STUDY OF IN VITRO METHODS TO EVALUATE IMMUNOSUPPRESSION

TBTC effects on primary human bone marrow long term cultures

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STUDY OF IN VITRO METHODS TO EVALUATE IMMUNOSUPPRESSION

TBTC effects on primary human bone marrow long term cultures

Onderzoek naar *in vitro* methoden voor het vaststellen van immuunosuppressie

Effecten van TBTC op primaire humane beenmerg kweken

(met een samenvatting in het Nederlands)

Studio di metodi i*n vitro* per valutare l'immunosoppressione

Effetti del TBTC su colture primarie di midollo osseo a lungo termine

(con riassunto in Italiano)

Proefschrift

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Chapter one

GENERAL INTRODUCTION

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EU legislation in relation to immunotoxicity testing

Directive 86/609/EEC (Council Directive, 1986) on the protection of animals used for experimental and other scientific purposes requires that the European Commission and the Member States "should encourage research into the development and validation of alternative techniques, which could provide the same level of information as that obtained in experiments using animals but which involve fewer animals or which entail less painful procedures,..". In addition, the implementation of the new EU regulatory framework for Registration, Evaluation, Authorization and restriction of Chemicals (REACH) should be based on the use of alternative test methods, suitable for the assessment of health and environmental hazards of chemicals, wherever possible (Regulation EC n° 1907/2006). The European Commission is actively supporting measures to reduce the number of animal tests, by funding research into alternative testing methods and by steering the process of making suitable scientifically-validated methods accepted at regulatory level via the European Centre for Validation of Alternative Methods (ECVAM).

Most toxicological endpoints require an integrated testing approach such as the combination of *in vitro* and *in silico* methods or the use of test batteries, owing to the fact that many alternative methods are not suitable as purely stand-alone methods. For evaluation of immunotoxicity test batteries are also necessary, because the functionality of the immune system is determined by complex interactions between many different cells.

Immunotoxicity evaluation is a growing concern for regulatory authorities. The World Health Organization (WHO) together with the International Programme on Chemical Safety (IPCS) are working on a harmonized guidance on Immunotoxicity Risk Assessment (WHO/IPCS, 2008). Recent guidelines from ICH (International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use) emphasize the need to assess immunotoxic effects during the preclinical

phase of drug development, and recommend specific tests to measure immunotoxicity (ICH, 2006).

The CFU-GM (colony forming unit-granulocyte macrophage) assay to predict acute neutropenia (Pessina et al., 2003) is the only validated alternative method presently available for immunotoxicity assessment. No other method has yet been validated. Given the importance to develop alternative methods for immunotoxicity assessment, the present work aimed to:

- evaluate the predictivity of several *in vitro* tests (e.g. proliferation assays and cytokine production using both human and rodent cells), which could be used to detect immunosuppression;
- develop an *in vitro* method which could be applied to study toxic effects on human immune cell differentiation, mimicking the bone marrow microenvironment.
- use this *in vitro* method to test tributyltin chloride (TBTC, a chemical known for its imunotoxicity) on two different kinds of cells: adipocytes and lymphocytes.

Immunotoxicity

Many chemicals, including drugs and environmental agents, can interfere with the functionality of the immune system and induce immunotoxicity. Based on the clinical experience, immunotoxic effects can be divided into four categories: immunosuppression, immunostimulation, hypersensitivity, and autoimmunity (Putman et al., 2003). The search for alternative methods presented in this thesis was focused on immunosuppression.

Immunosuppression

Immunosuppression is the consequence of a reduced immune response. Two major types of clinical adverse effects may be associated to

immunosuppression: impaired resistance against microbial pathogens and development of malignancies.

