Food for Thought …
Immunotoxicology: Challenges in the 21st Century and *In Vitro* Opportunities

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Summary

Over the last two decades, little has changed in the practice of immunotoxicity testing for regulatory purposes, especially for immunosuppression, and autoimmunity is still a challenge. Current guidelines still rely on animal tests, which include some immune endpoints in repeated dose tests and trigger dedicated tests only when certain alerts indicate a problem. At the same time, however, a wealth of in vitro approaches has been developed, but few have been adopted for routine testing. The extent to which immunotoxicity of chemicals represents a health problem for the human population at low levels of exposure is unclear: it appears that responses of healthy individuals to immunological challenges differ widely and most immunomodulators have few adverse effects except when they coincide with an infectious or malignant challenge or when early in life exposure is expected, in which cases the odds of progressing into infection, autoimmune diseases, or cancer can be changed. The enormous overcapacity of immune defense, the presence of compensatory mechanisms, and their fast restoration each contribute to limiting health threats for the individual, though on a population base also minor immunomodulation may result in increased morbidity. In vitro alternative approaches may allow screening for problematic substances and prioritize them for in vivo testing. New approaches are emerging from mapping pathways of immunotoxicity. Increasingly, the contribution of inflammatory and infectious components to the adverse outcome pathways of chemicals is recognized for various hazards, urging inclusion of tests for proinflammatory and immunomodulatory properties of chemicals into integrated testing strategies.

Keywords: immune system, xenobiotics, toxicity testing, alternative methods, toxicology for the 21st century

Introduction

Do we need yet another review on alternative methods in immunotoxicology? The authors have contributed to many that have already been published (Gennari et al., 2005; Corsini, 2006; Carfì et al., 2007; Corsini and Roggen, 2009; Galbiati et al., 2010; Lankveld et al., 2010; Pfaller et al., 2010). Immunotoxicology in general is also well covered in reviews and book chapters (Descotes, 2004, 2006; Kadow et al., 2009; Dietert, 2010; House, 2010; Rooney et al., 2012), and is even the subject of entire textbooks (Dean et al., 1994; House et al., 2006), though arguably the latter may no longer be entirely up to date. That is why this review is not another attempt to sum up the state of the art but to ask some fundamental but out of the box questions.

Cambridge Advanced Learner's Dictionary & Thesaurus defines "immunology" as "*the study of how the body fights disease and infection*"[1]. Toxic immunomodulation would then mean either excess or deficiency in fighting disease and infection. Excess situations could be seen, for example, in cases of allergy and sensitization, where essentially harmless antigens trigger excessive defense. These are extensively covered in the above-mentioned reviews; here, only autoimmunity and pyrogenicity/inflammation will be touched on as examples of inappropriate immunoactivation. Defective situations could be seen for example in cases of primary and secondary immunodeficiency, which result in increased susceptibility to pathogenic infection and cancer (Gallagher et al., 2010; Riminton et al., 2011). This review will primarily focus on toxic immunosuppression.

The only remark concerning hypersensitivity we want to make is that allergic contact dermatitis is to a considerable extent a preventable disease. There is a pressing need for alternative, non-animal methods to reduce and ultimately replace animal tests for this endpoint, as also required by some European regulations (i.e., Cosmetics Regulation and REACH). The correct detection of skin sensitizers, the characterization

[1] http://dictionary.cambridge.org/dictionary/british/immunology

of potency, the understanding of human skin exposure, and the application of adequate risk assessment and management strategies can all contribute to a reduction of allergic contact dermatitis. A range of *in vivo* methods exist that have been proven to be very accurate in terms of the predictive identification of sensitizers. The challenge is to obtain the same quality of information using *in silico* or *in vitro* methods (Basketter et al., 2012). Even if no validated tests are yet available, important progress in the last decades has resulted in the development of alternative methods that could lead to the replacement of the existing animal models in the near future. At present, three non-animal test methods, namely the Direct Peptide Reactivity Assay (DPRA), the Keratinosens™ and the human Cell Line Activation Test (hCLAT) are under formal validation at EURL ECVAM for their potential to predict skin sensitization potential, while the Myeloid U937 Skin Sensitisation Test (MUSST) has been discontinued due to transferability problems. Results are expected at the end of 2013. It has been predicted, however, that it will take at least another 7-9 years for the full replacement of the *in vivo* animal models for sensitization (Adler et al., 2011). It is expected that *in vitro* data will be integrated into a testing strategy along with peptide reactivity data, bioavailability data, and some informed rating of structural alerts in order to establish an acceptable exposure level (Jaworska et al., 2011; Hartung et al., 2013, a t^4 workshop report on ITS is in preparation).

Immunotoxicology can be defined as the study of adverse effects on the immune system resulting from occupational, inadvertent, or therapeutic exposure to drugs, environmental chemicals and, in some instances, biological materials (House et al., 2006).

What does the immune system comprise in vertebrates?

1. Innate immunity: a) physical barriers, b) chemical barriers (i.e., pH, lipids, enzymes), c) cells (i.e., granulocytes, monocytes/macrophages, natural killer cells);
2. Adaptive immunity: a) humoral (B cells, antibodies), b) cell mediated (T cells, lymphokines).

We can look, on the one hand, at possible toxic effects on these components. Beside the direct and indirect effects on immune cells, we need to consider effects of chemicals on the barriers. We should be aware that one of the most critical, i.e., the barrier of the gastrointestinal tract, is very little studied. It is constantly exposed to foreign antigens present in food and the human gut contains about 1-2 kg of bacteria whose possible translocation to the systemic circulation is a permanent immunological threat.

No immunotoxicity tests have been conducted on 86% of high production volume (HPV) chemicals (EDF, 1997). Although *in vitro* or *ex vivo* immune function tests are available for many components of the immune system, animal studies or, occasionally, occupational and clinical studies *ex vivo* or *post mortem* are used more commonly. However, it is not clear whether a certain immunomodulation observed in a study model will result in a clinical manifestation, especially in a low dose, prolonged exposure scenario. This is a very critical issue, because an extrapolation from the high doses used in the laboratory model to the low doses of mixtures of compounds entering the human system through environmental and/or occupational exposure can be very difficult (Colosio et al., 2005).

On the other hand, we can look at the immune system as a whole and measure the impact of chemicals on its overall function, i.e., does chemical exposure lead to infection and cancer? Clinical grade immunosuppression indeed results in increased cancer manifestations: 5 years of cyclosporine A treatment increases the risk for any kind of cancer 3-4 fold (Sodeman et al., 2011). Any significant change induced by xenobiotic exposure on the functionality of immune cells must be considered a hazard, whose effective risk for human beings should be carefully evaluated during the risk assessment phase. If a chemical is immunotoxic and, in particular, if it affects NK cell activity and cell-mediated immune responses, this may represent a risk for decreased immune surveillance and cancer. Immunotoxicity assays might therefore play a role in future integrated testing strategies for carcinogenicity, representing a non-genotoxic mechanism of carcinogenicity (Basketter et al., 2012).

