Development, evaluation and prevalidation of an immunotoxicity test based on human whole blood cytokine release

Thesis

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Summary

There are several ways how xenobiotics can influence the human immune system. Besides being stimulated, resulting in sensitization, autoimmunity or inflammatory reactions, the immune system can also be suppressed, possibly leading to infectious disorders. Human whole blood was suggested to model the human immune system \textit{in vitro}. In fact, over the years it was shown that human whole blood responds to stimulants such as lipopolysaccharide (LPS) and staphylococcal enterotoxin B (SEB), by the release of cytokines and other immune mediators, similar to \textit{in vivo}.

The first publication of this thesis ‘Whole blood cytokine response as a measure of immunotoxicity’ develops the basic concept and illustrates opportunities of an whole blood cytokine response based immunotoxicity test. One of the experiments presented, shows that an \textit{ex-vivo} study of animal blood gives a representation of the \textit{in vivo} treatment with an immunomodulating agent. Circulating immune cells appear to reflect \textit{ex vivo} the immunotoxicity exerted \textit{in vivo}. Immunosuppressing compounds were shown to potently suppress cytokine responses to LPS \textit{in vitro}, as compared to higher concentrations required of non-immunotoxic compounds. In contrast, stimulation of cytokine release with LPS could be enhanced by pro-inflammatory cytokines in therapeutic use such as GM-CSF or IFN-\(\gamma\).

In the second publication of this thesis ‘Evaluation and prevalidation of an immunotoxicity test based on human whole blood cytokine release’, this concept of a immunotoxicity test based on human whole blood cytokine response was optimised, standardised and prevalidated. The potency of pharmaceutical compounds to modulate cytokine release by human blood monocytes and lymphocytes \textit{in vitro} was determined by measuring the release of interleukin 1\(\beta\) (IL-1\(\beta\)) and interleukin 4 (IL-4), respectively. It was shown that certain pharmaceutical agents
can also exert an immunostimulating effect on monocytes, resulting in an increased release of IL-1β. SC₄ (i.e. 4-fold stimulating concentration) values were determined to quantify this stimulating effect. The IC₅₀ (i.e. 50% inhibiting concentration) values of immunosuppressing compounds were determined and compared to in vivo peak plasma concentrations to see whether the IC₅₀ values would fall into the range of the therapeutic plasma concentrations. This held true in general, for the compounds that do not require metabolic activation and which do not cause cytotoxicity at the IC₅₀ value. Although most compounds were suppressing IL-4 release by lymphocytes more strongly, there were a few agents that suppressed IL-1β release by monocytes more pronouncedly, indicating that some compounds have a specificity for a certain immune function.

The test method was evaluated according to the guidelines for development and prevalidation of an in vitro alternative method. The inter- and intra-laboratory reproducibility was good, and correlation with human and animal in vivo data showed that the results of the test were meaningful for the in vivo situation. The results demonstrate the feasibility of such an human based in vitro immunotoxicity test. A standard operating procedure (SOP) was developed, describing the scope, limitations and methodology of the immunotoxicity assay, and can be found in Annex I of this thesis.

The immunotoxicity test method, as presented here, offers the possibility to screen compounds for immunotoxic properties against monocytes and Th2 lymphocytes of the immune system. Overall, the method has proven to offer reproducibility and transferability of results and to discriminate between immunotoxicants and non-immunotoxicants. Furthermore, also immunostimulating compounds can be identified. The procedure was evaluated and appears to be suitable for further validation of the method.
Deutsche Zusammenfassung

Xenobiotika beeinflussen das menschliche Immunsystem auf vielfältige Weise: Neben einer Stimulation, die zu Sensitivierung, Autoimmunität oder Entzündungsreaktionen führen kann, kann das Immunsystem auch supprimiert werden, was die Gefahr von Infektionen mit sich bringt. Die Verwendung von einem Modell für das menschliche Immunsystem auf der von humanem Vollblut wird hier vorgeschlagen; in der Tat konnte in den letzten Jahren gezeigt werden, dass humanes Vollblut auf Stimuli wie Lipopolysaccharid (LPS) und Staphylokokken Enterotoxin B (SEB) ähnlich mit der Freisetzung von Zytokinen und anderen Botenstoffen des Immunsystems reagiert wie \textit{in vivo}.


In dem zweiten Manuskript dieser Dissertation "Evaluation and prevalidation of an immunotoxicity test based on human whole blood" wurde diese Konzept eines Immuntoxizitätstestes basierend auf der Zytokinantwort von menschlichem Vollblut optimiert,
standardisiert und prävalidiert. Die Potenz verschiedener pharmakologischer Wirkstoffe, die Zytokinbildung von menschlichen Blutmonozyten und -lymphozyten in vitro zu modulieren, wurde durch Messung von Interleukin-1β (IL-1β) bzw. Interleukin-4 (IL-4) ermittelt. Es konnte gezeigt werden, dass bestimmte Pharmaka einen immunstimulatorischen Effekt auf Monozyten ausüben, was in einer gesteigerten Freisetzung von IL-1β resultiert. SC₁₄-Werte (d.h. die Konzentration, die zu einer vierfachen Steigerung führt) wurden ermittelt, um diesen stimulatorischen Effekt zu quantifizieren. Der IC₅₀-Wert (d.h. die zu 50% inhibitorische Konzentration) der immunsuppressiven Stoffe wurden bestimmt und mit den klinischen maximalen Plasma-Konzentrationen verglichen, um zu sehen, ob die IC₅₀-Werte in den Bereich der therapeutischen Spiegel fallen. Dies stimmt im allgemeinen für die Substanzen, die nicht durch Metabolisierung aktiviert werden und die in diesem Konzentrationsbereich noch nicht zytotoxisch sind. Obwohl die meisten Substanzen die IL-4-Bildung des Lymphozyten stärker hemmten, gab es doch einige Substanzen, die die IL-1β-Bildung des Monozyten deutlicher unterdrückten, was auf eine Spezifität einzelner Substanzen für die verschiedenen Immunfunktionen hinweist.

Der Test wurde entsprechend den Richtlinien für die Entwicklung und Prävalidierung von Ersatzmethoden zum Tierversuch evaluiert. Die Intra- und Interlaborvarianz war gering und die gefundene Korrelation mit in vivo-Daten von Mensch und Tier zeigen, dass die Resultate aussagekräftig für die in vivo Situation sind. Die Ergebnisse zeigen die grundsätzliche Machbarkeit eines solchen human-basierten in vitro Immunoxizitätstestes. Ein Standardprotokoll (SOP) wurde entwickelt, das die Anwendung, die Limitationen und die Methodik des Immunoxizitätstestes beschreibt; dies liegt als Anhang I dieser Dissertation bei. Die hier präsentierte Immunoxizitätsthemethode bietet die Möglichkeit, immunoxische
1 General Introduction

Xenobiotics (i.e. pharmaceutical compounds, environmental pollutants, pesticides etc.) can impair the functioning of the human immune system. Some xenobiotics impair the immune system unintended while others are designed to suppress the immune system, such as pharmaceuticals used for organ transplantations or for suppression of allergic reactions.

Human blood cells respond to certain stimuli. Endpoints of determination can be cellular (i.e. cell proliferation, cytotoxicity, NK activity, phagocytosis, antibody production, surface markers), on protein level (i.e. cytokines or enzymes) or molecular (i.e. intracellular calcium fluxes, activation of transcription factors, mRNA expression, gene chip or mRNA protection assay). Cytokine production can be measured easily with Enzyme Linked Immuno Sorbent Assays (ELISAs), and cytotoxicity with Alamar blue reduction. By comparing effects of compounds on cytokine release and on cell viability, it is possible to discriminate between general toxicity and specific immunotoxicity.

1.1 Human immunotoxicity

Immunotoxic compounds can cause adverse effects on the human immune responses against infections. There are two types of immunotoxic drugs, immune stimulators and immune suppressors.

Immunosuppressive drugs can induce two major types of adverse affects in direct relation to their pharmacological effects;

- Infectious complications
- Lymphomas and virally induced neoplasia

There are many pharmaceutical compounds known for their immunosuppressing action.

Immunostimulating agents can induce:

- Impairment of hepatic biotransformation pathways
- Flu-like reactions
- Acute cytokine syndrome
- Autoimmune diseases
- More frequent allergic reactions

Examples of immunostimulating agents are cytokines.

1.2 Cells of the human immune system

There are several blood cell populations present in human blood, each responding in different ways to immunologic triggers. Blood cells that play a role in the immuneresponse are the leukocytes. This blood cell population consists of lymphocytes, phagocytes and auxiliary cells.

1.2.1 Lymphocytes

Lymphocytes consist of T, B and null cells, and constitute 20-40% of the body’s white blood cells. They circulate in the blood and lymph and are capable of migrating into the tissue spaces and lymphoid organs. All three cell types are small (6µm), motile and non-phagocytic cells. B and T lymphocytes that have not interacted with antigen are resting cells in the G0 phase of the cell cycle. Without antigens these cells undergo programmed cell death. If an antigen is
present, they are rescued from cell death, and enter the cell cycle by progressing from G0 into G1 and subsequently into the S, G2 and M phase. When active they are 15µm diameter lymphoblasts. These cells have more cytoplasm and organellar complexity than small lymphocytes. The lymphoblasts proliferate and differentiate into effector- or memory cells.

B-cells are made in the bone marrow and contain membrane bound immune globulin’s (antibody), which serve as receptors for antigens. Most B cells express class II MHC molecules, the cell is also classified as an antigen-presenting cell (APC). Mature B cells also express receptors for complement. The B-cells produce antibodies.

T-cells have membrane receptors for antigen. These T-cell receptors are different from the IgG (antibodies) of the B-cells, but have some structural features in common most notably in the structure of its antigen-binding site. The T cell recognises an antigen only when it is associated with a self-molecule encoded by genes within the major histocompatibility complex (MHC). The antigen may be displayed together with MHC molecules on the surface of antigen-presenting cells or on virus-infected cells, cancer cells, etc. The T-cell system will eliminate the self-cells. T-cells express distinctive membrane molecules like Thy-1, CD4 and CD8. T cells that express CD4 recognise antigen associated with class II MHC molecules, whereas T cells expressing CD8 recognise antigen associated with class I MHC molecules. The CD4\(^+\) cells generally function as T helper (Th) cells and are class II restricted; CD8\(^+\) cells generally function as T cytotoxic (Tc) cells and are class I restricted. Th cells proliferate extensively following recognition of and antigen-class II MHC complex on an antigen-presenting cell. Th cells secrete a variety of cytokines, sometimes called lymphokines, which play a central role in
the activation of B, Tc, and a variety of other cells that participate in the immune response. The Tc cell is activated by interaction with an antigen-class I MHC complex on the surface of an altered self-cell (e.g. virus infected cell) in the presence of appropriate cytokines. This activation generates cytotoxic T lymphocytes (CTLs), which mediate killing of altered self-cells. Another subpopulation of T lymphocytes, called T-suppressor (Ts) cells, has been postulated.

Null cells fail to express the membrane molecules that distinguish B and T lymphocytes. These cells also fail to display antigen-binding receptors of either the T- or B-cell lineage and therefore lack the attributes of immunologic specificity and memory. One functional population of null cells called Natural Killer cells (NK) are large, granulated lymphocytes (LGL’s). NK cells play an important role in host defence against tumour cells. In some cases the NK cell makes direct membrane contact with a tumour cell in a non-specific, antibody-independent process. Some NK cells, express CD16, a membrane receptor for the carboxyl-terminal end of the antibody molecule. These NK cells can bind to anti-tumour antibodies bound to the surface of tumour cells and subsequently destroy the tumour.

1.2.2 Phagocytes

This cell population exists of neutrophils, monocytes and eosinophils. They serve to activate the lymphocytes, to increase the effectiveness of antigen clearance by phagocytosis, or to secrete various immune effector molecules.
Monocytes will circulate in the blood for 8 hours, and then they will migrate into tissues and differentiate into macrophages. They are normally in resting state, but in case of an infection they will become active. The activity can be further enhanced by cytokines secreted by activated Th cells, by mediators of the inflammatory response, and by bacterial cell-wall products. One of the most potent activators of macrophages is interferon gamma (IFN-\(\gamma\)), secreted by activated Th cells. The activated macrophages secrete various cytotoxic proteins and also higher levels of MHC class II molecules, allowing them to function more effectively as antigen-presenting cells. Thus macrophages and Th cells exhibit an interacting relationship during the immune response, each facilitating the activation of the other. The cell factors that are secreted by activated macrophages are;

- **Interleukin;** Induces activation of Th cells following interaction with antigen-MHC complexes.
  - Promotes inflammatory response and fever.
- **Complement proteins;** Promote elimination of pathogens and inflammatory response.
- **Hydrolytic enzymes;** Promote inflammatory response.
- **Interferon alpha;** Activates cellular genes resulting in the production of proteins that confer an antiviral state on the cell.
- **Tumour necrosis factor;** Killing of tumour cells.
- **Interleukin 6;** Promotes inducible hematopoiesis.
Neutrophils are produced in the bone marrow during hematopoiesis. They are released into the blood and circulate 7-10 hours before migrating into the tissues where they can survive for 3 days. Neutrophils are the first cells that will arrive at the site of inflammation. It are phagocytes that are able to kill bacteria with their lytic enzymes.

Potent stimulators of Neutrophils are IL-8, NAP-2 (neutrophil-activating peptide) and formyl-peptides (fMLP).

Eosinophils are mobile, phagocytic cells that can migrate from the blood into the tissue. Their major role is the defence against parasitic organisms. In infection the eosinophils secrete the contents of the eosinophilic granules which damages the parasitic membrane. These cells are significantly less important than the neutrophils.

### 1.2.3 Auxiliary cells

Basophils, mast cells and platelets belong to the auxiliary cell population. They excrete a variety of mediators that cause inflammation in surrounding tissues. Especially mast cells play an important role in allergy development. The main purpose of inflammatory reactions is to attract leukocytes and the soluble mediators of immunity towards a site of infection.

### 1.3 Immune response after infection with LPS or SEB

The immune response is depending on the kind of infection. Different antigens all cause activation of some kinds of blood cells, resulting in the production of different cytokines. For *in vitro* experiments these infections can be simulated by the addition of stimuli to human
whole blood and can be used to study several pathways in the immune response. Lipopolysaccharides (LPS), Staphylococcal Enterotoxin B (SEB) amongst others such as Concanavalin A, TSST, OKT3 etc. can be used to stimulate human blood cells *in vitro*.