Infectious complications have often been described in patients treated with corticosteroids (Klein et al., 2001), radiation or immunosuppressive drugs in the post-transplantation period (Sia and Paya, 1998; Del Pozo et al., 2009). In extreme cases of immunosuppression (e.g. treatment with anti-cancer drugs) opportunistic infections occur that normally do not develop in immunocompetent individuals (Meckler and Lindemulder, 2009). The possible occurrence of infectious diseases has not been extensively studied in healthy humans exposed to occupational or environmental chemicals or physical agents that are known to be immunosuppressive in animals. However, trends towards more frequent infections have been found in exposed humans. For example, Inuit infants exposed to organochlorine pesticides in the environment were found to be at a greater risk of developing otitis media (Dewailly et al., 2000). In Dutch preschool children the effects of perinatal background exposure to polychlorinated biphenyls (PCBs) and dioxins persist into childhood and might cause a greater susceptibility to infectious diseases. In fact, PCB body burden was associated with a higher prevalence of recurrent middle-ear infections and of chicken pox (Weisglas-Kuperus et al., 2000). It was shown that polyhalogenated aromatic hydrocarbons (PHAHs), including PCBs accumulate in the aquatic food chain and are found in high concentrations in seals and other marine mammals. In seals fed the contaminated Baltic herring it was observed an impairment of natural killer cell (NK) activity, T lymphocyte function, and antigen-specific lymphocyte proliferative response *in vitro*. Additional feeding studies in rats using the same herring batches indicated that an effect on the thymus may be responsible for changes in cellular immunity, and that virus-specific immune response was impaired. Authors showed that PCBs levels in the aquatic food chain are immunotoxic

for mammals. This could result in diminished host resistance and an increased incidence and severity of infectious disease (Ross et al., 1996).

Contamination of organotins particularly butyltins (BTs), has been suspected to cause immunosuppressive effects leading to subsequent infectious diseases or opportunistic infections by pathogens in marine mammals. A study, on finless porpoises (Neophocaena phocaenoides) showed a significant association between BT levels and parasitic infection status of lung nematodes. This study suggests that BTs could be a factor affecting parasitic infection, especially the presence or severity of lung nematodes in finless porpoises. Since chemical exposure may alter the susceptibility of organisms to infectious diseases, the interaction of chemical contamination with infectious diseases needs to be investigated in greater depth to understand the risk of population decline due to these factors in marine mammals (Nakayama et al., 2009).

The chronic use of immunosuppressive agents (such as Cyclosporin A, Mizoribin, Cyclophoshamide) to prevent allograft rejection increases the long-term risk of malignancy. Along with graft vasculopathy, malignant neoplasias have become a significant limiting factor for long-term survival of heart transplant recipients (Rinaldi et al., 2001). Epidemiological studies showed that lymphoproliferative disorders are 30- to 50- fold more frequent in renal transplant patients than in the general population (Penn, 1995).

In vivo methods in immunotoxicity

Immunotoxicity evaluation is an important component of safety assessment within preclinical toxicology studies as indicated by ICH guideline S8 (ICH, 2006). Comparable guidelines have been issued by regulatory agencies like OECD (Organization for Economic Cooperation and Development) and US EPA-OPPTS (US Environmental Protection Agency-Office of Prevention, Pesticides and Toxic Substances) regarding the safety assessment of chemicals and pesticides. The OECD Test Guideline No. 407 includes

parameters of immunotoxicological relevance as part of a repeated 28 day oral dose toxicity study in rodents (OECD, 2008). This guideline indicates that it is possible to obtain information about immunotoxicity through the analysis of parameters accepted in standard toxicity studies (STS) such as: total and absolute differential leukocyte counts, globulin serum levels, organ weight, gross pathology and histology of lymphoid organs (thymus, spleen, draining lymph node and at least one additional lymph node), histology of bone marrow, BALT (bronchus-associated lymphoid tissues), and NALT (nasal-associated lymphoid tissues). Such tests that are suitable for incorporation in standard toxicity studies are generally considered Tier I tests. Only in case of concern additional immunotoxicity studies are needed, as indicated by the US EPA-OPPTS 870.7800 (EPA, 1996[a]) and ICH S8 (ICH, 2006).