Immunocompetence is only one parameter beside the physiological and genetic factors, and exposure to infectious agents at different doses and degrees of virulence. It changes the odds in the lottery of infection. This is likely the reason why immunotoxicity does not follow a threshold model – missing a single virus or bacterium can result in an infection. The probability is low for the individual, but on the level of populations we might see an effect (Germolec, 2004). Still, it is difficult to assess whether mild to moderate immunotoxicity caused by chemical exposure predisposes to infections of the upper respiratory tract or re-activation of latent viruses (herpes), the most common manifestations of therapeutic immunosuppression. As cited below, there is not much such evidence in humans: Immunosuppression induced by pesticides may explain the increased incidence of infections in humans observed in several studies (reviewed in Corsini et al., 2013) and two recently published studies indicate that perfluorinated compounds, an important class of environmental contaminants commonly detected in blood samples from both wildlife and humans, may lower vaccine protection in children (Grandjean et al., 2012; Granum et al., 2013). Further studies are clearly needed to address this critical point.

Consideration 1: What is immunotoxicity beside clinical immunosuppression and inappropriate immunostimulation?

The immune system can be seen as a more or less concerted system of armies carrying different varieties of weapons to act against different enemies. It defends the body but can also turn against it. It is among the fastest dividing organs together with gut mucosa (under permanent digestion) and hair follicles, as evidenced by the typical and dose limiting side effects of chemotherapy, the most extreme (therapeutic) intoxication of humans performed on a regular basis, with a lethality of up to 10%.

Chemotherapy primarily hits fast dividing cells and thus results in hair loss, nausea, and immunosuppression. But we also know that it takes strong doses, strict adherence to the dose regimen, and often combinations of substances to achieve therapeutic immunosuppression, as the immune system recuperates quickly if there is a window of reduced immunosuppression as evidenced in cases of transplant rejection.

What we typically see is that there is a fine balance between achieving therapeutic immunosuppression to prevent transplant rejection and increased incidence of infectious complications. This is not surprising, as defense against infections is a key function of the immune system. So we can ask, is there any evidence of immunosuppression caused by environmental chemicals that manifests in increased infections?

Infection is the Achilles' heel of the human body and has only been outrun by other diseases as the main cause of death with the help of modern medicine. Therefore, the immune system is under tremendous evolutionary pressure, which has also led to tremendous interindividual and interspecies differences. The immune system also has enormous overcapacities, as it must be the last to fail. This characteristic will predictably buffer chemical impacts on this organ system and indeed a functional deficit often only manifests under the additional stress of mass infection of an animal.

The focus on immunosuppression in the study of immunotoxicity has been questioned by experts in the field: "*Present methods of evaluating immunotoxicity are primarily focused on immunosuppression, even though unexpected immunosuppression has rarely been a cause for concern*" (Descotes, 2006). In industrialized countries hypersensitivity reactions represent the most frequently reported immunotoxic effects of chemicals. Immunostimulation or "immunoenhancement", i.e., an exaggerated immune response, is known primarily in sensitization (allergies, including contact dermatitis) and autoimmunity. The clearest disturbance of the immune system, indeed, is the dramatically increasing incidence of allergies. According to CDC, for example, the prevalence of food allergies in children aged 0-17 years in the US increased between 1997 and 2011 from 3.4% to 5.1%, and during the same period skin allergies increased from 7.4% to 12.5%. There was no significant trend in respiratory allergies (around 17.0%)[2]. Some allergies are responses to chemicals and thus it seems logical to blame chemical exposure. But is this correct? Pollen allergy (hay fever) and food allergy are similarly increasing. Many people feel threatened by the ever-increasing number of chemicals they are exposed to, but is there a causal link to sensitization? First, it appears that the incidence of allergies has been increasing continuously over the last century, not really supporting the hypothesis that a few specific chemicals have changed our likeliness to develop sensitization. Could instead the exposure to many chemicals with sensitizing or immunomodulating potential be causing this? The fact that about 35% of all chemicals tested in mice or guinea pigs produce skin sensitization (Casati et al., 2005) seems to support this alternative hypothesis. However, this might again be a high-dose phenomenon, as it corresponds with clinical experiences, where work exposure and prolonged contact with the skin are typically required to result in manifestations. Still, the majority of humans do not develop allergies, while our test animals react sufficiently uniformly to allow a testing regimen with relatively small groups. In susceptible human individuals, drugs and chemicals may initiate, facilitate, or exacerbate pathological immune processes, resulting in autoimmunity and allergy. In principle, they can induce mutations or influence the regulation of genes coding for immunoregulatory factors, they can modify immune tolerance and regulation, leading to immunostimulation as well as immunosuppression. Advances over the last decade now bring integrated testing strategies using *in vitro* and *in silico* approaches to replace the animal tests into reach (Basketter et al., 2012). Drug allergy is still a significant problem and among the most common causes of new drugs being withdrawn from the market.

Autoimmunity is the other most relevant activating derailment of the immune system. This group of diseases is extremely varied including, among others, autoimmune thyroiditis, thrombocytopenia, hemolytic anemia, hepatitis, systemic lupus erythematosus (SLE), insulin-dependent diabetes mellitus, myasthenia gravis, multiple sclerosis, and Sjögren's syndrome. There are examples of drugs associated with autoimmune phenomena: autoimmune hepatitis (dihydralazine, halothane, tienilic acid), drug-induced lupus (dihydralazine, procainamide, propylthiouracil), glomerulonephritis (gold thiomalate), and occulomucocutaneous syndrome (practolol). This side effect can be quite frequent, e.g., 10-20% of patients receiving procainamide and 5-20% receiving hydralazine develop systemic lupus erythematosus (Dean et al., 2007). There is some evidence for food and environmental chemicals causing autoimmunity, e.g., autoimmune thyroiditis (iodine), scleroderma (L-5-hydroxytryptophan), and SLE (alfalfa seeds). Vinyl chloride, trichloroethylene, aniline (Spanish toxic oil syndrome), tryptophane, silica, paraffin, and silicones are among chemicals leading to autoimmune manifestations, especially sclerotic and lupus-like diseases (Kilburn and Warshaw, 1994). Kosuda and Bigazzi (1996) list more than a hundred xenobiotics associated with autoimmune disease. Pesticides also have been suggested to play a role (Holsapple, 2002).

A number of syndromes similar to those in humans can be mimicked in animals (Pieters, 2007; Lam-Tse et al., 2002), but the diversity of autoimmune diseases limits their utility as a screening tool (Luster and Gerberick, 2010). The difficulties to study xenobiotic-induced autoimmunity in animal models recently have been reviewed (Germolec et al., 2012). These models have been used to ascertain a role for mercury and pristine (TMPD) as inducers of autoimmunity and there is some evidence for silica, gold, trichloroethylene (TCE), and dioxin (TCDD). It appears that chemical-induced autoimmunity is rarely organ-specific but more likely systemic. While basic research into autoimmunity also uses *in vitro* models, e.g., for

[2] http://www.cdc.gov/nchs/data/databriefs/db121.htm

multiple sclerosis (van der Star et al., 2012; Goebels, 2007), there is essentially no *in vitro* or *in silico* model to screen for autoimmune effects of xenobiotics yet. Given the complexity and diversity of the different autoimmune diseases, including multiple genetic, environmental, and stochastic processes, it is rather unlikely that such alternative methods will become available in the near future. Mapping the respective pathways of toxicity in the established cases might be a first step to develop a toolbox for testing such effects.