### 1.3.1 Lipopolysaccharides

Lipopolysaccharides (LPS) is a B-cell mitogen. After activation by LPS several immune responses can occur;

*humoral response (antibody production)*

When antigen presenting cells like B lymphocytes, monocytes, macrophages, langerhans cells thymic, dendritic and epithelial cells are stimulated with LPS, these cells can interact with Th cells by presenting the antigen (LPS) on a MHC class II molecule on the cell membrane. When bacteria are involved namely Th2 cells are activated. Antigens taken up by B lymphocytes take 1-3 hours to appear with MHC class II on the cell surface, via early endosomes.

B-cell + LPS ----> B-cell + MHC class II-LPS complex

This complex will attach to a Th cell that contains the receptor for it. Interaction of Th cells with antigen (via MHC class II) initiates a cascade of biochemical events that induces the resting Th cell to enter the cell cycle (from G0 to G1 transition) and cumulates in expression of the high affinity receptor for IL-2 and secretion of IL-2. In response to IL-2 (and in some cases IL-4), the activated T-cell progresses through the cell cycle, proliferating and differentiating into memory cells or effector cell. Following antigen recognition, cytokines are produced. The
Th cell will excrete IL-2, IL-4, IL-5, IL-6 and IFN-γ. mRNA expression for IFNγ starts 30 minutes after antigen recognition. Interferon γ is then secreted.

B-cell + MHC class II-LPS complex + Th cells ----> IL-2, IL-4, IL-5, IL-6 and IFNγ

When this process is completed the B lymphocyte is able to differentiate further into plasma cells, which are excreting antibodies, and B-memory cells.

*bacterial septic shock*

Lipopolysaccharide can also cause bacterial septic shock. Macrophages will produce TNFα, IL-1, IL-6, IL-11, GM-CSF, G-CSF and M-CSF.

**1.3.2 Staphylococcal Enterotoxin B**

Staphylococcal Enterotoxin B (SEB) is an exogenous superantigen and is a T cell mitogen. It cross-links a T cell to a class II MHC molecule (CD4+/Th cells) in an antigen independent matter, resulting in the activation of a distinct set of Vβ-expressing T cells, namely human Vβ 3, 14, 15, 17 and 20. Estimates are that 1 out of 5 T cells can be activated by Staphylococcal Enterotoxins, resulting in the release of abnormally high levels of cytokines. The high levels released can *in vivo* lead to shock and death.

Cytokines produced by Th cells following SEB treatment are IFNγ, IL-2, IL-2r, IL-3, IL-4, IL-6 and IL-13.
When proliferation of Th cells is blocked (e.g. by alkylating agents) the production of cytokines (IFN$_{\gamma}$ amongst others) will be less. This will affect B-cell proliferation too, since one of the growth factors of B-cells is IFN$_{\gamma}$.

### 1.4 Mechanisms of immunotoxicity

There are several mechanisms of action of compounds, which may lead to immunotoxicity. These mechanisms can be general towards all immune cells, or can be more specific towards certain immune functions.

#### 1.4.1 General mechanisms of action

Some mechanisms of action, causing a suppression of cytokine release in all blood cells are now well known, such as regulation of gene expression, alkylation of DNA, inhibition of purine synthesis, inhibition of pyrimidine synthesis and inhibition of kinases and phosphatases. Each of the factors is essential for a well controlled immune response. Test compounds were chosen to cover each of these mechanistic groups.

#### 1.4.2 Specific mechanisms of action

Some mechanisms of action are more specific, and can result in the inhibition of cytokine response from certain blood cell types.

Chloroquine for example is an anti-malarial drug. It inhibits mitogen-induced lymphocyte proliferation and decreases leukocyte chemotaxis, lysosomal enzyme release and generation of
toxic oxygen metabolites. It also specifically reduces the generation of IL-1. Some of these effects may follow from the fact that it has a lysosomotrophic action, raising the pH of the lysosomes, particularly in phagocytic cells such as macrophages, and thus interfering with the action of the acid hydrolases. It may also intercalate in the DNA and inhibit DNA and RNA synthesis.

1.5 Importance of cytokine release

Cytokines are 15-28 kDa large, and are effective in pg/ng amounts. Cytokines travel from blood to the organs and back again to the blood.

The importance of cytokine release may be illustrated by analysing the effect of cytokines on the functioning of the T helper (Th) cell population. A shift between T helper cell populations may be caused by the cytokine profile of ‘natural immunity’ evoked by different offending agents (such as immunotoxic compounds).

Modulation of Th cell responses is one means by which xenobiotics may cause immunotoxicity. Th cells consist of Th1 and Th2 cells, which each produce their own cytokine pattern. The Th1 cells produce IFNγ, IL-2, and TNFβ, which mediate delayed-type hypersensitivity responses, provide help for the production of certain antibody subtypes (including complement-fixing antibodies), activate macrophages, and may be particularly important for dealing with antigens expressed on cell surfaces. Hence, Th1 cells appear to have a major role in host defences against viruses and other types of intracellular pathogens and against tumours. Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which are responsible for strong antibody production by promoting, IgG4, IgA, and IgE responses, enhance eosinophil
differentiation and inhibit several macrophage functions, thus providing phagocyte-dependent protective responses. These cells seem to be particularly important in responding to certain parasitic infections and also play a major role in immediate-type hypersensitivity, including reactions to common allergens such as pollen and dust mite and atopic asthma.

A shift from Th1 to Th2 responses can enhance both infectious and allergic disease. Many factors, including exposure to xenobiotic compounds, can modulate the balance between Th1 and Th2 cells. Cytokines produced by Th1 cells stimulate Th1 and inhibit Th2 development and proliferation.

1.6 Aim

The aims of this study were to evaluate if human whole blood cultures could be used for human immunotoxicity testing, by using a medium size throughput system and simple endpoints as cytokine and cytotoxicity measurements. The test-model was pre-validated by testing pharmaceutical compounds and determining the immunosuppression and/or immunostimulation towards monocytes and lymphocytes, quantitatively. The relevance of the immunotoxicity of the test compounds, as tested in vitro, was determined by comparing the in vitro results with in vivo plasma concentrations.
2 Whole blood cytokine response as a measure of immunotoxicity

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2.1 Abstract

Immunotoxicity, although increasingly recognized as a potential hazard, still lacks standardized \textit{in vivo} and \textit{in vitro} models. The considerable species differences and species-specific effects in immune responses prompt the development of human in vitro test systems. Immunotoxic reactions comprise \textbf{activation} (inflammatory processes, autoaggressive processes, pyrogenicity), \textbf{sensitization} (priming, idiosyncratic reactions) and \textbf{impairment} of immune responses (anergy, immunosuppression). We have previously studied a human whole blood system which allows the study of the release of inflammatory cytokines in response to a variety of stimuli. This model allows the assessment of this basic immune mechanism without preparation artifacts and relatively small inter-individual variances. We have used this model
previously to assess pyrogens, namely type (1) immunotoxic reactions. The model also proved to be suitable for immunopharmacological studies in vitro as well as ex vivo. Here, we studied the suitability of the test system to study type (3) immunotoxic effects. In order to also allow ex vivo studies, we have transferred the system to murine blood. This report summarizes our own use of this model with special emphasis on immunotoxicological studies. Our own listed bibliography gives access to the variety of applications of the human whole blood model since its introduction in 1982.

**Keywords:** Immunotoxicology; Leukocytes; Cytokines; Whole blood; Lipopolysaccharide (= endotoxin)

### 2.2 Introduction

The immunotoxicity of drugs and other compounds in human use is increasingly recognized as a potential hazard. To date, only very few in vivo and in vitro models are available to address immunotoxicity in a standardized manner. The considerable species differences in the response of the host immune system as well as the species specificity of some agents to be tested (e.g. biotechnology-derived products such as cytokines, antibodies, vaccines) prompt the development of human in vitro test systems.

In general, three types of immunotoxic reactions have to be considered:

1. **activation** of the immune system (inflammatory processes, autoaggressive processes);
2. **sensitization** (priming) of immune response mechanisms (idiosyncratic reactions);
3. **impairment** of immune response mechanisms (anergy, immunosuppression).
Pyrogenicity can be perceived as a prototype (1) case. Foreign material of either bacterial, fungal, viral or chemical origin initiates a sometimes adverse immune response (fever). Adverse side-effects of pro-inflammatory immunomodulators such as GM-CSF or interferon-\(\gamma\) can be taken as examples of type (2) reactions, which prompt a more vigorous response to subsequent stimulation but do not induce a reaction by themselves. However, classical idiosyncratic reactions such as allergic phenomena (e.g. hapten- or drug-induced T cell activation) cannot be mimicked by the model. For type (3), immunotoxic drugs such as cyclosporin, dexamethason and azathioprin are well-known model substances. The immune response varies considerably between different species. Unfortunately, human immune cell lines only partially reflect the responses of primary cells. As blood of healthy donors and patients is easily accessible, numerous studies have been carried out employing isolated human peripheral blood cells. The purification process, however, is very laborious and thus difficult to standardize. Furthermore, the highly sensitive leukocytes are easily affected and/or modulated by these procedures. Therefore, the simple approach of employing human whole blood is becoming increasingly popular.

Whole blood cytokine release (or more general mediator release since eicosanoids, NO or degranulation products can be determined using the same approach) was found to be more homogenous between different donors than the respective models using isolated cells. Apparently, the human organism has to control the responsiveness of its leukocytes very carefully: any hypo-responsiveness will result in infectious complications and any hyper-responsiveness in inflammatory disorders. A major concern against whole blood models is the inter-individual variation in leukocyte numbers. In fact, in healthy donors the normal range of leukocyte numbers is within a fairly tight window — few methods to count and adjust cell
numbers in routine practice are as precise. Furthermore, using a differential blood cell count, responses can be normalized to the number of a given leukocyte population in the experiment. A number of applications in pharmacology and toxicology have emerged in recent years: the model can be used employing a standardized stimulus (such as an endotoxin preparation) to assess in vitro any acute immunomodulatory properties of test agents (1-4). We currently evaluate the system for assessing direct immune toxicity as well as metabolism-mediated toxicity by adding P450-transfected cell lines to the system. In addition, it is possible to perform identical experiments with animal blood allowing comparison of the responses of different species.

As the model is very sensitive to stimulation by endotoxins [e.g. less than 10 pg/ml of the World Health Organization (WHO) reference preparation induces cytokine release] and reacts also to non-endotoxin pyrogens, we suggested employing this as a pyrogenicity test (5-10). This model actually uses the human fever reaction to determine pyrogenic contamination.

The model can be used to monitor immune functions ex vivo, for example, of volunteers treated with immunomodulatory agents (11-14) of patients exposed to toxins or of patients in order to characterize the course of disease as well as the effect of treatment. It has proven to be very advantageous that the same model can be used in vitro and ex vivo.

Notably, we have also developed a whole blood lymphokine response model employing bacterial superantigens and a simplified test version using prepared reaction vials which allow bedside testing in a hospital setting (15-19).

In conclusion, the very simple model of whole blood cytokine release offers a variety of opportunities to assess immune functions in an highly standardized manner. The predictive value of these approaches is currently under investigation.
2.3 Materials/methods/procedures

Heparinized blood from healthy donors was diluted 1+4 (v/v) immediately after venous puncture with cell culture medium RPMI 1640 (Biochrom, Berlin, Germany) or clinical grade saline for pyrogen testing. The latter allows only assessment of early cytokine release (e.g. IL-1β, TNF-α, IL-6). The standard stimulus was endotoxin, Lipopolysaccharides from Salmonella abortus equi (10 mg/ml) purchased from Sigma (Deisenhofen, Germany). Putative effectors were already included into the medium before the addition of blood. Incubations were carried out in open polypropylene reaction tubes (Eppendorf, Hamburg, Germany) at 37°C and 5% CO₂ for 24 h. Cell-free supernatants obtained by centrifugation at 3000 g for 1 min. were stored at -80°C until measurement. Mediators were measured with ELISAs based on commercial antibodies (Pharmingen, Hamburg, Germany).

GM-CSF was from Sandoz/Essex (Nürnberg/Munich, Germany, respectively) and IFN-γ was obtained from Thomae (Biberach, Germany). Immunotoxic (azathioprin, dexamethason) and general toxic controls (furosemid, galactosamin, paracetamol) were from Sigma. Cyclosporin A was obtained from Sandoz. All substances were dissolved in saline or dimethyl sulfoxide (DMSO) in final concentrations controlled to exert no effect on the blood cytokine response to endotoxin, that is, a maximum of 0.1% DMSO final concentration.

BalbC mice were obtained from the Tierforschungsanlage of the University. Murine blood was taken by heart puncture under terminal pentobarbital (Nembutal) anesthesia and injection of 300 IE heparin. The same incubation protocol was followed as described for human blood. IL-
11 (a gift from Schering-Plough, USA) was injected sc at 100 mg/kg in saline 24 h before blood withdrawal.

### 2.4 Results

Lipopolysaccharides (LPS) from Gram-negative bacteria is a well-characterised stimulus of inflammatory leukocyte responses such as TNF-\(\alpha\) release from monocytes and IFN-\(\gamma\) release from lymphocytes (Fig. 1).

![Fig. 1. Kinetics of the LPS-inducible cytokine release in human whole blood. Heparinized blood was withdrawn from five healthy donors, diluted fivefold in RPMI-1640 and incubated in the presence of 10 mg/ml LPS from S. abortus equi at 37°C at the time points indicated. TNF-\(\alpha\) and IFN-\(\gamma\) were determined in the cell-free supernatants by ELISA. Data represent mean ± S.E.M.](image)

When the known immunoactivators GM-CSF or IFN-\(\gamma\) were present in the incubations stimulated by LPS, a concentration-dependent increase in TNF-\(\alpha\) release was found (Fig. 2),
demonstrating the pro-inflammatory effect of either recombinant protein \textit{in vitro}. These agents represent pro-inflammatory immunomodulators.