The EPA proposes in its guidelines EPA-OPPTS 870.7800, EPA-OPPTS 880.3550 (EPA, 1996[b]) and EPA-OPPTS 880.3800 (EPA, 1996[c]) more functional Tier II tests if dysfunction or impairment of the immune system was detected in Tier I tests, and to be used along with data from routine toxicity testing. Tier II assays include, for instance, specific host resistance assays (Listeria monocytogenes, Trichinella Spiralis) and the so-called SRBC-assay. In this latter assay, rats and/or mice are immunized with sheep red blood cells (SRBC) approximately 4 days prior to the end of the exposure to the chemical of concern. At the end of the exposure period, the anti-SRBC plaque-forming cell (PFC) assay or enzyme-linked immunosorbent assay (ELISA) is performed to determine the effects of the test substance on either splenic IgM PFC response, or serum IgM levels, respectively.

Flow cytometry analysis of leukocytes distributions may be included in Tier I tests. For instance, expression of phenotypic markers for major lymphocyte populations (total T (CD3), total B (CD-45R), NK (using a marker specific to the species and strain of animal used), and T subpopulations (CD4 and

CD8)) can be used to determine the effects of the test substance on either splenic or peripheral-blood lymphocyte populations.

These guidelines are used for conventional chemicals registered for food and non-food used pesticides (EPA-OPPTS 870.7800, EPA, 1996[a]) or for biochemical pesticides (EPA-OPPTS 880.3550, EPA, 1996[b]; EPA-OPPTS 880.3800, EPA, 1996[c]).

Also the ICH guideline S8 indicates a set of additional immunotoxicity tests in case of causes of concern. These additional studies include tests to determine: T-cell dependent antibody response (TDAR), the distribution of leukocyte populations (to identify the specific cell populations affected), natural killer cell activity, host resistance, macrophage/neutrophil function, and cell-mediated immunity (ICH, 2006).

Despite the efforts in defining a common strategy in assessing immunotoxicity, detection of potential immunotoxicity may still not be adequately addressed (Gennari et al., 2005).

In vitro methods in immunotoxicity

In 2003, ECVAM hosted a workshop to review and discuss the possible use of *in vitro* systems for evaluating immunotoxicity. A report of the workshop was published in 2005 (Gennari et al., 2005). Based on knowledge collected over the last 20-30 years on immunotoxicity of chemicals, an important distinction was made between myelotoxicity and lymphotoxicity, because in view of immunotoxicity these address different levels of immune system's ontogeny and functionality.

Compounds that are capable of damaging or destroying the bone marrow will often have a profound immunotoxic effect, since the bone marrow contains precursors of major effector cells of the immune system (e.g. granulocytes, macrophages). Thus, if a compound is myelotoxic, there may be no need to proceed with additional evaluation since the material will be immunotoxicant. The methodology for evaluating myelotoxicity *in vitro*

(CFU-GM assay) using bone marrow or cord blood cell culture systems has been validated by ECVAM in 2006 (Pessina et al., 2003). Compounds that are not myelotoxic may still selectively damage or destroy lymphocytes, which are responsible for acquired immunity. Lymphotoxicity may result from inhibition of differentiation or activation but also from killing of lymphocytes. Detection of cell death (necrosis or apoptosis) should therefore be the initial test. Inhibition of activation could be determined by assessment of the effect on proliferation in a non-antigen specific proliferation assay. T cells may be stimulated by mitogens (PHA, ConA) or by a combination of anti-CD3 and anti-CD28. For B-cells an optimum system has not been developed yet, but is expected to be similar to the murine system incorporating a combination of antibodies and cytokines, i.e. anti-IgM antibody and IL-2 have been shown to induce human B-cell proliferation (Jamin et al., 1995).

Besides proliferation, cytokine release reveals the ability of immune cells to be activated in response to immunomodulatory agents like mitogens. The possibility to include in immunotoxicity assessment the evaluation of other types of leukocytes such as phagocytes could be informative as well. For example, TNF-α-release by monocytes, or INF-γ-release by lymphocytes have been shown to be good indicators of immunotoxicity (Langezaal et al., 2001).

Other approaches in the immunosuppression evaluation are:

- *Determination of potential effects on antibody induction/production*. In animals, the TDAR is considered to be the "gold standard". However, there are currently no good systems for *in vitro* antibody production using human cells. Development of human *in vitro* systems will require optimization of many aspects, including determination of optimum stimulation, and the actual endpoint to be used (Herzyk and Holsapple, 2007).