Consideration 2: Do environmental chemicals cause relevant immunotoxicity?

In 1994, Ernest Tucker stated: "*Currently, the lack of clear evidence that humans suffer significant immunosuppression or defective immune responsiveness from xenobiotics calls for well-designed cohort studies to effectively evaluate their effects on immune functions in humans*." (Dean et al., 1994). 16 years later, Luster and Gerberick (2010) similarily stated: "*it is relatively difficult to determine the contribution of chronic low-level immunosuppression or the cumulative effect of modest changes in immune function to the background incidence of disease in the human population*". The human epidemiological database is very limited owing to the lack of validated assays of sufficient sensitivity, the difficulty of accurately determining infectious disease incidence, and the high costs involved. The impressive reduction in infectious diseases over the last century due to hygiene, nutritional status, vaccines, antibiotics, etc. does not really allow us to discern the contribution of chemical-induced immunosuppression leading to infectious complications. In April 2010, the US President's Cancer Panel published the report "Reducing Environmental Cancer Risk" (Reuben, 2010) which stated that "overall cancer incidence and mortality have continued to decline in recent years". Thus there is at least no evidence of an increasing frequency of diseases which could be attributed to immunotoxicity. This obviously says nothing about specific exposed or sensitive groups or specific types of infectious disease or cancer. This situation must be considered in the context that life expectancy has tripled (Kirkwood, 2008) during the period in which these chemicals were introduced. The pattern of environmental chemical exposure, however, is continuously changing. We apparently now have the cleanest water and air since decades but manufactured food and consumer products expose us to new substances on an almost daily basis, prohibiting us from drawing definite conclusions on the chemicals' effects on our health.

In case of the immune system the problem is even more difficult than for other hazards: first, we are not talking about one organ but bone marrow, thymus, spleen, about 1000 lymph nodes and a similar number of Peyers' patches in the gut, the lymphoid tissue associated with skin, mucosa, bronchi, gut and the genitourinary tract as well as the peripheral leukocytes. The second unique aspect is the capacity for self-restoration and emergency recruitment of leukocytes. If we see the immune system as the armies of self-defense, we understand that any change to their capacities and reactivity will only become visible with the appropriate challenge. An impairment of the navy will only be visible when the enemy attacks by ship… Third, and even more complicated, the "balance of powers" typically comes with advantages and disadvantages when moving the equilibrium. The T_H1/T_H2 hypothesis, for example, suggests that we either defend well against bacteria or against worms. Impairing one branch of the immune system would strengthen the other. Fourth and last, genetic determinants appear to play a major role in immune responses as evidenced, e.g., by the greatly different level of sensitivity of different inbred mouse strains towards certain pathogens. This will dramatically impair our ability to identify health threats in any animal model relying on such reduced biological background. Any strategy to cover genetic diversity, however, will boost animal or human study subject numbers.

The most important aspect is the continuum of immunotoxicity (Kaminski et al., 2007): it is based on the recognition that immune responses in the normal human population vary considerably. This means that immunomodulation does not necessarily take an individual out of a healthy response pattern. Figure 1 shows the population distribution of immune response strength (if something like this can be defined…) and how an individual within this population is shifted by an immune response modifier (toxicant) toward immunosuppression or immunoactivation; only if this immunotoxic effect pushes the individual to the extremes of this distribution (out of normal, i.e., disease or significantly increased likelihood of disease), will we really see a manifestation of immunotoxicity. This means that we have a broad distribution of strength or type of immune responses that must be considered normal. Changes, induced, for example, by chemicals acting as immune response modifiers (toxicants), can affect either the strength of a com-

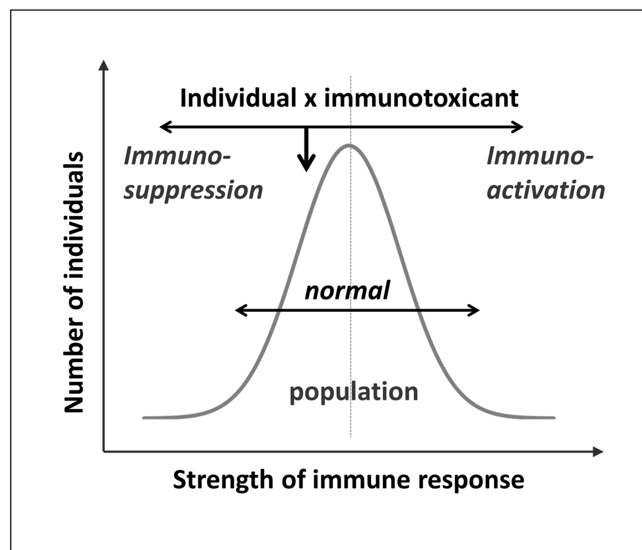

Fig. 1: Schematic representation of the population's variability in immune response and an individual within the population changing strength of immune response because of exposure to an immunotoxicant

ponent or the pattern of responses and will normally not lead to disease manifestation. But, increasingly, at both extremes of the distribution, exposure and circumstantial factors, such as nutrition, comorbidities and others, will add up to compound the likelihood of disease manifestation. The hypothesis is put forward that this system very strongly buffers broad variations of immune responses, but that extreme dysregulation greatly changes the odds of disease manifestation owing to the permanent pressure of infectious agents at the barriers and the enormous resources of the immune system that, once activated can turn against the host.

There seems to be tremendous buffer and networked self-control against overshooting and attenuated immune response. Only extreme alterations appear to result in clinical symptoms as the majority of immune cells are never challenged by supposed threats, i.e., pathogens as well as correctly or not identified malignant constituents of the body. We lack thresholds of adversity for immunomodulation that do not represent an increased incidence of disease when the immune response is inadequate. This would be a prerequisite for establishing any screening for immunotoxic instead of immunomodulatory properties.

We know from the clinics that it usually takes drastic treatments to compromise the human immune system to result in therapeutic immunosuppression. Some examples of doses of immunosuppressants that achieve immunosuppression as the main or a side effect:

- cyclophosphamide: 1-3 mg/kg/day (high bioavailability)
- azathioprine: 1.5-3 mg/kg/day (high bioavailability)
- methotrexate: 5-15 mg/kg/7 days (variable, moderate bioavailability)
- glucocorticoids: 0.1-1 mg/kg/day (high bioavailability)
- cyclosporin: 2.5-15 mg/kg/day (variable, moderate bioavailability)
- tacrolimus: 0.1-0.3 mg/kg/day (variable, moderate bioavailability)

We might argue that these agents are optimized with regard to bioavailability and efficacy. Thus, we might conclude that general industrial chemicals not targeting the immune system are unlikely to be more effective than intentional immunosuppressants. We might derive from this a threshold of toxicological concern in the mg/kg/day range, which means that substances at less than this concentration are unlikely to result in immunosuppression in humans.