![Graph showing cytokine release](image_url)

**Fig. 2.** GM-CSF and IFN-\(\gamma\) increase LPS-inducible TNF-\(\alpha\) release in whole blood. 20% whole blood was incubated for 24 h in the presence of 10 mg/ml LPS in the presence of human recombinant cytokines GM-CSF or IFN-\(\gamma\) at the concentrations indicated. Data represent mean cytokine levels formed of five donors.

When LPS stimulated incubations were carried out in the presence of various xenobiotics, the immunotoxic model compounds proved to be more immunosuppressive by several orders of magnitude compared to the general toxic compounds: dexamethasone blunted monokine (TNF-\(\alpha\), Fig. 3) and lymphokine (IFN-\(\gamma\), Fig. 4) release at nanomolar concentrations; Azathioprin as well as cyclosporin A significantly attenuated IFN-\(\gamma\) formation in the low micromolar range, while they had no effect on TNF-\(\alpha\) at this concentration, which is in line with their well-known effect on (proliferating) lymphocytes, namely the formation of the lymphokine IFN-\(\gamma\) but not of the monokine TNF-\(\alpha\) was attenuated. Comparable effects of
unspecific toxins were observed in the millimolar range only. The latter effects represent most probably cytotoxic reactions while no toxic effects of the low concentrations of dexamethasone, azathioprine and cyclosporin were observed employing FACS analysis (data not shown).

Fig. 3. Effect of toxic and immunosuppressive compounds on TNF-α release in LPS-stimulated whole blood. 20% whole human blood was incubated in the presence of 10 mg/ml LPS from S. abortus equi plus various compounds (black symbols indicate clinically used immunosuppressant agents, open symbols indicate agents without known immunotoxic effect). Data represent mean ± S.E.M. TNF-α release of blood from five donors determined by ELISA.
Fig. 4. Effect of toxic and immunosuppressive compounds on IFN-γ release in LPS-stimulated whole blood. 20% whole human blood was incubated in the presence of 10 mg/ml LPS from S. abortus equi plus various compounds (black symbols indicate clinically used immunosuppressant agents, open symbols indicate agents without known immunotoxic effect). Data represent mean ± S.E.M. IFN-γ release of blood from five donors determined by ELISA.

In order to also allow ex vivo studies, an analogous murine whole blood model was set up: the kinetics of TNF-α release showed a maximum at 4 h after LPS stimulation, which was chosen thereafter as the standard incubation time. When mice were pretreated sc with IL-11, the incubation of blood withdrawn 24 h later in the presence of LPS resulted in reduced formation of TNF-α (Fig. 5). Notably, the white blood cell count was not affected. This implies that IL-11 exerted an anti-inflammatory effect on monocytic TNF-α formation.
Fig. 5. LPS-inducible TNF-α-release in whole blood *ex vivo* from mice pretreated with IL-11. Mice were injected sc 100 g/kg of human recombinant IL-11 or human serum albumin as control 24 h before heparinized blood was withdrawn by cardiac puncture under terminal anesthesia. Blood was diluted fivefold by RPMI-1640 and stimulated in the presence of 10 mg/ml LPS from *S. abortus equi* for 4 h. Data represent mean ± S.E.M. of six animals.

### 2.5 Discussion

The putative hazard to humans by immunotoxic agents is a subject of increasing concern. As no standardized methodology is available, authorities have encouraged research in this area to set up appropriate test systems. In order to avoid additional animal utilization for this type of safety assessment, two approaches are feasible: the first strategy is to set up *in vitro* test systems preferably of a human nature. Here, we have adopted a practical test system based on primary human leukocytes, which are used increasingly in immunopharmacology for this purpose. We have previously documented the feasibility of this cell system for the detection of
immune stimuli such as pyrogens. Here, the effect of drugs and toxins in vitro on the response to a standard stimulus was used as a readout of immunotoxic properties. Two well-documented pro-inflammatory recombinant proteins (IFN-γ, GM-CSF) in clinical use were shown to increase the inflammatory cytokine release in vitro. In contrast, the immunosuppressive effects of three drugs on their respective target leukocyte population was demonstrated, namely the suppression of all mononuclear leukocytes by dexamethason and selectively of lymphocytes by cyclosporin A and azathioprin. These data encouraged a more detailed ongoing prevalidation carried out at the European Centre for the Validation of Alternative Methods (ECVAM, Ispra, Italy) under the supervision of Dr. Sandra Coecke. A careful comparison with a number of in vitro as well as in vivo studies will be necessary to estimate the predictive value of this model.

A second strategy is based on the implementation of additional endpoints to address immunotoxicity in animals used for general toxicity studies. These assays have to be very simple to allow performance in conjunction with histopathology. Whole blood incubations might offer this opportunity since they avoid any cell separation. Here we demonstrated this principle using IL-11, which is under development for a hematological indication. The ex vivo found immunosuppressive, anti-inflammatory, potential of this compound represents an interesting side-effect of the agent. Further developments of these assays will include the implementation of additional endpoints (e.g. granulocyte products, additional lymphokines) and transfer to a rat system (since rats are more commonly used for general toxicity). Furthermore, current studies at ECVAM include the combination of the model with drug-metabolising systems such as microsomes, primary hepatocytes and P450-transfected cell lines, which will further broaden the applications of the model.
3 Evaluation and prevalidation of an immunotoxicity test based on human whole blood cytokine release

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3.1 Abstract

Immunotoxicology is a relatively new field in toxicology of emerging importance because immunotoxicity appears to contribute to development of cancer, autoimmunity, allergy and other diseases. At present, there is a lack of human cell-based immunotoxicity assays to predict the toxicity of xenobiotics towards the immune system in a simple, fast, economical and reliable way. Existing immunotoxicity tests are mainly performed in animals while species differences are favoring human-based testing.

Whole blood cytokine release models have attracted increasing interest and are broadly used for pharmacological \textit{in vitro} and \textit{ex vivo} studies as well as for pyrogenicity testing. We adapted those methods for immunotoxicity testing, allowing potency testing of immunostimulating and immunosuppressive agents. After stimulation with lipopolysaccharide or staphylococcal enterotoxin B, interleukin-1\textbeta{} and interleukin-4 are released by monocytes and lymphocytes, respectively. 31 Pharmaceutical compounds, with known effects on the immune system, were used for the optimisation and standardisation of the method by analysing their effect on
cytokine release. The *in vitro* results were expressed as IC$_{50}$ values for immunosuppression and SC$_{4}$ values (4-fold increase) for immunostimulation and were compared to therapeutic serum concentrations of the compounds in patients and *in vivo* LD$_{50}$ values from animal studies. Results show that *in vitro* data are correlating with *in vivo* data and thus the test appears to reflect immunomodulation. Results were reproducible (CV = 20 ± 5%) and the method could be transferred to another laboratory ($r^2 = 0.99$). We therefore propose this method for further validation and for use in immunotoxicity testing strategies.

**Key words:** Human whole blood, cytokines, interleukin-1β, interleukin-4, monocytes, lymphocytes, immunotoxicity, immunosuppression, immunostimulation, immunomodulation, *in vitro, in vivo*, pre-validation.

### 3.2 Introduction

Immunotoxicology is an emerging field in toxicology that lacks versatile, standardised *in vitro* test models. Toxicity of a compound towards immune functions can contribute to autoimmunity, hypersensitivity reactions or direct immunotoxicity (20, 21). Autoimmunity and hypersensitivity are a result of immunostimulation, and testing is currently mostly performed *in vivo*. The most promising method for autoimmunity testing is the Popliteal Lymph Node Assay (PLNA) and for allergenicity testing the Local Lymph Node Assay (LLNA) (22). Because of the complexity of events leading to sensitisation, it is not yet possible to substitute these tests by an alternative *in vitro* test. Immunosuppression may lead to development of lymphoma’s and acute leukaemias (23). Direct immunotoxic effects can lead to both suppression and
stimulation of responses that are crucial for a normal immune response like inflammation, immune cell proliferation, cytokine production and antibody formation (20). In these fields, testing is mainly performed in vivo with complex methods such as histopathological determinations (OECD guideline # 407, adopted in 1981 and modified in 1995) (24-26), or ex vivo by determination of lymphocyte proliferation (27, 28) or antibody formation (29-31). Testing of cell-mediated immunotoxicity is regularly done with delayed-type hypersensitivity reactions and overall immunotoxicity is determined with host resistance assays in vivo (20).

Some in vitro tests exist for lymphocyte proliferation (32), antibody production and NK activity (33), but there is an urgent need for more validated, simple and human based in vitro tests for hazard identification (34-37). Here we test whether the simple in vitro human whole blood cytokine release model can judge the potency of a compound to alter primary functions of the human immune system, which is not feasible in vivo. Advantages of such human blood cell-based in vitro tests are:

1. Species differences between humans and animals are avoided.
2. Human primary cells are employed in their physiological proportions and environment, avoiding preparation and cultivation artifacts.
3. In vitro testing is less expensive and time-consuming than in vivo testing.
4. The number of compounds and concentrations tested can be increased.
5. The amount of substance required is dramatically reduced, allowing testing at earlier stages of drug development.
6. Effects on different blood cell populations can be tested in a single model.
7. Changes of cellular immune response can be quantified, enabling potency testing.

We propose here a new in vitro test that is developed in compliance with the criteria necessary
for further validation (38), which may be routinely used, is simple to perform and is human-based. The test is based on the well-known human whole blood method, adopted by us in 1996 for pyrogen testing (39), which uses the release of endogenous pyrogens (e.g. cytokines) in the presence of exogenous pyrogens to test medical devices, pharmaceuticals, vaccines etc. for contamination with pyrogens. Substances, that are toxic for the white blood cells, alter cytokine release in response to stimulation. Using whole blood for potency testing of immunotoxicity, with cytokine release by different blood cells as endpoint, allows assessment of the influence of chemical substances and can be measured easily with a sandwich ELISA (36, 40).
### 3.3 Materials and methods

#### 3.3.1 Principle of the method

When treating human blood with LPS or SEB, monocytes and Th2 lymphocytes will release IL-1β and IL-4, respectively. Cytokine release is measured in the supernatant after a 40-hour incubation time, in the presence or absence of immunotoxic and non-immunotoxic test compounds. Quantification of IL-1β or IL-4 with an ELISA and calculation of IC$_{50}$ or SC$_{4}$ values allows potency testing for immunotoxicity.

#### 3.3.2 Human whole blood incubations with test-compounds

The human whole blood method was modified for use in 96-well plates to allow a higher throughput. Blood from healthy volunteers was collected into heparinised tubes (Sarstedt, Verona, Italy), and was kept at room temperature for maximal 4 hours until use. Stock solutions of test compounds were prepared in pyrogen-free polypropylene reaction tubes (Eppendorf, Milan, Italy). Water-soluble compounds were solved in RPMI 1640 culture medium (Life Technologies, Milan, Italy), supplemented with 2.5 IU/ml heparin (Hoffmann La Roche, Grenzach-Whylen, Germany), 100 IU/ml Penicillin/Streptomycin and 200 mM L-glutamine (Life Technologies, Milan, Italy). The stock solution was 40-fold of the highest concentration, from which a serial dilution was made in pyrogen-free 24- or 96-well plates. Then 5 µl compound were transferred into 96-well plates, with a total volume of 155 µl cell culture medium in each well. If not water-soluble, the compounds were solved in ethanol or DMSO and a serial dilution in RPMI at 1.3 times of the final concentration was made in 6-well plates. 155 µl of these dilutions were pipetted into each well. Any problems with solubility
were documented. The final solvent concentration never exceeded 0.1%. Then 5 µl of 4 µg/ml LPS from *Salmonella abortus equi* (Phenol extract, Sigma, Milan, Italy) or SEB (Sigma, Milan, Italy) was added to each well in 96-well plates, resulting in a final concentration of 100 ng/ml. Finally, 40 µl human whole blood was added to each well (1 + 4 dilution) and the plates were incubated for 40 hours at 37 °C with 5% CO₂. On each plate, 7 concentrations could be tested in triplicate with 2 donors. The donor blood was controlled in several ways:

1) Blood was incubated in the presence of stimulus and two concentrations (0.002 and 0.008 µM for IL-1β and 0.04 and 0.005 µM for IL-4) of dexamethasone, which is known to inhibit all cytokine release strongly (41). This was done to control if the donor showed a normal cytokine release pattern. Blood in presence of dexamethasone should show cytokine release between 20% and 50% for the highest concentration and between 50% and 80% for the lowest concentration, as compared to the LPS control.

2) Blood was incubated without stimulus or test-compound to control spontaneous cytokine release. Values higher than 0, as calculated with the standard curve, indicated that the donor could not be used.

3) Blood was incubated with test compound, without further stimulus to control if the compound itself was capable of inducing cytokine release. If so, the compound might be contaminated with e.g. endotoxins, or might be stimulating cytokine release by itself.

After the blood incubation, 130 µl of the supernatant was collected and stored at –80 °C until ELISA analysis.
3.3.3 Cytotoxicity measurements

The rest of supernatant in the wells (70 µl) contained all blood cells, which were re-suspended by gentle tapping against the sides of the plate. Alamar Blue (Space, Milan, Italy) was diluted 10-fold with pre-warmed non-supplemented RPMI 1640 from which 150 µl were added to each well and incubated for 2 hours at 37 °C. Resorufin formation, as an endpoint for cell viability, was detected at 544 nm excitation and 590 nm emission wavelengths with the Ascent fluoroscan (Dasit, Cornaredo, Italy).

3.3.4 Cytokine measurements

Antibody pairs for IL-1β, IL-2, IL-8, IL-13, IFN-γ and recombinant cytokines were from Endogen, purchased from Tema Ricerca (Bologna, Italy). The antibody pair for IL-4 was from Pharmingen, purchased from Polymed (Florence, Italy), and peroxidase substrate solution was purchased from Dianova (Hamburg, Germany).