- *Determination of potential effects on cytotoxic T lymphocytes (CTL).* CTL are a population of $CD8^+$ lymphocytes characterized by specific cytotoxicity for target cells in an antigen and MHC Class I-restricted manner. Assessment of CTL function may reveal deficits not only in the effector phase of the immune response, but also functional abnormalities in cellular activation and regulatory pathways (Carreno and Collins, 2003).
- *Determination of potential effects on NK cells*. Large granular lymphocytes or NK cells are involved in non-specific immunity and, as such, should be included in any *in vitro* immunotoxicology study design. NK cells are enumerated based on surface markers (mainly CD56) and their cytotoxic function is usually assayed *in vitro* using (^{51}Cr) labeled target cells or the four-color flow cytometry-based cytotoxicity assay (FCC) (Kim et al., 2007). As NK cell assays are extremely sensitive to modulation by toxic substances, they are usually included in toxicological evaluations (ICH, 2006; EPA, 1996[a]; EPA, 1996[b]).

As mentioned, this thesis focuses on immunosuppression, and as immunosuppression may be directed toward early immature leukocytes, a brief overview is given on immune system ontogeny.

The immune system ontogeny

The cells of the immune system originate in the bone marrow, where many of them also differentiate and mature. Then they migrate to circulate in the blood and in the lymphatic system to patrol peripheral tissues.

All the cellular elements of immune system derive from the same precursor cells, the pluripotent hematopoietic stem cells in the bone marrow. Blood cells and their precursors are packed in the extravascular spaces of the medullary vascular sinuses, which form a dense network in the bone marrow. The bone marrow contains adherent cells, known as stromal cells

(phagocytic cells, endothelial cells, and adipocytes), which create microenvironmental niches that maintain blood cell viability and supply the requisite factors for their development. Using different molecular markers to define progenitors, it has been shown that primitive hematopoietic progenitors are uniformly scattered throughout the bone marrow (Hirose et al., 2002).

The pluripotent hematopoietic stem cells give rise to stem cells of more limited potential: the myeloid progenitor and the common lymphoid progenitor (Fig. 1). The myeloid progenitor is the precursor of erythrocytes, megakaryocytes, granulocytes and macrophages. Macrophages are the mature form of monocytes, which circulate in the blood and differentiate continuously upon migration into tissues.

The common lymphoid progenitor gives rise to the lymphocytes. There are two major types of lymphocytes: B lymphocytes, which when activated differentiate into plasma cells that secrete antibodies; and T lymphocytes, which differentiate into cytotoxic T cells, helper T cells or regulatory T cells. Both B and T lymphocytes originate in the bone marrow but only B lymphocytes mature there, T lymphocytes migrate to the thymus to undergo maturation.

Once they have completed their maturation, both types of lymphocytes enter the bloodstream, from which they migrate to the peripheral lymphoid organs: lymph nodes, spleen, and gut-associated lymphoid tissues (GALT) (Janeway and Travers, 1997).

