nilsson, G., Lovik, M., Dastych, J. and Vandebriel, R. J. 2005. Detection of immunotoxicity using T-cell based cytokine reporter cell lines ("Cell Chip"). *Toxicology* 206:257–272.

Ross, P., De Swart, R., Addison, R., Van Loveren, H., Vos, J. and Osterhaus, A. 1996. Contaminant-induced immunotoxicity in harbour seals: Wildlife at risk? *Toxicology* 112:157–169.

Rougier, N., Redziniak, G., Mougin, D., Schmitt, D. and Vincent, C. 2000. *In vitro* evaluation of the sensitization potential of weak contact allergens using langerhans-like dendritic cells and autologous T-cells. *Toxicology* 145:73–82.

Rustemeyer, T., De Ligter, S., Von Blomberg, B. M., Frosch, P. J. and Scheper, R. J. 1999. Human T-lymphocyte priming *in vitro* by haptenated autologous dendritic cells. *Clin. Exp. Immunol*. 117:209–216.

Sakaguchi, H., Ashikaga, T., Miyazawa, M., Kosaka, N., Ito, Y., Yoneyama, K., Sono, S., Itagaki, H., Toyoda, H. and Suzuki, H. 2009. The relationship between CD86/CD54 expression and THP-1 cell viability in an *in vitro* skin sensitization test–human cell line activation test (h-CLAT). *Cell Biol. Toxicol*. 25:109–126.

Seed, M. J., Cullinan, P. and Agius, R. M. 2008. Methods for the prediction of low-molecular-weight occupational respiratory sensitizers. *Curr. Opin. Allergy Clin. Immunol*. 8:103–109.

Sia, I. G. and Paya, C. V. 1998. Infectious complications following renal transplantation. *Surg. Clin. North Am*. 78:95–112.

Sly, R. M. 1999. Changing prevalence of allergic rhinitis and asthma. *Ann. Allergy Asthma Immunol*. 82:233–248.

Sleijffers, A., Garssen, J. and Van Loveren, H. 2002. Ultraviolet radiation, resistance to infectious diseases, and vaccination responses. *Methods*. 28:111–121.

Thurm, C. W. and Halsey, J. F. 2005. Measurement of cytokine production using whole blood. *Curr. Protoc. Immunol*. Chapter 7:Unit 7.18B.

Thyssen, J. P. and Menné, T. 2010. Metal allergy–a review on exposures, penetration, genetics, prevalence, and clinical implications. *Chem. Res. Toxicol*. 23:309–318.

Tuschl, H., Kovac, R. and Weber, E. 2000. The expression of surface markers on dendritic cells as indicators for the sensitizing potential of chemicals. *Toxicol. In Vitro* 14:541–549.

van de Sandt, J. J., Roguet, R., Cohen, C., Esdaille, D., Ponec, M., Corsini E., Barker, B., Fusenig, N., Liebsch, M., Benford, D., de Brugerolle de Fraissinette, A., and Fortasch, M. 2002. The use of human keratinocytes and human skin models for predicting skin irritation. The report and recommendations of ECVAM workshop. *Altern. Lab. Anim*. 27:723–743.

van Och, F. M., Van Loveren, H., Van Wolfswinkel, J. C., Machielsen, A. J. and Vandebriel, R. J. 2005. Assessment of potency of allergenic activity of low molecular weight compounds based on IL-1alpha and IL-18 production by a murine and human keratinocyte cell line. *Toxicology* 210:95–109.

van Schayck, C. P. and Smit, H. A. 2005. The prevalence of asthma in children: A reversing trend. *Eur. Respir. J*. 26:647–650.

Verstraelen, S., Nelissen, I., Hooyberghs, J., Witters, H., Schoeters, G., Van Cauwenberge, P. and Van Den Heuvel, R. 2009. Gene profiles of THP-1 macrophages after *in vitro* exposure to respiratory (non-)sensitizing chemicals: identification of discriminating genetic markers and pathway analysis. *Toxicol. In Vitro*. 23:1151–1162.

Vial, T. and Descotes, J. 2003. Immunosuppressive drugs and cancer. *Toxicology* 185:229–240.

Weigt, H., Muhlradt, P. F., Larbig, M., Krug, N. and Braun, A. 2004. The Toll-like receptor-2/6 agonist macrophage-activating lipopeptide-2 cooperates with IFN-gamma to reverse the T_H2 skew in an *in vitro* allergy model. *J. Immunol* 172:6080–6086.

Wohlsen, A., Martin, C., Vollmer, E., Branscheid, D., Magnussen, H., Becker, W. M., Lepp, U. and Uhlig, S. 2003. The early allergic response in small airways of human precision-cut lung slices. *Eur. Respir. J*. 21:1024–1032.

Yoshida, Y., Sakaguchi, H., Ito, Y., Okuda, M. and Suzuki, H. 2003. Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naive THP-1 cell line. *Toxicol. In Vitro*. 17:221–228.