ELISA plates (Life Technologies, Milan, Italy) were coated overnight at 4°C with 50 µl/well coat antibody in 0.1 M NaHCO₃, pH 8.2. Plates were blocked with 200 µl/well PBS supplemented with 3% bovine serum albumine (Serva, Heidelberg, Germany), pH 7.0 for 2 h at room temperature. The plates were washed twice with PBS/0.05% tween-20. Sample (50 µl/well) and tracer-antibody (50 µl/well) in PBS/BSA 3% were added and incubated for 2 h. After 6 wash cycles, plates were incubated for 30 min. with streptavidin-peroxidase (Dianova, Hamburg, Germany; 1 µg/ml in PBS/BSA 3%, 100 µl/well). After 8 washes, 100 µl/well TMB liquid substrate solution (Sigma, Milan, Italy) was added and incubated at room temperature for 10 to 20 min. After addition of 50 µl/well stop solution (1 M H₂SO₄), absorption was
measured at 450 nm using a reference wavelength of 690 nm, with the Spectramax 250 spectrophotometer, and concentrations of cytokines were calculated with Softmax pro version 3.1 software (Molecular Devices, Milan, Italy). Detection limits for IL-1\(\beta\) were 7 pg/ml and for IL-4 1 pg/ml. Values lower than detection limit were set at 0.

### 3.3.5 IC\(50\) and SC\(4\) calculations

For IC\(50\) or SC\(4\) (i.e. the four-fold stimulating concentration) calculations, as a measure for immunosuppression or immunostimulation, respectively, the data had to meet following criteria:

- The LPS or SEB stimulated control value had to be higher than background.
- The linear part of the curve for the test compound had to contain at least 4 points.
- The goodness of fit in terms of \(r^2\) of the linear regression had to be at least 0.8.
- The IC\(50\) or SC\(4\) value had to be in the linear part of the curve.

When the data were consistent with these criteria, a linear correlation was calculated for the linear part of the curve, and the IC\(50\) or SC\(4\) values were calculated for each donor (Microsoft Excel).

### 3.3.6 Compounds used

*Presumed non-immunomodulating agents (42)*

Acetaminophen, Cyclophosphamide, Digitoxin, Digoxin, Dimethylsulfoxide (DMSO), Ethanol, Mizoribine, Sodium chloride, Ouabain, and Warfarin.
**Immunostimulators**
Cimetidine (43), Levamisole (44, 45), interleukin 2 (46), Interferon gamma (47), Isoniazid (48).

**Immunosuppressors**
Acrolein, Azathioprine, Chlorambucil, Chloroquine, Cyclosporin A, Dexamethasone, Fluorouracil, FK506, Leflunomide (49), Actinomycin D (42).

**Microtubule disrupting agents**
All compounds were purchased from Sigma in Milan, Italy, except for FK506 (Tacrolimus), which was generously provided by Fujisawa (Munich, Germany).

### 3.3.7 Sources of in vivo data

*In vivo* therapeutic plasma concentrations were obtained from original scientific publications, from which citations are available upon request. The *in vivo* LD₅₀ values were obtained from scientific publications, the dictionary of substances and their effects (54) and a database from the European Commission (55).

### 3.3.8 Statistics

Inter- and intra-donor variations in cytokine release were calculated with coefficients of variation (CV) in Microsoft Excel, by dividing the standard deviation by the mean.
Significance of this difference was tested by a one-way parametric ANOVA in Graph Pad Prism for windows, version 3.00 (San Diego, California, USA). Significance of correlation between IC$_{50}$ values of 2 laboratories, between IC$_{50}$ values of IL-4 and IL-1β release and between in vivo LD$_{50}$ and in vitro IC$_{50}$ values were calculated according to Pearson in Graph Pad Prism, and the correlation coefficient ($r_p$) was determined.

3.4 Results

3.4.1 Cytokine release after stimulation with LPS or SEB, choice of endpoints

For the determination of the suitable stimulus concentration, incubation time and selection of the endpoint, the release of six cytokines was determined during a time course from 15 to 100 hours. Human blood was stimulated with 0 to 500 ng/ml lipopolysaccharide (LPS) from Salmonella abortus equi or 0 to 500 ng/ml staphylococcal enterotoxin B (SEB), and cytokines produced by monocytes (IL-1β), Th1 lymphocytes (IL-2 and IFNγ), Th2 lymphocytes (IL-4 and IL-13) or several blood cell populations (IL-8) were measured. Incubations were performed at 37 °C with 5% CO$_2$. Maximum amounts of cytokine were formed after stimulation with 100 ng/ml LPS or SEB, which concentrations were used further on. After 40 hours incubation there was occasionally a spontaneous release of IL-8 and IFNγ and after 50 hours of IL-8 and IL-2 (data not shown). After stimulation with LPS, only IL-1β, IL-8, IFNγ and only very late IL-2 were released (Figure 1A) while stimulation with SEB released all six cytokines (Figure 1B). IL-4 and IL-13 were only detected after stimulation with SEB, indicating that those cytokines were only produced by lymphocytes. Although IL-4 was
released less, this cytokine was favored above IL-13 because release of IL-4 started earlier and it has a well-known effect on B lymphocyte activation and antibody formation. IL-1β was found in optimum amounts very soon after stimulation with LPS, while after stimulation with SEB it was produced in much smaller amounts with an optimum at longer incubation times, suggesting a direct stimulation by LPS and indirect by mediators released in response to SEB. IL-2 and IFNγ were released faster and in much greater amounts after stimulation with SEB, as compared to stimulation with LPS. IL-8 was detected in very high amounts after stimulation with both LPS and SEB. In previous work we showed that after stimulation with LPS, TNFα and IL-1β were exclusively produced by monocytes and IFNγ predominantly by cytotoxic T cells and that LPS-inducible IFNγ is depending on TNFα release (56). IL-1β was chosen as an endpoint for immunotoxicity against monocytes and IL-4 for immunotoxicity against lymphocytes. The time course experiments (Figures 1A and B) indicated that from 40 hours incubation onwards both IL-1β and IL-4 showed maximum levels.
Figure 1: Mean cytokine release after stimulation with 100 ng/ml LPS (A) or SEB (B), +/- standard deviation, for three blood donors.
3.4.2 Reproducibility

Cytokine release in LPS- or SEB-stimulated blood was determined on different dates, for 13 blood donors, to control if intra-donor differences were different from inter-donor differences. The average IL-1β release between donors varied from 220 to 940 pg/ml, and intra-donor variations had a coefficient of variation (CV) of 56% +/- 24%. No significant difference was found in cytokine release between most donors, as determined with a non-parametric, one-way ANOVA analysis. The average IL-4 release between donors varied between 10 and 76 pg/ml, and intra-donor variations had a CV of 18% +/- 11%. For this cytokine there was a significant difference in the amounts of cytokine release between donors. We wanted to know if the amount of cytokine release was influencing our IC₅₀ calculations. Therefore, the average IC₅₀ values of chloroquine for IL-4 release were calculated for 4 donors, from which there were sufficient IC₅₀ values (n ≥ 4). Variations in IC₅₀ values within those donors were low, with a CV of 20% +/- 5%, and the non-parametric one-way ANOVA confirmed that there was no significant difference in IC₅₀ values between donors.

3.4.3 Transferability

Several chemicals (chloroquine, chlorambucil, colchicine, cyclophosphamide, dexamethasone, FK506 and warfarin) were sent to the University of Konstanz (Laboratory B), where blood incubations were performed with the same protocol and material as in Ispra at the JRC (Laboratory A). There was a good correlation between IC₅₀ values from both laboratories for IL-1β (Figure 2A) and IL-4 (Figure 2B). The data were significantly correlating for both IL-1β and IL-4 with a coefficient of \( r_p^2 = 0.99 \), as calculated according to Pearson.
Figure 2: Correlation of IC\textsubscript{50} values from chlorambucil, chloroquine, colchicine, cyclophosphamide, dexamethasone and warfarin between the two laboratories, as measured for IL-1\(\beta\) (A) and correlation of IC\textsubscript{50} values from chloroquine, colchicine, cyclophosphamide, dexamethasone, FK506 and warfarin between Laboratory A (Ispra, Italy) and Laboratory B (Konstanz, Germany) as measured for IL-4 (B). Correlation was calculated with the mean if more than one replicate was measured.
3.4.4 Cytotoxicity versus immunotoxicity: identification of non-specific immunotoxicity

Cytotoxicity of compounds against blood cells was measured to determine if the test could discriminate between immunotoxicity and mere cytotoxicity and to identify compounds with cytotoxic mechanisms. Alamar blue reduction was used as a measure for cell viability (57). Isoniazid and 17β estradiol interfered with alamar blue, the other compounds allowed this cytotoxicity measurement. Chlorambucil (Figure 3A), acrolein, cyclophosphamide and DMSO were cytotoxic in the concentration range that was suppressing both IL-1β and IL-4 release in the supernatants. Acetaminophen, colchicine and ethanol were cytotoxic in the concentration range that was suppressing only the IL-1β release, while they showed lower IC₅₀ values for IL-4 release at non-cytotoxic concentrations. Chloroquine was cytotoxic in the concentration range that was suppressing only the IL-4 release. Dexamethasone represents an example of a compound that was not cytotoxic at immunotoxic concentrations (Figure 3B).
Figure 3: Examples of cytotoxicity and immunotoxicity towards monocytes and lymphocytes, as measured with alamar blue reduction and cytokine release for chlorambucil (A) and dexamethasone (B).

3.4.5 In vitro IC$_{50}$ values as a measure of immunosuppression, and their correlation with human in vivo serum concentrations

In order to establish a test system for immunotoxic potency, IC$_{50}$ values of pharmaceuticals including immunostimulators, immunosuppressors and compounds without suspected effect on immune functions were calculated. By determination of IC$_{50}$ values of compounds against both IL-1β and IL-4 release, information can be obtained about the specificity of compounds to suppress monocytes or lymphocytes, and thus the potency of the compound to suppress specific immune functions. When correlating results for IL-1β and IL-4 the IC$_{50}$ values were very similar (Pearson; p < 0.0001, r$_p$ = 0.91), suggesting a more general toxicity towards leukocytes instead of cell-type selectivity. However, chloroquine, cimetidine, dexamethasone,
levamisole and warfarin suppressed the IL-1β formation stronger, while the other agents, especially FK506, had more specificity for IL-4 formation (Figure 4A). The compounds digitoxin, digoxin, ouabain, mizoribine and vinblastine could not be plotted because they were suppressing IL-4 release and stimulating IL-1β release.

![Graph showing IL-4 versus IL-1β](image)

**Fig 4A:** Comparison of immunosuppression by compounds towards monocytes (IL-1β) and lymphocytes (IL-4). Triangles present immunomodulating compounds and circles non-immunotoxic compounds. The bisecting line indicates equality of IC_{50} values.
Based on these results it was decided to determine immunosuppression of compounds by measuring IL-4 release by lymphocytes, excluding the cytotoxic compounds. Immunotoxic compounds were found in 2 groups, group P with log IC$_{50}$ values $< 1$ µM and group Q with log IC$_{50}$ values $> 10$ µM. Non-immunotoxic compounds were also found in 2 groups, group R with log IC$_{50}$ values $< 1$ µM and group S with log IC$_{50}$ values $> 100$ µM (Figure 4B).

Figure 4B: Distribution of average Log IC$_{50}$ values of non-cytotoxic test compounds, with IL-4 release by lymphocytes as endpoint. Immunotoxic compounds are represented as triangles and non-immunotoxic compounds as other symbols.
as circles, as classified according to the information available in the literature. Cytotoxic compounds are excluded from the graph. AZA = Azathioprine and WAR = Warfarin.

Group R comprises the cardiac glycosides, which were presumed to be non-immunosuppressors, but in our test system they strongly suppressed IL-4 release by lymphocytes. Some IC$_{50}$ values of immunotoxic compounds from group Q were overlapping with non-immunotoxic compounds from group S: Colchicine (microtubule disruptor), cimetidine (known to increase lymphocyte proliferation), isoniazid (sensitizer that may cause autoimmunity) and levamisole (lymphocyte proliferation) were expected to stimulate the immune response in vivo (44, 48, 50, 58). Acetaminophen, mizoribine and warfarin are not known to have any immunosuppressing or stimulating activities in vivo (42). Thus immunostimulating compounds were not found to be immunosuppressing when measuring reduction in IL-4 release, underlining the reliability of the choice of endpoint.

In order to judge the relevance of immunotoxic effects, it was assumed that known immunosuppressing compounds from the clinic should exhibit IC$_{50}$ values below therapeutic plasma concentrations (Figure 5). All non-immunotoxic compounds were found in lower concentrations in human plasma than the IC$_{50}$ value. From the immunosuppressants, cyclosporin A, dexamethasone and FK506 were found in higher concentrations in plasma, in contrast to chloroquine, chlorambucil and azathioprine. Although cardiac glycosides were suppressing IL-4 release to a high extent (Figure 4B), they were found only in lower concentrations in plasma. Carrying out the same analysis for IL-1β release, chloroquine was picked up as positive as well, at non-cytotoxic concentrations. According to these data, we calculated a sensitivity of 67% and a specificity of 100% for the combined endpoints in the test. Please note that the number of test compounds is limited. Increasing sensitivity by
employing IC$_{25}$ values was not very effective (data not shown), however, did additionally allow classification of fluorouracil as immunosuppressing.

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<th>IC$_{50}$ IL-4 in vitro &lt; Therapeutic plasma concentration</th>
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<td>Non-immunotoxict</td>
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<td>Acetaminophen</td>
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<td>(Cyclophosphamide)</td>
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<td>Cyclosporin A</td>
<td>Dexamethasone</td>
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<td>FK506</td>
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Figure 5: Classification of immunosuppressing and non-immunotoxic test compounds, by the test method, compared to therapeutic plasma concentrations. Compounds in brackets were cytotoxic (Alamar blue reduction) at the IC$_{50}$ concentration for IL-4 suppression. Chloroquine was classified positive in the test based on the result for IL-1β only.

Interestingly, IC$_{50}$ values for both IL-1β and IL-4 correlated with LD$_{50}$ values in both rat and mice, with the best correlation between in vitro IL-4 and in vivo intravenous mouse values (Figure 6). Compounds that need metabolism before being immunotoxic, like cyclophosphamide and mizoribine, tend to be more toxic in vivo than in vitro. Vinblastine and colchicine, which are microtubule-blocking agents that stimulate IL-1β release, also seem to be more toxic in vivo than in vitro. On the contrary, ouabain, digitoxin and digoxin and cyclosporin A were found to be more toxic in vitro, as compared to in vivo LD$_{50}$ data.
### In vivo data (LD<sub>50</sub>)

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<th>IC&lt;sub&gt;50&lt;/sub&gt; IL-1β</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; IL-4</th>
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Figure 6: Correlation between *in vitro* immunotoxicity and *in vivo* general toxicity, as determined with correlations according to Pearson.