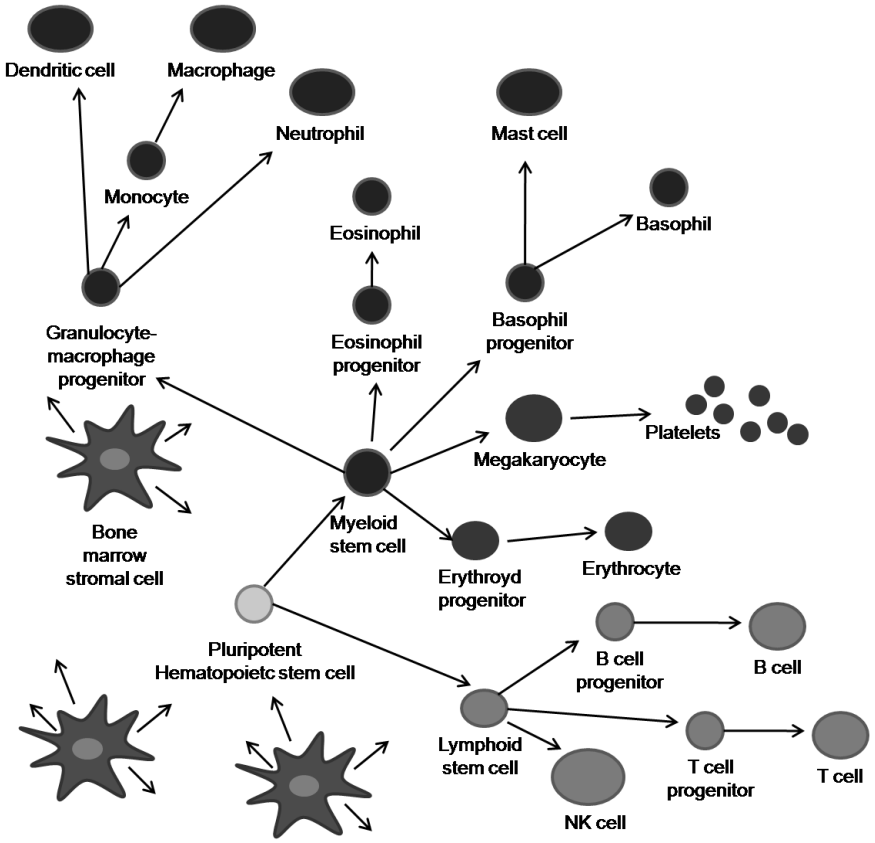

Figure 1. Hematopoiesis starts from pluripotent hematopoietic stem cells, which differentiate into lymphoid and myeloid stem cells. The lymphoid stem cells give rise to NK cells, B cells and T cells. The myeloid stem cells differentiate into different progenitors: granulocyte-monocyte, eosinophil, basophil, megakaryocyte, erythroid. The granulocyte-monocyte progenitors give rise to macrophages, neutrophil and dendritic cells. The eosinophil progenitors give rise to eosinophil cells. The basophil progenitors differentiate to basophils and mast cells. Megakaryocytes are platelet precursors and the erythroid cells are erythrocyte precursors. Each differentiation process requires specific cytokines and growth factors produced by the bone marrow stromal cells.

B cells development

Early B cell development depends on stromal bone marrow cells. The stroma provides the necessary support for B lymphopoiesis through the expression of adhesion contacts proteins and growth factors like stem cell factor (SCF) and interleukines. B cells develop via various B lineage-restricted precursors into immature B cells (Hystad et al., 2007).

The stages in primary B cell development are defined by the sequential rearrangement and expression of heavy- and light-chain immunoglobulin genes (Fig. 2), which are part of the B cell receptor (BCR). It consists of μ heavy chain (HC), light chain (LC) and CD79α/CD79β heterodimer (Igα/Igβ). The physical association of CD79α/CD79β heterodimer with μHC is necessary for the efficient transport of μHC to cell surfaces as well as for signal transduction through the BCR.

The earliest B lineage cells are known as pro-B cells, in which rearrangement of heavy chain immunoglobulin gene segments takes place. D_H to J_H joining at the early pro-B cell stage is followed by V_H to DJ_H joining at the late pro-B cell stage; CD79α and CD79β are present in the cytoplasm and only after the expression of μ-chain they migrate to the cell surface (Koyama et al., 1997). Productive VDJ_H joining leads to expression of an intact μ-chain, which is the hallmark of the next main stage of development, the pre-B cell stage. The μ-chain in large pre-B cells is expressed transiently at the cell surface in combination with a surrogate light chain as part of a pre-B cell receptor and this permits the cell to divide further before giving rise to small pre-B cells. The proliferating large pre-B cell gives rise to non-dividing small pre-B cells in which the μ heavy chain is found intracellularly. At this point the pre-B cell receptor is no longer displayed, and light-chain gene rearrangements proceed. Once a light chain gene is assembled and a complete IgM molecule is expressed on the cell surface, the cell is defined as an immature B cell. Then the cell is subject to selection for self tolerance and survival in the periphery. As the surviving B cells emerge into the periphery, they undergo further differentiation to become mature B cells expressing immunoglobulin D in addition to immunoglobulin M. These cells are called naïve B cells, until they encounter and are activated by foreign antigen.