Immunosuppressive compounds can interact directly with immunocompetent cells, resulting in alteration of the status and/or functionality of the immune system. Characterization of how immunotoxicants interfere with cell signaling may lead to a better understanding of their molecular mechanism of action. Different mechanisms can lead to immunotoxicity:

1. Chemicals can kill immune cells, resulting in bone marrow toxicity and immunosuppression. Compounds that can damage or destroy the bone marrow will often have a profound immunotoxic effect, since the effectors of the immune system will no longer be available. Antitumor drugs, benzene, and ionizing radiation are examples of myelotoxic compounds.
2. Chemicals can interfere with general or immune specific signaling pathways, resulting in changes in the expression of surface markers, cytokine production, cell differentiation and activation. Immunotoxic compounds can act via a receptor mediated or non-receptor mediated effect. Examples of chemicals acting through a receptor-mediated effect include glucocorticoids, polycyclic aromatic hydrocarbons, and cannabinoids, while immunotoxic compounds acting through a non-classical receptor mediated event include calcineurin inhibitors, metals, and some pesticides.

Many substances exert immunosuppressive effects by inhibiting bone marrow stem cell proliferation (cyclophosphamide, methotrexate), or spleen/thymus (organotin, TCDD), or by directly affecting mature leukocytes (glucocorticoids, TCDD, cyclophosphamide, methotrexate), e.g., by inhibiting lymphocyte proliferation or triggering their apoptosis. Cyclophosphamide was shown to selectively deplete a regulatory T cell population (Weir et al., 2011). Noteworthy, humans appear to be much less sensitive to the immunosuppressive effects of TCDD and, apparently, some others than rodents or even monkeys. Because of the non-specific nature of some of these immunosuppressants, several modes of action are observed, e.g., for steroids suppression pro-inflammatory cytokine and chemokine production, upregulation of TGF-β, shift to anti-inflammatory T_H2-responses, suppression of NK cell functions, and impaired dendritic cell activation and differentiation. Noteworthy, cytotoxic drugs also have various immunostimulatory effects (Zitvogel et al., 2008), such as increases in effector T cell stimulation and tumor immunogenicity as well as decreases in tumor induced immune suppression (Weir et al., 2011).

Immunosuppression of the granulocytes of the innate immune system appears to be rare, likely due to the rapid renewal of granulocytes from bone marrow, which can be dramatically accelerated by the induction of colony-stimulating factors (Hartung, 1999; Hareng and Hartung, 2002). The rare (2-9 patients per million inhabitants per year) but potentially life-threatening disease agranulocytosis, if not caused intentionally by chemotherapy, is attributable to 70-97% to drugs (Garbe, 2007). Its pathogenesis is still incompletely understood, but immune mediation and damage to granulocytes or their hematopoietic precursors by reactive drug metabolites appear to play a role. There is no evidence for environmental chemicals playing a major role, though case reports can be found (Knutsen, 1978). They did not warrant test development *in vivo* or *in vitro*, but a QSAR has been developed to predict such effects (Díaz et al., 2003).

Therefore, macrophage effects might be more critical as they control granulocyte recruitment and activation. The most advanced test here is the whole blood monocyte cytokine release assay (see below), though variants using isolated peripheral blood mononuclear cells (PBMC) are also available for similar evaluation.

So far, we are not systematically collecting information on immunomodulatory effects of chemicals and drugs. A database of immune effects of xenobiotics might be a first step to estimate the role of chemicals in immune associated health burden. There is a National Toxicology Program (NTP) database which contains information on the NTP studies that have been conducted and one can request access to this. NTP and EPA currently are working on a new database that will be publicly available.

Tab. 1: Immunotoxic alerts in standard toxicology studies
(i.e., 28-day repeated dose toxicity testing study in rodents)

- Changes in total and differential white blood cell counts, i.e., leukocytopenia/leukocytosis, granulocytopenia/granulocytosis, or lymphopenia/ lymphocytosis;
- Changes in clinical chemistry, i.e., serum immunoglobulin levels and albumin/globulin ratios;
- Alterations in organ weights, i.e., thymus and spleen, and/or histology of primary and secondary lymphoid organs, i.e., bone marrow, thymus, spleen, draining and distant lymph nodes;
- Increased incidence of infections;
- Increased occurrence of tumors, in the absence of genotoxicity, hormonal effects, or liver enzyme induction;
- Chemical retention in organs/cells of the immune system.

Consideration 3: Determining immunosuppression *in vivo* and *in vitro*

In vivo testing for immunotoxicity

There are relatively few guidelines for testing compounds for immunotoxicity. The earliest guidelines were developed for pesticides in 1996 by US EPA (OPPTS 880.3550 followed by 880.3800 and 870.7800). They reflect the NTP's tier-testing approach (Luster and Gerberick, 2010; Basketter et al., 2012) and typically request *in vivo* tests in rodents (Descotes, 2006).

For drugs, International Conference on Harmonization (ICH) Safety Number 8 guidance recommends a "weight-of-evidence approach", i.e., alerts of immunotoxicological potential in standard tests should trigger specific tests. Table 1 lists changes that should trigger tier II testing on a case-by-case basis. Noteworthy, activation of the immune system (hypersensitivity and autoimmunity) is not covered by S8. Guidance is also available from FDA[3].

For environmental chemicals, no dedicated OECD test guidelines exist, but extensions were made to 28-day repeat dose toxicity testing (TG 407) (Institóris et al., 1998). The European REACH program does not require immunotoxicity as a standard information requirement. However, under short-term (28-day studies) and sub-chronic (90-day studies) repeated dose testing it requests: "*Further studies shall be proposed by the registrant or may be required by the Agency in accordance with Article 40 or 41 in case of: ...indications of an effect for which the available evidence is inadequate for toxicological and/or risk characterisation. In such cases it may also be more appropriate to perform specific toxicological studies that are designed to investigate these effects (e.g. immunotoxicity, neurotoxicity)…*"[4]. The most recent guidance document comes from WHO 2012 as Harmonization Project Document No. 10 Guidance for Immunotoxicity Risk Assessment for Chemicals[5].

Animal tests constitute the current gold standard for immunotoxicology. Interpreting data from animal immunotoxicology studies for risk assessment has proven challenging, especially when the immunological effects are minimal-to-moderate in nature (Germolec, 2004). Attempts were made to complement or replace them with *in vitro* methodologies. The workshop "*Immunotoxicology and in vitro possibilities*" (Sundwall et al., 1994) analyzed the then current status of *in vitro* methods for assessing immunotoxicity. At that time, experts agreed that there was no immediate way to replace the whole animal for immunotoxicology studies. On the other hand, much progress has been achieved regarding the reduction in the number of animals used, since *in vitro* models can be used for prescreening. At present, a tiered approach has been proposed, since useful information can be obtained from regular 28-day general toxicity tests if increased attention is paid to the study of the histopathology of a large variety of lymphoid tissues, coupled with immunohistochemical measurements and the determination of antibody classes. Furthermore, it was established that the *in vitro* test should be validated against information gained from humans rather than the results from laboratory animal species. We followed this workshop with a more extensive one in 2003 (Gennari et al., 2005). The recommendations made then still stand and those not addressing sensitization are reproduced in Table 2.