#### 3.4.6 In vitro SC₄ values as a measure of immunostimulation

As a measure for immunostimulation, we calculated the concentration at which IL-1β release was 4 times stimulated (SC₄ value) as compared to the LPS control. This threshold was deduced from the average and median stimulating effect of 20 compounds that showed any stimulating capacity (Figure 7A). Those compounds were found to stimulate IL-1β release with an average of 4.2-fold (n = 152), and a median value of 2.7-fold. The majority of values, namely 67% exerted a stimulation of 3.6-fold or lower. Therefore a 4-fold stimulation will only be reached by compounds that are specifically stimulating the immune response. Interestingly, some compounds stimulated LPS-inducible IL-1β formation at concentrations at which IL-4 release was decreased. From all test compounds, only recombinant IL-2 and IFNγ were found to increase release of both cytokines, others stimulated IL-1β release only. Therefore, IL-1β
release is suggested to be a suitable endpoint for detection of immunostimulating properties of compounds. In Figure 7B the SC$_4$ values of all compounds that increased IL-1$\beta$ release at least 4-fold at average are plotted against the extent of stimulation observed. Surprisingly, colchicine and digoxin exhibited such stimulatory effects. Therefore, more cardiac glycosides (digoxin, digitoxin and ouabain) as well as microtubule disruptors (colchicine, taxol, 17$\beta$ estradiol, 2-methoxy estradiol, nocodazole and vinblastine) were tested. The stimulatory effect extended also to the other agents with the same mechanism of action, suggesting that the immunostimulation can be related to the class of agents as a whole. Cardiac glycosides were stimulating at concentrations between 0.01 and 0.03 µM, and the microtubule blocking agents between 40 and 160 µM. The extent of stimulation was differing substantially for different compounds. From both SC$_4$ and the extent of stimulation it can be seen that the cardiac glycosides are the most potent stimulators of IL-1$\beta$ release. Besides cardiac glycosides and microtubule blocking agents, also DMSO, FK506 and mizoribine exerted an immunostimulatory effect and SC$_4$ values could be determined (Figure 7B, triangles). The IL-1$\beta$ immunostimulating agents were in general not found to be suppressing IL-4, except the cardiac glycosides, which had this opposite immunomodulatory effect.
Median at 2.7-fold stimulation

Average at 4.2-fold stimulation

n = 152,
20 compounds
Figure 7; Immunostimulation by test compounds.

Average and median stimulation of all stimulating compounds (A). Average immunostimulation (x-fold, n > 3) and SC₄ values of several compounds, as measured by IL-1β release by monocytes, +/- standard deviations where n > 3. Triangles represent compounds with various mechanisms, circles represent the cardiac glycosides and squares the microtubule blocking agents. Single SC₄ values for 17β estradiol, 2-methoxy estradiol, mizoribine and nocodazole. Downwards error bars were omitted, to keep a better overview (B).
3.5 Discussion

Potency testing for hazard identification of immunomodulating compounds becomes increasingly important, since it is now recognised that disturbances in immune response can contribute to severe diseases (59). Cytokines are released as one of the first steps of immune response and quantitative alterations can be used as a measure of immunomodulation. Depending on the type of inflammogen (stimulus), human blood cells release different cytokine patterns, originating from several blood cell populations. Stimulation with lipopolysaccharide (LPS) leads to the release of interleukin-1β (IL-1β) by the monocytes. Experiences from the development of the whole blood pyrogen test have shown that IL-1β, which induces inflammation, fever and septic shock, qualifies as an endpoint for monocytes with minor differences to IL-6 or TNFα. We have previously shown that employing SEB and prolonging the incubation period from 48 to 72 hours, the whole blood model can be extended to determine also the release of various lymphokines (60, 61). Staphylococcal enterotoxin B (SEB) is a superantigen that can link specific T cell receptor Vβ regions of the T-lymphocytes to MHC class II molecules present on antigen presenting cells (APC’s). This leads to the activation of both APC’s and T-lymphocytes and to release of various lymphokines such as IL-2, IL-4, IL-13 and interferon gamma (IFNγ) (62, 63). IL-4 was chosen as lymphokine read-out, since it is not produced by monocytes and reflects activation of B-cells by Th2-cells thus allowing assessment of the interplay of two major lymphocyte populations (64, 65). The results support that IL-1β and IL-4 are suitable endpoints for toxicity measurements against monocytes and lymphocytes, respectively. Because LPS is not capable of initiating the release of IL-4 or IL-13, we assume that it is not capable of stimulating the Th2 lymphocytes, which release these cytokines. Although IL-4 is produced by Th2 lymphocytes, eosinophils,
neutrophils and basophils (66-69), this cytokine appears to represent the performance of the Th2 lymphocytes, because the other blood cell populations do not possess T cell receptors (CD3), which are required for SEB activation. IL-4 increases major histocompatibility complex class II expression on antigen presenting cells and is therefore regarded as an important cytokine for antigen-specific immune response. The lack of tests studying immunotoxicity against this humoral immune function also favors the choice of IL-4 (64, 70).

In our test, lymphocytes were more sensitive for immunosuppression than monocytes (Figure 4A). This is most likely caused by the fact that lymphocytes are producing and releasing their cytokines later than monocytes, and the fact that they proliferate in vitro after stimulation with SEB, giving toxic compounds more time to exert any toxicity against the lymphocytes, resulting in lower IC_{50} values. However, results showed that certain compounds, such as chloroquine, suppress IL-1β release more than IL-4 release, showing more specificity for monocytes.

By determination of cytotoxicity, non-specific immunosuppressive compounds were identified and it was remarkable that acrolein, chloroquine, chlorambucil and cyclophosphamide were all alkylating agents. Their action in vivo is mainly on proliferating T and B-lymphocytes and antibody formation (49), which was not the endpoint of our method. The results suggest that alkylating agents have a mechanism of action that cannot be identified as immunotoxicity in the assay. Cytotoxic compounds will have to be tested for cytotoxicity against other cells e.g. fibroblasts, before immunotoxicity against blood cells can be excluded.

We have shown that the method is transferable to another laboratory, from which a high correlation of IC_{50} values was obtained for several test compounds. We found a difference in
amounts of cytokine production between donors, which is due to several factors e.g. amount of white blood cells, age, number of receptors, polymorphisms etc. However, these inter-donor differences had a minimal influence on IC₅₀ value determinations, and did not impair testing, indicating that the test method is robust.

We used information from databases and publications for the identification of suitable test compounds, on the basis of their clinical use to suppress or stimulate the human immune response, as well as drugs causing immunomodulation as an unwanted side effect. We compared the toxicity of those compounds with others that are known or at least presumed to be harmless for the immune system. Allison et al. (49) classified immunosuppressing pharmaceutical compounds as capable of regulating gene expression, alkylating DNA, blocking purine synthesis, blocking pyrimidine synthesis or blocking phosphatase and kinase synthesis. We determined immunosuppression, expressed as IC₅₀ values of IL-4 release, for most agents with these mechanisms of action in the following order from most to least immunosuppressing: 1) regulators of gene expression (actinomycin D and dexamethasone), which strongly inhibit both monokine and lymphokine release. 2) Inhibitors of kinase and phosphatase (cyclosporin A and FK506), which strongly inhibit lymphokine release and moderately inhibit monokine release. 3) Inhibitors of novo purine synthesis (azathioprine), which moderately inhibits both monokine and lymphokine release. 4) Inhibitors of de novo pyrimidine synthesis from which leflunomide moderately inhibits monokine release and fluorouracil was not immunotoxic. 5) Alkylating agents (acrolein, cyclophosphamide, chlorambucil, chloroquine), which were not found to be immunotoxic but cytotoxic.

The comparison between in vitro IC₅₀ values and in vivo therapeutic plasma concentrations, in order to examine the in vivo relevance of results, clarified that non-immunotoxicants were not
found to be immunosuppressing with our test method, indicating that there are little false positive results. Three out of six immunosuppressing compounds were found with lower concentrations in plasma than *in vitro*, namely chloroquine, chlorambucil and azathioprine. Chloroquine is a cytotoxic and alkylating agent, which is known to specifically inhibit IL-1β release (58). In fact it was picked up as immunosuppressing when IL-1β was taken as the endpoint. Chlorambucil is a cytotoxic and alkylating agent as well, which was not well soluble in the incubation mixture. Alkylating agents exert their effects mainly on proliferating cells (49), therefore cytokine release might not be a suitable endpoint for compounds with such mechanisms. Azathioprine is rapidly converted to the active metabolite mercaptopurine (71). Thus the therapeutic plasma concentration of the parent compound is underestimating the amount of active agents. Cyclosporin A (CSA), dexamethasone and FK506 were found in higher concentrations in plasma, than the IC₅₀ *in vitro*. CSA and FK506 are used in organ transplant patients, where the aim is a block of immune response, and have the same mechanism of action (72). FK506 (Tacrolimus) is *in vivo* 10 to 100 fold more potent than CSA (73), which was also the case in our *in vitro* test where the IC₅₀ values were 0.01 µM and 0.46 µM for FK506 and CSA, respectively. Lowering the IC₅₀ value of IL-4 release to IC₂₅ did additionally allow classification of only one compound (fluorouracil) as immunosuppressing, indicating that the detection limit is not the problem here.

Besides azathioprine, also leflunomide, cyclophosphamide, fluorouracil and mizoribine need metabolism to become immunotoxic. Also these compounds were found to be non-immunosuppressing by this test, because metabolism is lacking in the whole blood culture. In preliminary experiments, the introduction of microsomes showed no interference with the blood incubations, giving option for introduction of metabolism in the test system. On the
contrary, co-cultures of genetically engineered V79 cells, expressing cytochrome P450 enzymes, interfered with cytokine release by monocytes and lymphocytes (data not shown). When an overview of the actions of a compound against specific cytokines is required, also other cytokines besides IL-1β or IL-4 could be measured. Cimetidine for example, is a lymphocyte proliferating agent that stimulates IL-2 release via the H₂ receptor on T lymphocytes (74, 75). Lymphocyte proliferation can be stimulated in vitro by SEB and LPS, although first effects can be measured for LPS only after 3 days of culture (28). IL-2 is the cytokine important for the activation of proliferation of lymphocytes, and might therefore be an interesting endpoint (76).

Among the first set of test compounds the supposedly non-immunomodulating agents colchicine and digoxin were included. A careful review of the literature showed that microtubule blocking or disrupting agents such as colchicine interfere with gene expression of cytokines with IL-1β being elevated in patients (50-53). In vitro we also measured increase of IL-1β and stimulation could be extended to the other microtubule disrupters nocodazole, vinblastine, 2-methoxy estradiol, 17β estradiol and taxol. Cardiac glycosides like digoxin were found unexpectedly to be strongly stimulating IL-1β release and suppressing IL-4 release. The mechanism of action of these compounds is the inhibition of cellular sodium/potassium pumps, causing a decrease of K⁺ and an increase of Ca²⁺ in the cells (77). Matsumori et al. (78) described that the interleukin 1β level in mice, after treatment with cardiac glycosides, was clearly elevated. This supports our findings in vitro concerning the clear stimulation of IL-1β release by cardiac glycosides (Figure 7B). After further literature study it became clear that cardiac glycosides are also capable suppressing the proliferation of mononuclear cells (79). The in vitro results clearly show that those compounds are pronounced modulators of immune
response. One hypothesis for the strong immunomodulating capacity of cardiac glycosides is that they initiate release of histamine, which blocks IL-2 gene expression (Th1 lymphokine) and increases IL-1β release (74, 80). The release of histamine by basophils (0.5% of white blood cells) is initiated in vivo after an increase of cytosolic calcium (58), which is one of the effects that cardiac glycosides have on cells.

3.6 Conclusion

Taken together, our findings indicate that this in vitro method is capable of determining immunosuppression and immunostimulation, favouring IL-1β release for stimulation and IL-4 release for suppression of cytokines. When unknown compounds are tested as to their potency to modulate the immune response, and the mechanism is not known, only conclusions may be drawn from compounds that are showing clear immunosuppression or -stimulation. Compounds found to be non-immunosuppressing will have to be tested for metabolic activation, and for effects on additional endpoints such as antibody formation, lymphocyte proliferation and sensitisation, before it can be concluded whether the compound is immunotoxic or not.

The method as described here could be used for routine screening of compounds like pharmaceuticals, chemicals or pollutants. The method might be used in the future as a potency test for existing and new immunotoxicants. It is at the moment capable of determining the immunotoxic potency towards monocytes and Th2 lymphocytes, and extension to other cell types and measurement of other cytokines is very well possible, e.g. IL-2 or IFNγ release by Th1 lymphocytes, which are important for T-lymphocyte proliferation and monocyte activation, respectively. The test can distinguish between stimulation and suppression of
immune functions by test compounds, allowing classification of compounds into immunostimulators or -suppressors. It might also be possible to calculate No Observed Adverse Effect Levels (NOAEL’s) for test-compounds, towards cellular immune response. In general, compounds with IC$_{50}$ values lower than 250 $\mu$M and an absence of evidence for cytotoxicity are suspected to be immunosuppressors.

We think that this test qualifies for validation and that it might represent an alternative or filter for \textit{in vivo} tests used so far. With such a test, immunosuppressing or -stimulating agents could be identified in an early stage of drug development. Since this \textit{in vitro} test determines the potency of compounds to suppress monokine or lymphokine release, it would fit into an \textit{in vitro} tier testing program together with determinations of lymphocyte proliferation, antibody formation and NK cell activation \textit{in vitro}.

3.7 Acknowledgements

We want to thank Gregor Pinski for his excellent technical help with the inter-laboratory transferability study and protocol optimisation.
4 Summarising discussion

The aim of this thesis was to adopt the model of stimulated human whole blood cytokine release for the detection of immunotoxic properties of compounds. The characteristics of the cellular system were explored, the test set-up was optimised and standardised. Next, the performance of the assay including intra- and inter-laboratory variability was assessed. Employing a variety of pharmacological agents and comparing \textit{in vitro} immunotoxicity data with literature data on their clinical use as well as toxicity in animals, the assay was prevalidated. Since the method fulfilled the prerequisites for further validation, a standard operating procedure (SOP) was deduced, which can be found in Annex I.