Final development from immature B cells into mature B cells can occur either in the bone marrow or in secondary lymphoid organs such as the

spleen. In humans and mice the immunoglobulin diversity, generated by the rearrangement of gene segments encoding immunoglobulin heavy and light chain, begins in the fetal liver and continues throughout life in the bone marrow, albeit at a diminishing rate (Janeway and Travers, 1997).

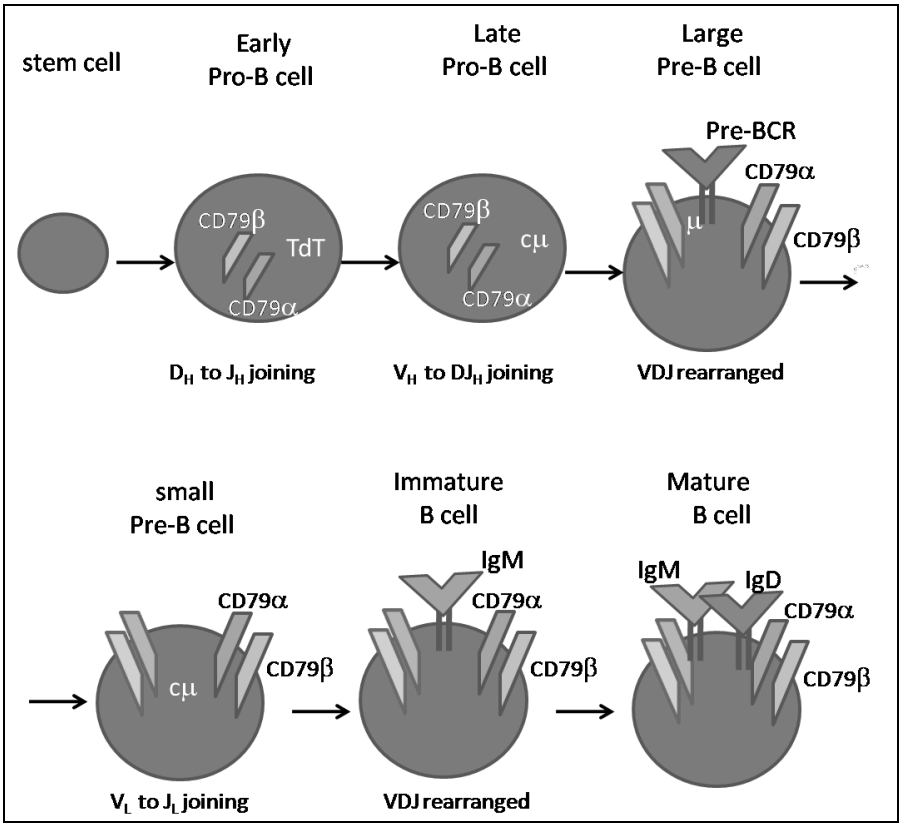

Figure 2. B cell differentiation from stem cell to mature B cell.

T cells development

T cells precursors complete their maturation in the thymus, which is one of the central lymphoid organs. The immature T cells or thymocytes proliferate and differentiate while passing through a series of discrete phenotypic stages that can be identified by distinctive patterns of expression of various cell surface proteins. It is during their development as thymocytes that the cells

undergo the gene rearrangements that produce the T-cell receptor (TCR), and the positive and negative selection that shape the mature receptor repertoire. These processes depend upon interactions of the developing cells with the cells of the thymic microenvironment, in particular epithelial and myeloid cells.

$CD34^{+}CD2^{+}$ T cell precursors migrate from bone marrow to the thymus. They are located in the outer cortical areas of the thymus, and are negative for CD4 and for CD8 (Double Negative, DN). As CD34 expression diminishes, there is a correlated increase in CD4 expression immediately followed by CD8 expression (Fig. 3). At the same time they start to rearrange the TCRβ (as well as the γ and δ) gene loci; this is the first step towards the expression of a functional TCR (Terstappen et al., 1992).

Productive rearrangement of the β chain is followed by its expression on the T cell membrane together with CD3 and the surrogate α chain. Signaling through the pre-T cell receptor causes the cells to stop rearranging β chain, undergo a period of proliferation, and begin expressing both CD4 and CD8, becoming double positive (DP) T cells. Importantly, successful rearrangement of the TCRβ chain is a prerequisite for survival and subsequent proliferation of thymocytes of the αβ-lineage (Bommhardt et al., 2004).