In vitro testing for immunosuppression

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, an initial evaluation of myelotoxicity should be performed (Tier 1). If a compound is myelotoxic, there may be no need to proceed with additional evaluation. The methodology for bone marrow culture systems is published and well characterized. *In vitro* bone marrow culture systems are commercially available, and they would probably only have to be modified slightly to accommodate *in vitro* exposure to test material. Assays of immunosuppression have been validated to predict the maximum tolerated dose (MTD) in humans. Their

3 http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM078748.pdf

4 http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2006R1907:20121009:EN:PDF

5 http://www.inchem.org/documents/harmproj/harmproj/harmproj10.pdf

Tab. 2: General recommendations from the ECVAM workshop

- Hypersensitivity and immunosuppression are considered the primary focus for developing *in vitro* methods in immunotoxicology. Nevertheless, *in vitro* assays to detect immunostimulation and autoimmunity are also needed. Although developmental immunotoxicity is an emerging concern, there are no *in vitro* test models available at this time.
- It is recommended to use a flow chart/decision tree approach to evaluate whether or not a compound is immunotoxic (initial screening). Detection of compounds as potential immunotoxicants can then be followed up by more detailed *in vitro* mechanistic assays (e.g., antigen-specific or redirected CTL).
- 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. With the exception of bone marrow assays, the source of cells should be PBL (peripheral blood leukocytes) from donors prescreened for health, immune reactivity, etc.
- 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. It is anticipated that most of these assays will be amenable to a microculture format, increasing efficiency and decreasing cost.
- 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. This could be accomplished by a coordinated effort from governmental agencies, medical institutions, and industry. Access to any extensive animal databases, when available, will also be helpful.

suitability for use in chemical-induced immunotoxicity should be determined and would require prevalidation. These assays are relatively expensive if human cells are used, but the standardized nature of commercial systems should provide good feasibility.

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 (Tier 2). 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. An *in vitro* test to determine lymphotoxicity should be carried out (cell death by necrosis or apoptosis). Such assays would require prevalidation to evaluate their reliability/reproducibility. After myelotoxicity and overt cytotoxicity are excluded as endpoints, basic immune cell functionality should be assessed by performing specific functional assays, i.e., proliferative responses, cytokine production, NK cell activity, etc. (Tier 3), using non-cytotoxic concentrations of the tested chemicals (viability >80%). For T cells, the stimulatory agent could be a combination of anti-CD3 and anti-CD28 or mitogens such as concanavalin A (ConA) and phytohemagglutinin (PHA); for B-cells an optimum system would have to be developed but would be expected to be similar to the murine system incorporating an anti-immunoglobulin and cytokine or KLH. This determination may require broadening to include other types of immunocytes (e.g., phagocytes). These assays are relatively inexpensive (source material is readily available); the feasibility is high owing to the wealth of published methodology.

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 doubt whether a primary immune response can actually be induced in human peripheral blood leukocytes. 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). Recently, Koeper and Vohr (2009) reported that using a modification of the Mishell-Dutton assay with female NMRI mouse splenocytes, all six immunosuppressive compounds tested (with the exception of cyclophosphamide) and all four non-immunotoxic compounds were correctly identified. Further use and development of this model is, therefore, recommended.

Potential effects of chemicals on cytokine expression should be determined. The role of cytokine transcription or production should be evaluated as well as the modulation of cytokine receptors. It should also be investigated if cytokine transcription or production is skewed (T_H1/T_H2 shift). It will require careful consideration which cytokines should be measured to obtain the most useful information (e.g., proinflammatory, specific immunoregulatory cytokines). It is recommended that a broader panel of cytokines than is currently used be investigated. Both basal and activated cytokine production should be measured, and for activated cytokine production, anti-CD3 and anti-CD28, LPS, or allergens should be used. The whole blood assay is the most promising option owing to its advanced stage of prevalidation.

Many assay systems are available for measuring cytokine expression (e.g., ELISA, flow cytometry, molecular biology techniques, such as RT-PCR). They are moderately expensive, and their feasibility is high due to wealth of published methodology and commercial standardization.

Potential effects on NK cells should be determined. Cytolytic function should be measured (this is important for innate immunity). There are a variety of systems available for measuring cytolytic function (e.g., whole blood, radiolabel release, flow cytometry); these systems are robust and well characterized. The immunoregulatory function of NK cells should be evaluated due to the key regulatory nature of these cells. At present, such a system is not well described, and would require method development. A feasible system would probably be a modification of

Tab. 3: Major limitations and future research needs in *in vitro* immunotoxicology

- *In vitro* exposure is most straightforward for direct immunotoxicants. However, materials that require biotransformation would require special culture systems (e.g., culture in the presence of S9 fraction).
- Physiochemical characteristics of the test material 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. In order to retain the viability of the cells at an acceptable level, *in vitro* exposures are often performed in 0.1% ethanol or 0.1% DMSO as maximum solvent concentration, thereby maximizing the exposure concentration of the xenobiotic. This is an additional limitation of *in vitro* systems.
- *In vitro* systems do not take into account the interactions of the different components. It is difficult to reproduce the integrity of the immune system *in vitro*.
- *In vitro* systems do not account for potential neuro-immuno-endocrine interactions. There is no anticipated resolution for this deficiency at present.
- The current state of technology does not allow evaluation of the induction of a memory response *in vitro*. Resolution of this deficiency will require the development of novel culture systems.
- The current state of technology does not allow evaluation of recovery (acute vs. long-term immunosuppression). Resolution of this deficiency will require the development of novel culture systems.
- The current state of technology does not allow for evaluation of toxic effects on lymphoid architecture that could lead to defects in cellular interactions necessary for induction of immune responses (e.g., lymph nodes). Future developments in tissue engineering may solve this problem, but this is a long-range possibility.
- Exclusive use of human cells may limit the ability to bridge to the preexisting database of animal immunotoxicology studies.
- The use of ’omics should be considered for the search of new parameters and for the possibility of gene profiling after hapten treatment.
- Determination of potential effects on antibody induction/production.
- In animals, production of a T-dependent antibody (such as SRBC (sheep red blood cells)) is considered to be the gold standard. However, there are currently no good systems for *in vitro* antibody production using human cells.
- Development of human *in vitro* systems will require optimization of stimulator (preferably using antigen relevant to human exposure, such as TT (tetanus toxin)), culture conditions, and assay endpoint(s). For these reasons, further research in this area is strongly recommended.
- There is a need for research to develop *in vitro* models to detect autoimmunity and immunostimulation.

the existing whole blood model or other cytokine methods. The systems currently in use are highly reliable and reproducible; implementation for *in vitro* exposure would require additional development. The cost of performing these assays depends on the assay endpoint, but is overall relatively inexpensive; feasibility is high due to extensive past use of this methodology. In addition, these systems will require prevalidation for exclusive *in vitro* exposure.

An assessment of the functionality of immune cells could also include the measurement of other mediators, e.g., histamine, cytokines, eicosanoids, or activation of the complement cascade leading to hypersensitivity reactions. The use of the whole blood assay can also address the release of mediators by basophils (histamine) and monocytes (cytokines). Finally, the use of mast cell models also needs to be considered. At the moment, there is no strong evidence for a role of eosinophils being directly activated by compounds. Models are available.