Human whole blood, in culture between 15 and 100 hours, was shown to produce and release cytokines \textit{in vitro} after stimulation with stimuli such as lipopolysaccharide (LPS) or staphylococcal enterotoxin B (SEB). Stimulating with LPS and subsequently measuring interleukin 1\(\beta\) (IL-1\(\beta\)) or tumor necrosis factor alpha (TNF\(\alpha\)) in the supernatant of the blood incubation, represents a measure of the activity of the monocytes. When stimulating with SEB and measuring interleukin 4 (IL-4) or interferon gamma (IFN\(\gamma\)), the activity of the Th2 or Th1 lymphocytes can be assessed, respectively. Measurement of cytokine release with a sandwich ELISA method offers a simple and quantifiable endpoint, which may be used routinely for large numbers of samples. The \textit{in vitro} test developed here makes use of this endpoint for the determination of toxicity of compounds towards the human immune system.

In a series of pilot experiments, it was shown that pharmaceutical compounds such as dexamethasone, cyclosporin A and azathioprine inhibit cytokine release by monocytes and lymphocytes. Cyclosporin A and azathioprine had a differential effect on monocytes and
lymphocytes, showing a stronger inhibition of the lymphokine IFNγ. Compounds having a more general toxic effect on cells, such as furosemid, paracetamol and galactosamine, inhibited cytokine release at 1000-fold higher concentrations. This led to the suggestion that immunotoxicity of compounds is detectable in vitro, with such a human based assay, thus avoiding species differences. Also ex vivo the effect of compounds on the immune system could be measured, as shown by treatment of mice with IL-11 and subsequent stimulation of blood ex vivo with LPS. Cyclosporin A, inhibiting IFNγ and IL-4 release by lymphocytes, did not have any effect on IL-1β or TNFα release by monocytes, in the concentration range used, after incubation with SEB or LPS, respectively. Release of monokines upon SEB stimulation appears to be mediated by the lymphokines, as suggested by the fact that lymphokines and monokines were always affected in parallel. Since lymphokines are released much later after stimulation with LPS (IL-2) or in smaller amounts (IFNγ), as compared to stimulation with SEB, this also supports the notion that LPS is specifically stimulating monocytes. Although a variety of cytokines could be measured in the supernatants of blood incubations, interleukin 1β and interleukin 4 were chosen as endpoints for determining effects on monocytes or lymphocytes, respectively. These two cytokines were favored because of their pronounced and well-defined effects in vivo. IL-1β for example plays a key role in the inflammatory reaction, by activating almost all immune cells. Similarly, IL-4 is a cytokine which is a very important mediator of the humoral immune response. When required, the testing for immunotoxicity against Th1 lymphocytes could also be included easily by determination of IL-2 or interferon gamma (IFNγ) in the supernatant of the blood incubations, after stimulation with SEB.
By measuring cytotoxicity by alamar blue reduction in parallel with impairment of an immune function such as cytokine release, a simple characterization of the immunotoxic properties of a compound can be achieved. Therefore, alamar blue reducing capacity of the white blood cells was measured after each blood incubation, making it possible to identify compounds exerting cytotoxic effects on the blood cells. For such compounds, incubation with another non-immune cell line, such as fibroblasts, might be necessary to test if the immune cells are more sensitive for the toxic compounds, in order to identify immunotoxicity by a cytotoxic mechanism of this compound.

Results of immunosuppression by compounds were expressed as IC$_{50}$ values. The 50% inhibition of compounds was found to be a sensitive measure of immunotoxicity, because when lowering sensitivity to 25% (IC$_{25}$) inhibition, only one additional compound out of 21 could be picked up as immunotoxic. The results of two laboratories (ECVAM in Ispra, Italy and University of Konstanz, Germany) were compared and it was concluded that the method was transferable and results were reproducible. There were some differences in coefficients of variation, but this may be attributed to the different levels of routine experience with the assay of the operators performing the blood incubations.

GM-CSF and IFN$_{\gamma}$ have shown in the pilot experiments to cause a concentration dependent increase of monokine release (TNF$\alpha$) after stimulation with LPS, when present in the blood incubations. Stimulation with 10 $\mu$g/ml LPS caused strong stimulation of monokine release, but still GM-CSF and IFN$_{\gamma}$ were capable of increasing the monokine release. This is showing the importance of pro-inflammatory cytokines in the immune response. Surprisingly, two compounds which were present in the first series of test compounds for prevalidation showed
the same effect on monokine release, as measured by IL-1β. When incubating more compounds of the same therapeutic class in the human blood test, it became apparent that cardiac glycosides and microtubule disrupting agents in general cause immunostimulation in a dose-dependent manner. For immunostimulating compounds the SC₄ value was determined, and there was a remarkable correlation between mechanism of action and SC₄ value. The percentage of increase was varying strongly between donors and compounds. The compounds alone were not capable of inducing cytokine release by monocytes or lymphocytes. It still has to be figured out what the clinical relevance of this increase is. For a patient receiving treatment with one of these compounds this would imply that when an infection occurs, the immune system will react more strongly, which might even favor clinical complications such as septic shock. This knowledge could also be used when using blood for pyrogenicity testing of materials or solutions. Compounds that increase cytokine release by monocytes (such as cardiac glycosides or microtubule blocking agents) during stimulation with the pyrogen, could make the testing for pyrogens more sensitive.

When comparing IC₅₀ values to the in vivo plasma concentrations of patients, it was found that compounds that were known to be immunosuppressants were determined as immunosuppressing in vitro in 67% of the cases. This result is only an indication of the relevance of in vitro results, and performance of the test. Although 31 compounds were tested, we could not find the in vivo plasma concentrations for all compounds, and not all compounds were suppressing the cytokine release for at least 50%. Furthermore, a compound may be activated to a more toxic metabolite in vivo, while metabolism is absent in vitro. The cardiac glycosides are not known to be immunotoxic, although they suppressed lymphokines and
stimulated monokines strongly, suggesting that these compounds should be considered as immunotoxic. Cardiac glycosides are very toxic in vivo, and therefore were found in lower plasma concentrations than the in vitro IC\textsubscript{50} value. The mechanism of action by these compounds should be investigated more in depth, to be able to conclude if these compounds should be regarded as immunotoxic, at relevant doses. Overall the test is capable to determine the potency of immunosuppression by compounds, and comparison with in vivo plasma concentrations gives a good indication of the correlation of the in vitro results for prediction of in vivo toxicity. IL-4 and IL-1\beta have to be both determined when testing for immunosuppressing capacity of compounds, because some compounds (such as chloroquine) have shown to suppress IL-1\beta release more specifically. IL-1\beta has also shown to be a good endpoint for determining immunostimulation of compounds.

IC\textsubscript{50} values for both IL-1\beta and IL-4 correlated with LD\textsubscript{50} values in both rat and mice, with the best correlation between in vitro IL-4 and in vivo intravenous toxicity in mice. Compounds that need metabolism before being immunotoxic, like cyclophosphamide and mizoribine, tended to be more toxic in vivo than in vitro, showing that metabolism may cause an increase in toxicity. In preliminary experiments, the introduction of microsomes showed no interference with the blood incubations, giving the option for introduction of metabolism in the test system. Vinblastine and colchicine, which are microtubule-blocking agents that stimulate IL-1\beta release, also seemed to be more toxic in vivo than in vitro. On the contrary, ouabain, digitoxin and digoxin as well as cyclosporin A were found to be more toxic in vitro, as compared to in vivo LD\textsubscript{50} data. Compounds which are found to be much more immunotoxic in vitro, than
generally toxic in vivo, form potential candidates for having immunotoxic side-effects, which is again pointing at the cardiac glycosides.

For the development of an in vitro alternative method for animal experiments, guidelines were created by ECVAM for test development, prevalidation and validation of an alternative method, and these were followed carefully. The following steps were taken; (1) First, the basis of the method was described, which is the use of cytokine release by human whole blood for the characterisation of compounds capable of altering this cytokine release by suppression or stimulation. (2) The scientific purpose of such a test is to allow a qualitative and quantitative testing of compounds, of which the immunotoxic properties should be identified. (3) The test is relevant, because no test exists at the moment using cytokine release for detection of alterations in the immune responses. Cytokines released by circulating blood cells, are essential for the immunological defense of an individual. Therefore, alterations in cytokine release will have consequences for both cellular and humoral immune responses. Compounds of all classes (pharmaceuticals, chemicals, pollutants, etc.) can be incubated with whole blood, to determine their effect on cytokine release. Other in vitro methods for immunotoxicity testing exist, employing lymphocyte proliferation, NK-cell activity or antibody formation as endpoint, and are often non human-based. (4) There is a need for such a test because there is a lack of in vitro tests in the field of immunotoxicity and no human-based in vitro test exists at the moment, which is easy to perform, allows to address large sample numbers, and is quantitative. Furthermore, the test was adopted to 96-well plates, and only simple pipetting steps have to be done before incubation. This should allow even high-throughput screening. (5) The application of this in vitro test is to use it for testing of environmental xenobiotics or
pharmaceutical compounds, from which a governmental institution or pharmaceutical company institution needs to identify the effects on the human immune system. By using human or animal blood, species differences can be determined. Furthermore, the test could be used when the effect of drug treatment on the immune system of a patient has to be monitored for research or clinical purposes. This *ex vivo* determination of the effects on cytokine release before and after drug treatment gives answers about toxicity of the compound or effectiveness of the treatment. 

6) A **protocol** was developed and optimised, and a standard operating procedure was deduced.  

7) The **endpoint** for this immunotoxicity test is cytokine release by monocytes (IL-1β) and lymphocytes (IL-4). Cytokines can be easily and quantitatively measured with a sandwich ELISA method. For potency testing the results are expressed as IC₅₀ values for immunosuppression (IL-4 or IL-1β) and as SC₄ values for immunostimulation (IL-1β). By parallel determination of cytotoxicity with alamar blue reduction, non-specific cytotoxicity can be identified. When high-throughput screening is required, a few concentrations of the compounds would be sufficient to characterise the immunosuppressing potency. This application has not yet been fully explored, and more immunotoxic compounds have to be tested to define the concentration ranges at which a compound should be considered as strong-, moderate- or non-immunotoxic.  

8) As a **control**, donor blood is incubated with and without stimulus, to identify individuals with spontaneous cytokine release (e.g. due to infection). Furthermore, blood is incubated with two concentrations of dexamethasone, which have to alter cytokine release in a defined pattern. In addition, the test compound itself is incubated with donor blood, without further stimulus, to test whether it is capable of inducing cytokine release on its own. 

9) **Limitations** of the test are that it is not possible to test compounds which are not soluble in water, DMSO or ethanol. At the moment, only the acute effect of a
test-compound on cytokine release is measured, chronic immunotoxicity testing is not possible. (10) **Reproducibility**, despite the use of different donors, was found to be acceptable. IC\textsubscript{50} values were not significantly different between donors, although the extent of cytokine release between donors was. This indicated that differences in amounts of cytokine production between donors do not influence the potency testing of the compounds. (11) **Transferability** towards another laboratory was possible. Although results were slightly different, no significant difference in IC\textsubscript{50} values between two laboratories could be detected.

Because this test method fulfills all the requirements needed for a good test development, already 31 compounds were tested, and the outcome of results shows that the results obtained are meaningful for the human *in vivo* situation, this test is believed to be a good candidate for a validation study with a positive outcome.
5 References


Potency testing of immunomodulating compounds using cytokine release by human blood cells as endpoint.

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1 INTRODUCTION

The immune system is one of the targets for xenobiotics. Most types of immune cells are found in the blood, from where they give signals for further immune response in case of any confrontation with antigens or pathogens, or are being mobilised to sites of infection. Compounds and their metabolites are transported through the blood before being eliminated from the body via urine, lungs or faeces, making blood cells an easy target. Many pharmaceutical compounds are administered intravenously, which makes the blood cells even the first to be in contact with the xenobiotics, and in the highest concentrations.

Pharmaceutical compounds may be administered to patients with the purpose to suppress or stimulate the immune response, but can also cause immunomodulation as an unwanted side-effect. Any unwanted quantitative change of an otherwise qualitatively normal immune response is called immunotoxicity (Descotes, 1999). It is very important to know which xenobiotic compounds are immunotoxic, and with which potency, since immunomodulation is related to the development of cancer, autoimmune diseases and allergic reactions.

Testing for immunotoxicity is to date mainly performed in vivo with complex methods such as histopathological determinations (OECD guideline # 407, adopted in 1981 and modified in 1995) (Institóris et al., 1998; Gopinath, 1996; Vos et al., 1998), or ex vivo by determination of lymphocyte proliferation or antibody formation (Ladics et al., 1995; Dean, 1997). Some in vitro tests exist, but there is an urgent need for more validated, more simple and human based in vitro tests that can give answers to specific questions (Ghezzi, 1994; Tucker et al., 1982; Langezaal et al., 2001, Balls et al., 2001). Therefore, we propose this new test that is developed in compliance with the criteria necessary for pre-validation as described by Curren et al., that may be routinely used, is simple to perform and is human-based.

The method proposed in this SOP is capable of measuring toxicity against the cellular and indirectly the humoral immune response. It is based on the cytokine release by monocytes and lymphocytes in human whole blood after stimulation with Lipopolysaccharides and Staphylococcal enterotoxin B, respectively (Katial et al., 1998; Krakauer, 1999; Ulmer et al., 2000). To be able to discriminate between immunomodulation and adverse cytokine production caused by cytotoxicity, alamar blue reduction can be determined as a measure for metabolic capacity of the cells (O'Brien et al., 2000). When alamar blue conversion is not reduced while the cytokine release is changed, the compound specifically alters the immune response using a non-cytotoxic mechanism.
2 PURPOSE

The present method may replace or add to existing immunotoxicity tests that determine the toxicity of compounds towards the cellular immune response. It is human-based, using the release of interleukin 1β and interleukin 4 as a measure for toxicity against monocytes and lymphocytes, respectively.
3 SCOPE / LIMITATIONS

This test is developed for immunotoxicity potency testing of compounds.

Compounds that are not water-soluble may be tested, but the exact amount of compound causing immunomodulation is then difficult to determine accurately.

Immunostimulating compounds may be contaminated with pyrogens, which are able to stimulate the blood cells to produce cytokines. When the stimulating capacity is higher than 4-fold only, this will be regarded as specific immunostimulation instead of contamination.

Solvents (DMSO or ethanol) in which the compounds are solved should not exceed a final concentration of 0.1%. A solvent control should always be taken up into the experimental design. Ether should be avoided as a solvent because it is too volatile, which makes it impossible to produce an accurate concentration range.

The stimulus is added together with the compound. Therefore, only the acute effect of the drug against the blood cells will be measured. When the stimulus is added later or earlier, other effects of the compound against the blood may be found.

IL-1β release by monocytes occurs earlier than IL-4 release by lymphocytes. The compounds therefore will have more time to exert their toxicity against lymphocytes, resulting in generally lower IC₅₀ values.
4 METHOD OUTLINE

A compound is tested for its immunomodulating capacity by adding different concentrations to a 1:5 diluted human whole blood culture in a microtiter plate. A comparison is made with the reference compound dexamethasone from which two concentrations are placed on the same microtiter plate. After stimulating the blood with lipopolysaccharide (LPS, a monocyte activator and B-cell mitogen) or staphylococcal enterotoxin B (SEB, a superantigen), IL-1β and IL-4 respectively will be produced by healthy cells, which makes it possible to measure the effect of the compound on the monocytes and lymphocytes, respectively. After an incubation of 40 hours, supernatants are collected and the amount of cytokines is determined using an ELISA method. The amount of cytokines can be quantitatively measured with the spectrophotometer at a wavelength of 450 nm. Supernatants are removed and stored at –80 ºC until detection of the cytokines with ELISA measurement. The remaining blood cells are re-suspended in an alamar blue solution (1:10 diluted in medium) and are incubated for 2 hours at 37 ºC in which time the mitochondria of the white blood cells will metabolise alamar blue into resorufin. This metabolite can be measured at 544 nm excitation and 590 nm emission wavelength.
### 5 DEFINITIONS / ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>degree centigrades</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>AB</td>
<td>Alamar blue</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal Enterotoxin B</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 Beta</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immuno Sorbent Assay</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumine</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumine</td>
</tr>
<tr>
<td>NADH</td>
<td>Adenosine 5´-(trihydrogen diphosphate)</td>
</tr>
<tr>
<td>POD</td>
<td>Streptavidin Peroxidase</td>
</tr>
<tr>
<td>RCF</td>
<td>Gravity (g)</td>
</tr>
<tr>
<td>M, mM, µM</td>
<td>Molar, milli molar, micro molar</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethyl-benzidine</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% inhibiting concentration</td>
</tr>
<tr>
<td>SC₄</td>
<td>4-fold stimulating concentration</td>
</tr>
<tr>
<td>FW</td>
<td>Formula weight</td>
</tr>
</tbody>
</table>
Annex I

6 MATERIALS

6.1 Whole blood incubations

Solutions

- Human heparinised blood from healthy donors
- RPMI 1640 Medium (Life Technologies, Milan, Italy, cat. nr. 31870)
- Heparin-Natrium 5000 U/ml (Hoffmann La Roche, Grenzach-Whylen, Germany)
- L-glutamine (Life Technologies, Milan, Italy, cat. nr. 25030)
- Penicillin Streptomycin (Life Technologies, Milan, Italy, cat. nr. 15070)
- LPS (Sigma, Milan, Italy, cat. nr. L 1887)
- SEB (Sigma, Milan, Italy, cat. nr. S 4881)
- NaCl (Sigma, Milan, Italy, cat. nr. S 9888)
- Human Serum Albumin (Sigma, Milan, Italy, cat. nr. A 3782)
- Dexamethasone watersoluble (Sigma, Milan, Italy, cat. nr. D 2915)

Materials

- 96-well mictrotiter plates with low-evaporation lids (Corning, Milan, Italy cat. nr. 3595)
- Pyrogen-free 6- and 24-well plates (Corning, Milan, Italy cat. nrs. 3506 and 3524)
- S Monovettes with lithium heparin (Sarstedt, Verona, Italy, cat. nr. 01.1608.001)
- Multifly (Sarstedt, Verona, Italy, cat. nr. 85.1638.035)
- Pyrogen free tips of 1000 and 200 µl (Eppendorf, Milan, Italy, cat. nrs. 0030.001.303 and 0030.001.311 (single packed), 0030.065.514 (200 µl in boxes))
- Pyrogen free Eppendorf cups of 1.5 ml (Eppendorf, Milan, Italy, cat. nr. 0030.121.589)
- Calibrated pipettes of 1000, 200, 100, 20 and 10 µl (Gilson, other companies are accepted)
- Calibrated multichannel pipettes of 50 and 200 µl (Labsystems, Cornaredo, Italy. Other companies are accepted)
- Multipette plus (Eppendorf, Milan, Italy)
- Combitips plus of 0.1 and 1 ml (Eppendorf, Milan, Italy, cat. nrs. 0030.069.404 and 0030.069.439)
- Sterile and pyrogen-free reservoirs (Corning, Milan, Italy, cat. nr. 4870)
Technical equipment

- Incubator with 5% CO₂ and 37 °C (Heraeus Hera Cell, Hanau, Germany, other incubators are accepted)
- Sonifier (Microson, ultrasonic cell disruptor)
Annex I

6.2 ELISA determinations

Solutions

- PBS
- PBS/BSA3%
- Tween 20 (Sigma, Milan, Italy, cat. nr. P 7949)
- KH₂PO₄ (Merck, Rome, Italy, cat. nr. 4873)
- NaH₂PO₄.H₂O (Merck, Rome, Italy, cat. nr. 6580)
- KCl (Merck, Rome, Italy, cat. nr.4936)
- NaCl (Sigma, Milan, Italy, cat. nr. S 9888)
- Streptavidin Peroxidase (Jackson immunoresearch)
- Anti human IL-1β coat, 1.04 mg/ml (Endogen, Tema Ricerca, Rome, Italy, cat. nr. M-421B-E)
- Biotin labeled Anti human IL-1β tracer (Endogen, Tema Ricerca, Rome, Italy, cat. nr. M-420B-B)
- Anti human IL-4 coat, 0.89 mg/ml (Endogen, Tema Ricerca, Rome, Italy, cat. nr. P-451-E)
- Biotin labeled Anti human IL-4 tracer (Endogen, Tema Ricerca, Rome, Italy, cat. nr. M-450-B)
- Bovine Serum Albumin (Boehringer, Ingelheim, Germany, cat. nr. 11930, lot 10204)
- H₂SO₄ (Merck, Rome, Italy, cat. nr. 100731)
- 3,3',5,5'-tetramethyl-benzidine (TMB) solution (Sigma, Milan, Italy, cat. nr. T 0440)

Materials

- Nunc Immuno plates (Life Technologies, Milan, Italy cat. nr. 439454)
- Non-sterile reservoirs (Corning, Milan, Italy, cat. nr. 4870)
- Humid chambers

Technical equipment

- Spectramax 250 spectrophotometer for microtiter plates (Molecular devices, Milan, Italy. Also other spectrophotometers are accepted)
- Computer program for the calculations of cytokine concentrations (Softmax Pro, Molecular devices, Milan, Italy)
- Microtiter plate washer (Tecan, Mannedorf, Switzerland, cat. nr. F 029011, also other washers are accepted)
6.3 Alamar blue reduction (cytotoxicity)

- Alamar blue (Space, Milan, Italy cat. nr. BUF012B)
- RPMI 1640 (Life Technologies, Milan, Italy cat. nr. 31870)
- Fluorometer with a 544 emission and 590 excitation filter (Labsystems, Fluoroscan Ascent, type 374, Dasit, Cornaredo, Italy. Also other fluorometers are accepted)

6.4 Analysis

- Computer with Microsoft excel (any version later than 1998) and SOFTmax® PRO (Version 3.0, Molecular Devices).
7 METHODS

7.1 Whole blood incubations

When testing a new compound for its immunomodulating capacity, the concentration range in which the compound is immunotoxic should be determined. This procedure describes first how the optimum concentration range should be defined and second, how the IC\textsubscript{50} value of the compound can be determined.

7.1.1 General preparations

1) RPMI complete medium:

- 484.75 ml RPMI 1640 medium
- 10 ml L-glutamine (200 mM), 4 mM final concentration
- 5 ml Pen-strep (5000 U/l)
- 250 µl Heparin-natrium (5000 U/ml)

This medium has to be stored at 4 °C and may be used for 2 weeks.

2) LPS and SEB solutions:

- Make NaCl 0.9% in pyrogen-free water. Add 1 g Human Serum Albumin to 100 ml of this solution (1% HSA).
- Solve 1 mg LPS in 1 ml NaCl/1% HSA solution. This has to be stored at –20 °C and may be thawed over and over again for 2 years.
- Solve 1 mg SEB in 1 ml pyrogen-free water, to get a 1 mg/ml solution. This has to be stored at 4 °C and may be used for 1 year.

3) Dexamethasone (Reference compound)

- Solve dexamethasone (FW = 392.5 g) in RPMI complete medium.
  Use water-soluble dexamethasone, which is containing 66.8 mg dexamethasone per gram. Solve 5.876 mg into 1 ml RPMI complete medium to get a 1 mM solution.

- Make 40 times concentrated solutions with 0.002, 0.005, 0.008 and 0.04 µM final concentrations (see table 1)
Annex I

<table>
<thead>
<tr>
<th>Final concentration (µM)</th>
<th>40 × concentrated stock (µM)</th>
<th>Amount of Dexamethasone</th>
<th>Amount of RPMI (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04</td>
<td>1.6</td>
<td>16 µl (1 mM)</td>
<td>9.984</td>
</tr>
<tr>
<td>0.008</td>
<td>0.32</td>
<td>2 ml (0.04 µM)</td>
<td>8</td>
</tr>
<tr>
<td>0.005</td>
<td>0.2</td>
<td>2 ml (0.008 µM)</td>
<td>1.2</td>
</tr>
<tr>
<td>0.002</td>
<td>0.08</td>
<td>2 ml (0.005 µM)</td>
<td>3</td>
</tr>
</tbody>
</table>

*Table 1; Concentrations of the reference compound dexamethasone.*

<table>
<thead>
<tr>
<th>96-well plate</th>
<th>96-well plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI complete medium</td>
<td>RPMI complete medium</td>
</tr>
<tr>
<td>20 ml</td>
<td>20 ml</td>
</tr>
<tr>
<td>LPS or SEB (4 µg/ml)</td>
<td>LPS or SEB (4 µg/ml)</td>
</tr>
<tr>
<td>400 µl</td>
<td>Blood</td>
</tr>
<tr>
<td>3.2 ml</td>
<td>Blood</td>
</tr>
</tbody>
</table>

*Table 2; Example of volumes needed per 96-well plate*

7.1.2 Procedure whole blood incubations

Use pyrogen-free tips, tubes and plates for every step.

4) Collect blood in heparinised S monovettes with a multifly needle. Store the blood at room temperature and use it within 4 hours from collection. The donors have to be in good health, not suffering from any bacterial or viral infections (including colds and influenza), and to have been free from any such infection for a period of two weeks prior to the donation of blood. Blood donors are not to be taking non-steroidal anti-inflammatory drugs, immunosuppressants, glucocorticoids or any other drugs known to influence the production of cytokines.

5) Solve the test compound in the appropriate solvent, preferably the medium from step 1. If the compound is not water soluble, it needs to be solved in solvents like ethanol, methanol or DMSO.

Water-soluble compounds;
Prepare a stock solution of minimal 20 mM, in RPMI complete medium.

Non water-soluble compounds;
Prepare a stock solution of minimal 1 M because the solvent concentration should not exceed 0.1%.
6) Use the stock solution of the test compound to make concentration range A or B for water-soluble or non-water soluble compounds respectively (Tables 1A or B). When the compound is not very well water soluble, use the 1.29 fold concentrated serial dilution (table 1A) and add 155 µl of the compound to each well. If the compound is not soluble at these concentrations, the final concentration of test compound should be decreased.

<table>
<thead>
<tr>
<th>Concentration number</th>
<th>A 5 µl/well (× 40) (µM)</th>
<th>155 µl/well (× 1.29) (µM)</th>
<th>Final concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0.0129</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>0.129</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>400</td>
<td>1.29</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>2000</td>
<td>12.9</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>4000</td>
<td>129</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>40000</td>
<td>1290</td>
<td>1000</td>
</tr>
<tr>
<td>7</td>
<td>400000</td>
<td>12903</td>
<td>10000</td>
</tr>
</tbody>
</table>

Table 1A: Concentration ranges that should be used for range finding experiments of water-soluble test compounds. Concentration range A may be 40 or 1.29 fold concentrated.

<table>
<thead>
<tr>
<th>Concentration number</th>
<th>B 155 µl/well (× 1.29) (µM)</th>
<th>Final concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.129</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>1.29</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>12.9</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>64.5</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>129</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>645</td>
<td>500</td>
</tr>
<tr>
<td>7</td>
<td>1290</td>
<td>1000</td>
</tr>
</tbody>
</table>

Table 1B: Concentration range B that should be used for range finding experiments of non water-soluble test compounds.

7) Label two 96-well plates for IL-1β (stimulation with LPS) or IL-4 (stimulation with SEB).
8) First add medium, reference compound dexamethasone and test compound to the 96-well plates. Use the volumes from the pipetting schedules in table 2A or B, depending on the solubility of the compound.

<table>
<thead>
<tr>
<th>40-fold concentrated range</th>
<th>Wells + LPS or SEB</th>
<th>Unstimulated wells (controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI complete Medium</td>
<td>150 µl</td>
<td>155 µl</td>
</tr>
<tr>
<td>Test compound</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Stimulant (LPS or SEB)</td>
<td>5 µl</td>
<td>-</td>
</tr>
<tr>
<td>Blood</td>
<td>40 µl</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

*Table 2A; Pipetting schedule for 40-fold concentrated test compounds in the wells of the 96-well plates that are going to be used for IL-1β and IL-4 determinations.*

<table>
<thead>
<tr>
<th>1.29 fold concentrated range</th>
<th>Wells + LPS or SEB</th>
<th>Unstimulated wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>-</td>
<td>5 µl</td>
</tr>
<tr>
<td>Compound</td>
<td>155 µl</td>
<td>155 µl</td>
</tr>
<tr>
<td>Stimulant (LPS or SEB)</td>
<td>5 µl</td>
<td>-</td>
</tr>
<tr>
<td>Blood</td>
<td>40 µl</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

*Table 2B; Pipetting schedule for 1.29-fold concentrated test compounds in the wells of the 96-well plates that are going to be used for IL-1β and IL-4 determinations.*

In figure 1 the position of the samples is shown.
### Annex I

#### Figure 1; Incubation schedule for LPS or SEB-stimulated whole blood incubations. In the supernatants IL-1β or IL-4 will be detected respectively.