Immunoregulation (e.g., adjuvants, superantigen)

It has been shown that lymphocytes can be directly activated by microbial products (superantigen concept), leading to release of cytokines and clinical effects. To date, there is no example of this type of effect being induced by low molecular weight chemicals. The existing whole blood assay should be considered to address this question.

Adjuvants may be included in vaccine formulations to enhance the immune response to a particular antigen(s). It is known that certain compounds can have adjuvant activity through direct or indirect activation of dendritic cells (DC). Indirect activation can also result from non-immune cells present at the site of exposure to the compound.

For direct activation, human DC models are suitable, as previously described. For indirect activation the use of other cell models should be encouraged, depending on the route of exposure, e.g., human lung epithelial cells, human keratinocytes, gastrointestinal tract. In the case of non-immune cells we recommend evaluation of cytotoxicity and proinflammatory mediator release, e.g., cytokines, chemokines.

The major limitations of *in vitro* immunotoxicology and future research needs initially identified (Gennari et al., 2005) still stand as, ten year later, progress has been rather limited. Limitations and needs are listed in Table 3.

The test systems under consideration for assessing chemical-induced immunosuppression have not really changed (Tab. 4).

Tab. 4: Test systems under consideration for assessing chemical-induced immunosuppression

Model	Comment	Reference(s)
Myelotoxicity CFU-GM assay	Validated for determining the starting dose of chemotherapies in human trials	Pessina et al., 2001; Negro et al., 2001
Lymphotoxicity and proliferation	Human peripheral blood lymphocytes (whole blood or PBMC) or rodent splenocytes are typically used. Polyclonal stimuli include anti-CD3, anti-CD28, plant lectins such as concanavalin A (ConA) and phytohemagglutinin (PHA), superantigens, etc. Mixed lymphocyte reactions possible but less used for immunotoxicity.	Carfì et al., 2007
Antibody production, e.g., Mishell-Dutton assay	Gold standard in animals *ex vivo* but highly variable *in vitro*, originally mouse splenocytes, later human cells	Mishell, 1967; Dutton and Mishell, 1967; Wood et al., 1992
Cytotoxic lymphocyte function (CTL)	Allogeneic cells stimulate rodent splenocytes (e.g., P815 murine mastocytoma cell line) or human blood lymphocytes (e.g., Jurkat leukemic cell line) for killing by CTL. Rarely used for *in vitro* immunotoxicity.	House and Thomas, 1995
NK cell function, e.g., K562 killing	Sensitive to toxicants; use also *ex vivo*. A number of chemicals have been shown to impair NK cell activity. However, direct evidence for clinically significant pathologic consequences, such as infections or immunosuppression-related cancer in human beings exposed to these chemicals, is lacking.	Morales and Ottenhof, 1983; Roder et al., 1980; Descotes and Ravel, 2005; Kim et al., 2007; Blom et al., 2009
Whole blood cytokine release	Pre-validated assay for both monocyte and lymphocyte cytokine release, simple to perform on primary human cells *in vitro* and *ex vivo*.	Langezaal et al., 2001, 2002
Dendritic cell function	Very well characterized for the *in vitro* assessment of contact allergens. Less well established endpoint for immunosuppression.	Coutant et al., 1999; Hymery et al., 2006

Cytokine production and lymphocyte proliferation have been pre-validated (Carfì et al., 2007). The human T cell activation assay was selected as the most promising of the investigated *in vitro* immunotoxicity tests. This assay is based on CD3/CD28-mediated T cell activation using proliferation and cytokine release (TNFα and IFNγ) as read-out parameters. To pre-validate the human T cell activation assay, 20 compounds were selected, of which 10 were immunosuppressive and 10 non-immunosuppressive. Statistical analyses revealed that the human T cell activation test had a "sensitivity" (correct prediction of immunosuppressive chemicals) of 76% and a "specificity" (correct prediction of non-immunosuppressive chemicals) of 83% (manuscript in preparation). The human T cell activation assay may be a promising candidate for *in vitro* evaluation of immunosuppressive activity.

Immunotoxicogenomics represents a novel approach to investigate immunotoxicity. Hochstenbach et al. (2010) have recently reported a set of 48 genes that can be used to distinguish immunotoxic from non-immunotoxic compounds using human peripheral blood mononuclear cells. These genes might be considered candidate biomarker genes for immunotoxicity screening. However, even if many of the annotated genes appear to be immunologically relevant, *in vivo* studies in the human population or in experimental models are necessary to demonstrate their relevance. More recently, the same group explored the possibility of identifying a gene signature of direct immunotoxicants by testing the effects of 31 test compounds on the transcriptome of the human Jurkat T cell line (Shao et al., 2013). They confirmed that diverse modes of action are involved in direct immunotoxicity and that a set of pathways or genes, rather than one single gene can be used to screen compounds for direct immunotoxicity.

One of the authors has mainly contributed to this field by the adaptation of the whole blood cytokine test, validated earlier as a pyrogen test (Hartung et al., 2001; Hoffmann et al., 2005; Daneshian et al., 2009; Schindler et al., 2009), while the other has used the whole blood assay as an *in vitro* assay to characterize the molecular mechanisms of action of some pesticides and as mean to assess the effects of pesticides in human exposed workers *ex vivo* (Corsini et al., 2005, 2007).

As a prototypic example the whole blood cytokine test shall be discussed in more detail here. Both monocyte and lymphocyte functions (Hermann et al., 2003) can be assessed using selective stimuli (Langezaal et al., 2001). This observation advanced to a prevalidation study (Langezaal et al., 2002). The *in vitro* results were expressed as IC_{50} values for immunosuppression, and SC_4 (4-fold increase) values for immunostimulation. The whole blood cytokine results reflected immunomodulation from

in vivo studies. 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. Advantages of the human blood cell based *in vitro* test include (Gennari et al., 2004):

- Species differences between humans and animals are avoided.
- Human primary cells are employed in their physiological proportions and environment, avoiding preparation and cultivation artifacts.
- Culture techniques are extremely simple, e.g., allowing incubation in thermoblocks.
- Cryopreserved blood overcomes problems of availability, standardization, and risk of infection.
- *In vitro* testing is less expensive and time-consuming than *in vivo* testing.
- The same test can be employed ex vivo and *in vitro*.
- The number of compounds and concentrations tested can be increased.
- The amount of substance required is dramatically reduced, allowing testing at earlier stages of drug development.
- Effects on different blood cell populations can be tested in a single model.
- Changes of cellular immune response can be quantified, enabling potency testing.

Noteworthy, cryopreserved whole blood has been developed and validated for the pyrogen test (Schindler et al., 2004, 2006) and is commercially available. However, this has not been adapted for immunotoxicity testing to a major extent, though the advantages of availability, standardization, and pretesting for both abnormal responses and infectious threats are evident.