* = µM Dexamethasone. 0.002 and 0.008 µM for IL-1β plates (stimulation with LPS) and 0.005 and 0.04 µM for IL-4 plates (stimulation with SEB)

9) Before use, sonify the LPS stock solution for 30 seconds at the highest capacity, and bring 8 µl (1 mg/ml) into 1992 µl complete medium to get 4 µg/ml. Add 5 µl of this LPS solution immediately to each well with a multipette, to get 100 ng/ml final concentration.

Add 8 µl SEB (1 mg/ml) to 1992 µl complete medium medium to get 4 µg/ml. Add 5 µl of this SEB solution to each well to get 100 ng/ml final concentration.

10) Finally add 40 µl donor blood to each well with a multipette, two donors on each 96-well plate and incubate for 40 hours at 37 ºC in an incubator with 5% CO₂.

<table>
<thead>
<tr>
<th></th>
<th>Donor 1</th>
<th>Donor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>0 µM</td>
<td>0 µM</td>
</tr>
<tr>
<td>X</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>X</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>X</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>X</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>X</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>X</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>X</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>
11) Transfer 130 µl supernatant with a multi channel pipette to a fresh and labeled 96-well plate. Change tips for every sample row, and store at –80 °C until determination of the cytokines with an ELISA.

### 7.2 Alamar blue reduction

1) Pre-warm un-supplemented RPMI 1640 medium. Dilute alamar blue solution 10 times with the medium.

2) Tap against all sides of the sample plates to resuspend the blood cells that are left in the 96 well plates after the blood incubations (see step 11 from chapter 7.1.2.)

3) Add 150 µl to the blood cells and incubate for 2 hours at 37ºC at 5% CO₂.

4) Detect resorufin at 544 nm excitation and 590 nm emission wavelength with the fluorometer.

### 7.3 ELISA determination

#### 7.3.1 General preparations for the ELISA

1) Coating buffer: 0.1 M NaHCO₃, pH 8.2
   Add 8.4 g NaHCO₃ to 1 liter MilliQ water. Adjust the pH to 8.2.

2) PBS:
   Add to 10 liter MilliQ water; 80 g NaCl
   11.6 g Na₂HPO₄·2H₂O
   2 g KH₂PO₄
   2 g KCl
   Adjust the pH to 7.

3) Blocking solution, PBS/3%BSA:
   Add 30 g BSA to 1 litre PBS, prepared in step 2. Mix and adjust the pH to 7.0. Then filter the solution, aliquot into 200 ml plastic bottles and store at -20 °C for maximum 1 year.

4) Washing buffer, PBS/tween 20:
   Add 5 ml tween 20 to 10 litre PBS.
Annex I

Store at room temperature for maximum 2 weeks.

5) Reconstitute the IL-1β recombinant protein (5 µg/vial) in 100 µl distilled water (= 50 µg/ml). Dilute further to 50 ng/ml (5 µl of 50 µg/ml in 5 ml PBS/BSA 3%). Aliquot into 1 ml sterile eppendorf tubes, 60 µl per tube and store them in the freezer (-80 ºC).

6) Reconstitute the IL-4 recombinant protein (5 µg/vial) in 1 ml distilled water (= 5 µg/ml). Dilute further to 10 ng/ml (10 µl of 5 µg/ml in 5 ml PBS/BSA 3%). Aliquot into 1 ml sterile eppendorf tubes, 50 µl per tube and store them in the freezer (-80 ºC).

7) POD (Streptavidin-Peroxidase); Solve 1 mg into 1 ml MilliQ water.

8) Stop solution, 1M H2SO4; Add 27 ml H2SO4 to 473 ml MilliQ water.

7.3.2 Procedure for the IL-1β ELISA

9) Coat ELISA immuno plates with anti-human IL-1β that should be used at a final concentration of 1 µg/ml. Add 5.3 µl (1.04 mg/ml) coating antibody to 5.5 ml coating buffer and bring 50 µl into each well. Tap gently against the sides of the plate until the fluid is equally distributed to the bottom of the wells. Leave the plates overnight in the fridge at 4 ºC in a humid chamber.

10) Remove the coat by flicking and add 200 µl blocking solution (PBS/BSA3%) to each well. Incubate for 2 hours in a humid chamber at room temperature.

11) Prepare the standard curve;
    IL-1β: Add 540 µl PBS/BSA to the 60 µl aliquot to get a 5000 pg/ml solution. Dilute this solution 1 + 2 by taking 200 µl of 5000 pg/ml and adding 400 µl PBS/BSA (=1667 pg/ml).
    In total there are 8 standard solutions, that will be added in duplicate to rows 1 and 2 on the 96-well plates.

15) Prepare the tracer solution;
    IL-1β tracer: Pipet 2.2 µl biotin labeled monoclonal IL-1β antibodies (0.5 mg/ml) into 5.5 ml PBS/BSA to get a 0.2 µg/ml solution.

16) Wash the plates 2 times with PBS-tween 20, using the ELISA plate washer.

17) Add 50 µl tracer to each well.

18) Add 50 µl standard solution to the first two rows of the 96 well plates (duplicate values). Such standard curve should be taken on every plate.

19) Add 10 µl sample to rows 3 to 12. Add 40 µl PBS/BSA3% to dilute the samples 5 times.

20) Incubate for 2 hours at room temperature in a humid chamber.

21) Wash the plates 4 times with wash buffer using the ELISA plate washer.

22) Add 2.5 µl (1 mg/ml) POD solution to 50 ml PBS/BSA3% to obtain a 50 ng/ml solution. Add 100 µl of this solution to each well and incubate for 30 minutes at room temperature.
23) Wash 8 times with wash buffer, using the ELISA plate washer.
24) Add 100 µl per well TMB substrate solution and incubate for 10 minutes at room temperature in the dark. Control the colour development (blue) and stop the reaction when the complete standard curve is well visible. After 10 minutes control how far the colour development is and stop the reaction when the standard curve is completely visible.
25) Stop the reaction by adding 50 µl 1M H₂SO₄ to each well. Avoid bubbles.
26) Measure the OD at 450 nm with the Spectramax spectrophotometer for microtiter plates.

7.3.3 Procedure for the IL-4 ELISA

27) Coat ELISA immuno plates with anti-human IL-4 monoclonal antibody. IL-4 coat should be used at a final concentration of 3 µg/ml, so add 18.54 µl (0.5 mg/ml) to 5.5 ml coating buffer. Add 50 µl coating antibody per well and tap the plates gently until the fluid is equally distributed on the bottom of the wells. Leave the plates overnight in the fridge at 4 ºC in a humid chamber.
28) Remove the coat by flicking and add 200 µl blocking solution (PBS/BSA3%) to each well. Incubate for 2 hours in a humid chamber at room temperature.
29) Prepare the standard curves;
   IL-4: Add 950 µl PBS/BSA to the 50 µl aliquot to get a 500 pg/ml solution. Dilute this solution 1 + 1 by taking 500 µl of 500 pg/ml and adding 500 µl PBS/BSA (=250 pg/ml). Dilute until 7.8 pg/ml and use 0 pg/ml as the blank.
   There are 8 standard solutions per cytokine.
30) Wash the plates 2 times with PBS-tween 20, using the ELISA plate washer.
31) Add 50 µl standard solution to the first two rows of the 96 well plates (duplicate values). Such standard curve should be taken on every plate.
32) Add 50 µl sample to rows 3 to 12. Incubate for 3 hours in the incubator of 37ºC.
33) Prepare the tracer solutions;
   IL-4 tracer: Pipet 2.75 µl Biotin labeled monoclonal IL-1β antibodies (0.5 mg/ml) into 5.5 ml PBS/BSA to get a 0.25 µg/ml solution.
34) Wash the plates 2 times with wash buffer using the ELISA plate washer.
35) Add 50 µl tracer antibody to all the wells and incubate for 45 minutes at room temperature.
36) Wash the plates 4 times with wash buffer using the ELISA plate washer.
37) Dilute 1 mg/ml POD solution with PBS/BSA3% (2.5 µl, 1 mg/ml + 50 ml PBS/BSA) to obtain a 50 ng/ml solution. Add 100 µl of this solution to each well and incubate for 30 minutes at room temperature.
38) Wash 8 times with wash buffer, using the ELISA plate washer.
39) Add 100 µl per well TMB substrate solution and incubate for 15 minutes at room temperature in the dark. After 15 minutes check how far the colour development is and the reaction should be stopped when the standard curve is completely visible.
40) Stop the reaction by adding 50 µl 1M H₂SO₄ to each well. Avoid bubbles.
41) Measure the OD at 450 nm with the Spectramax spectrophotometer for microtiter plates.
8 DATA ANALYSIS AND ASSOCIATED ERRORS

8.1 Determination of cytokine content

Measure the cytokine production at 450 nm in a 96-well plate spectrophotometer, using SOFTmax® PRO (Version 3.0, Molecular Devices) and a 4-parameter fit for the standard curve. According to the standard curve, the program will calculate the amount of cytokine that is found in the samples in pg/ml. It is possible, but not necessary, to multiply the amount of cytokine found in the samples by the dilution factor of the blood (5-fold) or by the dilution factor of the sample (in case of IL-1β 5-fold).

8.1.1 Primary controls

Control if the samples that were not stimulated with LPS or SEB or incubated with test-compound have a value equal or lower than the background. If this is not the case the donor might have an infection, or an error in the ELISA occurred. Repeat the complete blood incubation or only the ELISA measurement.

Control if the samples that were not stimulated with LPS or SEB but contain test compound are stimulating the cytokine release. This could indicate a contamination of the test-compound with LPS, SEB or another stimulating compound.

Control if the samples that were incubated with stimulus, but without test compound, contain a detectable cytokine level. If not, the donor is not producing sufficient amounts of cytokines or, an error occurred in the ELISA. Repeat the complete blood incubation or repeat the ELISA measurement only.

Control if the reference compound dexamethasone is capable of reducing the cytokine release to around 70% and 30%. Historical data (n ≥ 80) of the suppression of IL-1β and IL-4 by dexamethasone, gave the following values;

IL-1β: 0.002 µM; 78% +/- 26%
        0.008 µM; 33% +/- 16%

IL-4:  0.005 µM; 65% +/- 21%
        0.04 µM; 25% +/- 18%

Half (50%) of the cytokine release without sample should fall in between the two dexamethasone values.
Example;

<table>
<thead>
<tr>
<th>Sample</th>
<th>IL-1β (pg/ml)</th>
<th>IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM test compound</td>
<td>1000 (500 = 50%)</td>
<td>50 (25 = 50%)</td>
</tr>
<tr>
<td>0.002 µM Dexamethasone</td>
<td>800</td>
<td>-</td>
</tr>
<tr>
<td>0.008 µM Dexamethasone</td>
<td>400</td>
<td>-</td>
</tr>
<tr>
<td>0.005 µM Dexamethasone</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>0.04 µM Dexamethasone</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 3; Example control values. 50% of the 0 concentration should fall in between the dexamethasone control values.

If those controls are done, the data may be used for determination of IC₅₀ or SC₄ values.

8.1.2 IC₅₀ and SC₄ determinations

For the determination of IC₅₀ or SC₄ values, the data should be imported into Microsoft Excel.

1) Create an X-Y scatter graph from the data.
2) In this program it is possible to create a trendline through the data points, from which a formula for linearity will be calculated automatically, and the coefficient of correlation can be displayed (R²).
3) Lay the trendline through the linear part of the curve.

To be able to calculate the IC₅₀ or SC₄ values of compounds, as a measure for immunosuppression or immunostimulation, respectively, the choice of concentrations is very important. For this quantification the data should meet following criteria;

- The non-treated, LPS or SEB stimulated control value should be higher than background.
- Triplicate values should show the same shape of dose response curves.
- The linear part of the curve should contain at least 4 points, and the points should be spread around both sides of the trendline.
- The coefficient of correlation (R²) should be at least 0.8.
- The IC₅₀ or SC₄ value should be in the linear part of the curve.

When the data are consistent with these criteria, a linear correlation may be calculated for the linear part of the curve, and the IC₅₀ or SC₄ values can be calculated for each donor (Microsoft Excel).

Example;
0 μM test compound = 60 pg/ml cytokine
Formula of the trendline = \( y = -0.0001x + 33.127 \)
\( R^2 = 0.913 \)

Calculation of the IC\(_{50}\) value;

60 pg/ml divided by 2 = 30 pg/ml

30 = -0.1x + 33.127
x = 30 - 33.127/0.1
IC\(_{50}\) value is 31.27 μM

### 8.2 Cytotoxicity determinations

Calculate what the percentages of decrease are as compared to the control (0 μM test compound). If the compound is decreasing the resorufin formation by 20% or more, in the range of the IC\(_{50}\) value, the compound is considered as cytotoxic. The compound is then considered to have a cytotoxic mechanism, and toxicity should be compared for blood in comparison to other cells e.g. fibroblasts to see if the compound has more specificity for immune cells.
9 HEALTH SAFETY AND ENVIRONMENT

Wear gloves, safety glasses and protective clothing while working with blood and test-compounds.
Work sterile in a safety cabinet.

Bacterial endotoxin (LPS) is pyrogenic when inhaled.

9.1 Stop solution and TMB substrate solution

Avoid contact with the stop solution, which is acidic. Wear gloves and eye protection. If this reagent comes in contact with skin, wash thoroughly with water and seek medical attention if necessary. The TMB/substrate solution contains peroxide. Since peroxides are strong oxidizing agents, avoid any skin contact with the TMB solution.

9.2 Blood incubations

Blood-contaminated sharp objects, e.g. needles and combitips, should be discarded in a proper container.
Cultures of human material should be treated as biologically hazardous waste and disposed accurately. The procedure used to kill blood cells and to eliminate any infections is bleaching (by adding bleach to the blood cells, and cleaning the work bench with 10% bleach) or autoclaving for 40 minutes at 120 ºC.
10 REFERENCES