A very interesting opportunity, as mentioned above, is the fact that the very same test can be performed *ex vivo* after immunomodulatory treatment or intoxication (Hartung et al., 1995a; von Aulock et al., 2004; Elsässer-Beile et al., 1993). The example of the whole blood immunotoxicity assay shows that with the relatively easy access to human primary cells, the field is predestined to use cells from the target species of interest. Noteworthy, protocols for other immune function assays using whole blood incubations are available affording similar advantages (Fletcher et al., 1987; Bloemena et al., 1989).

It should be noted that the array of *in vivo*, *ex vivo*, and *in vitro* immunotoxicity assays is still incomplete. With the growth of our understanding of immunological phenomena, new needs emerge, e.g., the call for assessing effects on regulatory T cells (Corsini et al., 2011): "*The concept of regulatory or suppressor cells having a role in chemical-induced immune system toxicology has been somewhat understudied. However, it is now recognized that T_{regs} play a critical role in maintaining the careful balancing act that allows the immune system to respond appropriately in the face of infection or disease, resolve when the challenge has diminished, and fail to respond to self-antigens. As shown by the speakers in this symposium, various immunoregulatory T cell subsets may be induced by environmental chemicals and protein allergens*." Similarly, we might consider other axes of the immune system from complement and other humoral factors, such as antibodies and surfactants, to eosinophils, neutrophilic granulocytes, B-cells or tissue immune cells, etc. The hope must be that a limited number of populations and immune functions are sufficiently representative to allow us to create a battery of tests that covers the effects of xenobiotics on the immune system. Notably, also an immune challenge *in vivo* will only probe some of these defense lines, and therefore the reliance on whole animal studies does not necessarily overcome this problem.

Consideration 4: Is there developmental immunotoxicity?

A particular aspect of the immune system is that it develops rather late in life. For example, thymus development lasts at least until puberty. Over the last couple of years, the need to consider the special vulnerability of the developing immune system has been discussed (Burns-Naas et al., 2008; DeWitt et al., 2012; Collinge et al., 2012; Dietert, 2008). Developmental immunotoxicology might predispose children to diseases such as childhood asthma, allergic diseases, autoimmune conditions, and childhood infections, which have been on the rise in recent decades. Our knowledge, especially across species, is still small. There may be critical windows of vulnerability of the developing immune system, such as:

- Hematopoietic stem cell formation
- Migration of hematopoietic stem cells to fetal liver and thymus, early hematogenesis and migration of macrophages to tissues
- Establishment of the bone marrow as a primary site of hematopoiesis and bone marrow and thymus as primary lymphopoiesis sites for B and T cells, respectively
- Functional development and maturation of immunocompetence

However, there are no corresponding non-genetic immune syndromes in the clinic. Granted, infections in the very young are often more severe compared to adults, and delays or impairments of these processes could further increase vulnerability. An approach in which pregnant animals are continuously exposed to test chemicals is currently favored to address all critical windows of developmental immunotoxicity at once. However, in general, research into this has been very limited. It will only be of major importance if there are substances that are developmental immunotoxicants but do not affect adults. A framework for developmental immunotoxicity has been proposed that favors the rat (Holsapple et al., 2005) though immunological tools are more limited here, although they do allow inclusion into standard guideline assays. *In vitro* approaches are rare to non-existent.

The development of *in vitro* and *ex vivo* tools for developmental immunotoxicology will be of critical importance when transitioning from the two-generation to an extended one-generation study for reproductive toxicology, where one of the key extensions requested is developmental immunotoxicity. If this

aspect cannot be satisfied without the use of additional animal groups savings in animals and costs will be minimal compared to the two-generation study. It has to be recalled that this represents one of the largest animal consuming tests and a key burden of the REACH program (Hartung and Rovida, 2009; Rovida and Hartung, 2009; Bremer et al., 2007; Rovida et al., 2011).

Consideration 5: Is there an immune component in many other toxicities?

The answer is clearly yes, and it is an underdeveloped area of research. Although not associated with specific immunity, a third common immunotoxic effect is inflammation, which contributes to tissue and organ damage (Luster amd Rosenthal, 1993). Inflammation is triggered by necrosis, e.g., as a consequence of cytotoxicity. Tissue destruction is normally accompanied by an inflammatory reaction. One hallmark of this inflammatory process is the infiltration by different subsets of leukocytes from the circulation into the wounded site (DiPietro, 1995). Chemokines, a subgroup of cytokines, are responsible for this site-directed migration of immune cells. Interestingly, we know little about how this is achieved. The most potent inducers of chemokine release are bacterial components such as lipopolysaccharide (LPS) of Gram-negative bacteria. Even though bacteria are not always present in wounded tissue, e.g., in ischemia, sterile trauma or other disturbances of tissue homeostasis like fibrosis, chemokine release, and infiltration of leukocytes into the damaged tissue may occur, triggered by damage associated molecular patterns or DAMPs, which includes ROS, uric acid, hyaluronic acid fragments, ATP, etc.

Components of destroyed cells might act as inducers of inflammation and leukocyte migration. We carried out a rather simple series of experiments, where human blood leukocytes were brought into contact with human cells (Schneider and Hartung, 2001): no cytokine release was induced if the cells were intact. To investigate the effect of necrotic cells, we challenged human whole blood, with a cell-lysate of a human fibroblast cell line (IMR-90). Under these conditions, we found a concentration- and time-dependent, selective induction of the chemokines IL-8 and MCP-1 measured by ELISA. A similar release of these chemokines was measured in isolated human PBMC and elutriation-purified human monocytes after stimulation with the IMR cell lysate. To exclude possible contamination of the lysate or the cell culture by mycoplasma or endotoxin, which would also cause a chemokine secretion, mycoplasma were excluded by a commercial ELISA and endotoxin by Limulus amoebocyte lysate test as well as the lack of effect of polymyxin B, a LPS-neutralizing compound. It would be very interesting to identify the components of the cell-lysate responsible for the chemokine induction and to compare different human cell-lines or primary cells as to their ability to induce chemokines.

The strongest trigger of inflammation, however, is microbial stimulation. We often forget that the human body carries 10 times more bacteria than its own cells. The gut contains 1-2 kg of bacteria containing about 50 g of endotoxin (Ernst Rietschel, Borstel, personal communication). Many, especially topical, toxicities include breakdown of skin and mucosal barriers, allowing translocation of bacteria and LPS *inter alia*. The contribution of this to the manifestation of irritation by chemicals has to the best of the authors' knowledge not been addressed.

We might turn an argument around to substantiate the hypothesis that, especially in acute oral intoxications, the animals do not die from the toxin but from secondary effects to the gastrointestinal tract (GIT) as argued already in this series (Hartung, 2008): it has been documented in at least three major attempts that cytotoxicity correlates pretty well with acute oral toxicity (see Halle register, MEIC study and the more recent ICCVAM/NICEATM/ECVAM validation study). Actually, this makes little sense if we assume that the substances are taken up, distributed and metabolized with complex kinetics, and can affect more than 400 different tissues with various sensitivities. Might it be that the animal experiment simply measures cytotoxicity to the GIT epithelium, which results in translocation of bacteria? Ironically, this would mean that we can pretty well predict this animal test *in vitro*, but the animal test measures a phenomenon (cytotoxicity to the intestine) that is irrelevant for humans (we would vomit – which rodents cannot do – or attempt to remove the intoxication before it reaches the intestine, supply intensive care treatment, etc.). Instead of our 9 million € effort of A-Cute-Tox (http://www.acutetox.org/), a well-designed series of animal experiments might demonstrate that the reference method is meaningless.

There is also strong evidence for involvement of immune cells likely activated by translocation of bacteria (Su, 2002; Nolan, 2010) in the effects of classic hepatotoxins (Laskin and Pendino, 1995; Leist et al., 1998; Luster et al., 2001): data exist for paracetamol, cocaine, nitrosamine, galactosamine, lead withdrawal, thioacetamide, α-amanitin, actinomycin D, diethyl-dithiocabarmate, phalloidin, CCl_4, cyproterone acetate, 1,2-dichlorobenzene, cadmium, allyl alcohol, heliotrine, ischemia-reperfusion, microcystine, and others. Often TNF, a key early response cytokine to LPS and bacteria released by Kupffer cells, the macrophages of the liver, is key here: for example, the toxicity of CCl_4, the prototype of a directly acting hepatotoxin, is inhibited by scavenging TNF with recombinant soluble TNF-receptor constructs (Czaja et al., 1994). Leist et al. (1997) proved the role of TNF and hepatocyte apoptosis during the poisoning of mice with α-amanitin or with actinomycin D, respectively, as these toxicities were also inhibited by passive immunization of mice against TNF. Inflammation-induced increases in susceptibility to toxicity are not limited to liver but, e.g., also sensitize to the toxic effects on the respiratory tract, kidney, and lymphoid tissue (Ganey and Roth, 2001).

Immunomodulation also plays a key role in carcinogenicity. Immunosuppressive activity is important as neoplastic cells frequently have antigenic properties that permit their detection and elimination by normal immune system function. Two decades ago, Luster et al. (1992) investigated the relationship

between chemical-induced immunotoxicity and carcinogenicity. The concordance between immunotoxicity and carcinogenicity for the 27 compounds in which sufficient data were available was 81% (p=0.019), suggesting that if a compound is immunotoxic it is likely to be carcinogenic. On the contrary, if a compound is not immunotoxic, the carcinogenic status is less clear (Luster et al., 1992). Furthermore, chemicals that are immunotoxic are more likely to result in multiple cancer sites than are non-immunotoxic compounds. These data are indicative of a close relationship between chemical induced immunotoxicity and the risk of cancer. Chemicals affecting the activity of NK cells, NKT cells, macrophages, $CD8^+$ cytotoxic T lymphocytes, or altering cytokine production are likely to compromise cancer immune surveillance (Luster et al., 1993). Suppression of normal immune function can reduce the effectiveness of this immune surveillance. As discussed earlier, some immunosuppressive treatments are in fact linked to increased (secondary) cancer. Thus, tests for immunotoxicity should form an integral building block for any integrated testing strategy for carcinogenicity (Basketter et al., 2012) to cover non-genotoxic mechanisms. Furthermore, inflammation is considered a key promotor of carcinogenicity (Philip et al., 2004; Mantovani et al., 2008) once cell transformation has taken place. Thus, proinflammatory immunomodulation will lead to the promotion of (pre)-neoplastic cells to cancer, and these should be part of any integrated testing strategy for carcinogenicity (Basketter et al., 2012).

Pyrogenicity, i.e., fever inducing effects but more generally induction of inflammation, might be considered a special form of immunotoxicity, although it is typically not produced by the test material but by contaminations, mostly bacterial endotoxins. A series of cellular methods based on the activation for cytokine release of blood monocytes or derived cells has been validated (Hermann et al., 2003; Gennari et al., 2004; Schindler et al., 2004, 2006; Hoffmann et al., 2005) and accepted for regulatory use. One of the authors has been particularly involved in the development of a whole blood pyrogen test (Hartung and Wendel, 1995b; Daneshian et al., 2009; Schindler et al., 2009).

The enormous potency of endotoxins (bacterial toxins are designed by evolution to damage) also might lead to another often-overlooked phenomenon: we rarely test for endotoxin contaminations of test substances. For example, recombinant proteins can absorb endotoxins that are then difficult to trace or remove (Wakelin et al., 2006). Similarly, nanoparticles can carry pyrogenic contaminations that are difficult to detect but biologically highly active (Jones and Grainger, 2009). Nanoparticles represent a most interesting test material because of their large surface area. The Limulus assay, the most prominent alternative pyrogen test, has problems testing solid materials. The whole blood pyrogen test instead works with a cell suspension, which might be especially suited to test nanoparticles (Hartung 2010c; Hartung and Sabbioni, 2011). To which extent other xenobiotics are contaminated with pyrogens is unclear; obvious candidates are all materials isolated from biological sources (such as herbal extracts), but hardly any synthetic chemist works under pyrogen-free conditions.

Not only sterile inflammation appears to play a role in the toxic effects of chemicals. Only slowly, the interplay of toxic damage and infection is beginning to be addressed (Ilbäck and Friman, 2007; Feingold et al., 2010). Taken together, the inflammatory component of various toxicities is underappreciated. It might be one of the components we lack when reproducing the hazardous effects of test substances *in vitro*. Including tests of immunomodulatory and pro-inflammatory effects of substances in integrated testing strategies (ITS) must thus be very strongly encouraged.

Consideration 6: The future of non-animal immunotoxicity testing

Many areas of toxicity are currently embracing new concepts, which are based on new technologies and the integrated use of information. The infamous NRC report *Toxicity Testing for the 21st Century: A Vision and a Strategy*, following the strategic plan for the US National Toxicology Program, has initiated tremendous efforts as have legislations in Europe on cosmetic and chemical safety as summarized elsewhere (Hartung, 2009b, 2010a,b). Slowly, a roadmap is evolving that can show us how to put the various elements together (Hartung, 2009a; Basketter et al., 2012). Immunotoxicology is left a little aside, as it is not a routine testing requirement for chemical safety, which prompted most of these developments. Unfortunately, the new European biocide and plant protection product legislation does not place the same emphasis on new and alternative methods as, for example, REACH (Ferrario and Rabbit, 2012). However, the field is starting to embrace the new concepts (Luebke, 2012). Opportunities lie especially in the mapping of pathways of toxicity (Hartung and McBride, 2011), integrated testing strategies (Hartung et al., 2012), and organotypic cultures, as promoted under human-on-a-chip approaches (Hartung and Zurlo, 2012). Their integration toward a systems toxicology is still only emerging (Hartung at al., 2012), but there is tremendous potential for immunotoxicology. A combination of various *in vitro* tests to predict *in vivo* immunotoxicology has never been attempted, though a relatively small number of endpoints appear to reflect animal immunotoxicity.

Immunotoxicology appears to be less a concern as a standalone health effect but more as a mechanism contributing to many, if not all, manifestations of toxicity of chemicals. Thus we see less potential of promoting new information requirements by test guidelines but rather want to encourage the inclusion of mechanistic tests into the ITS to reflect the adverse outcome pathways for most manifestations of toxicity.

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