CD3 is present in the cytoplasm of all thymocytes, including $CD34^{+}CD2^{+}$ subset. Late thymic T-cell differentiation is characterized by high levels of surface CD3 and by a loss of the co-expression of the CD4 and CD8 antigen. T cells become single positive (SP) for CD4 and CD8 and they localize to the thymic medulla where they undergo several rounds of cell divisions before they finally migrate to peripheral lymphoid organs (Terstappen et al., 1992).

Besides αβ T lymphocytes, cells expressing γδ TCR also develop in the thymus. This thymocyte population is particularly prominent during fetal

development, while its frequency strongly declines after birth (Bommhardt et al., 2004).

The rate of T cells production by the thymus is greatest before puberty. The thymus reaches its maximum weight at puberty, after which it undergoes an involution process which is apparent as a decrease in size and weight. During the involution the epithelial component shrinks, resulting in scattered small lymphocytes in abundant adipose tissue (Nishino et al., 2006), and reduced production of new T cells. However, in both mice and humans, removal of the thymus is not accompanied by any notable loss of T-cell function although adult thymectomy in mice is accompanied by some loss of T cells (Mackall and Gress, 1997). Thus, it seems that once the T cell repertoire is established, immunity can be sustained without the production of large numbers of new T cells from the thymus.

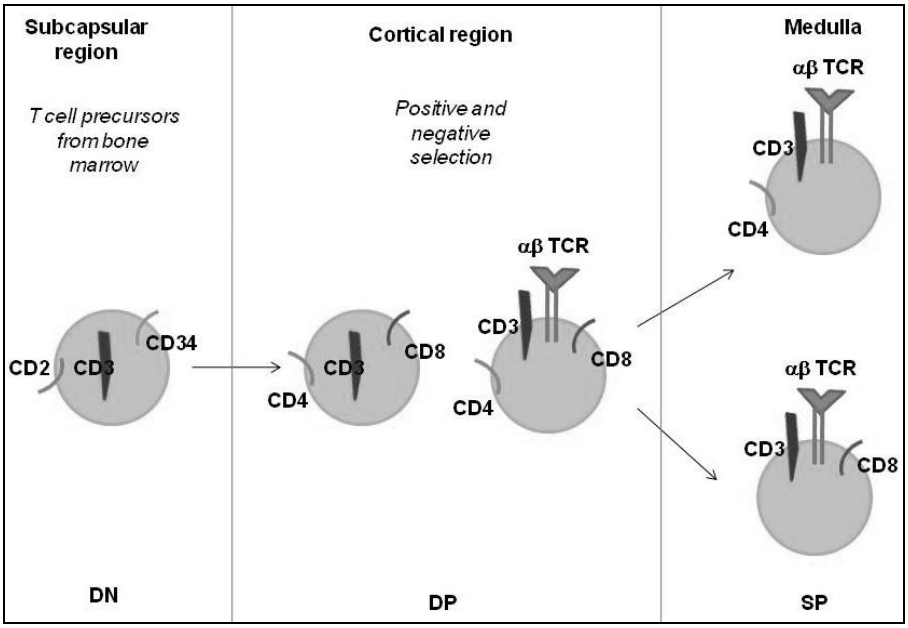

Figure 3. Schematic simplified representation of T cell differentiation in three main thymus regions: subcapsular, cortical and medulla. DN double negative; DP double positive; SP single positive.

Bone marrow adipocytes

Adipocytes originate from multipotent mesenchymal stem cells (MSCs) in the bone marrow, which are distinct from hematopoietic stem cells. From MSCs many kind of cells differentiate such as osteocytes, chondrocytes, myotubes, stromal cells and fibroblasts.

Within the bone marrow, the differentiation of MSCs into adipocytes or osteoblasts is competitively balanced; mechanisms that promote one cell's fate actively suppress mechanisms that induce the alternative lineage. This occurs through the cross talk between complex signalling pathways including those derived from bone morphogenic proteins (BMPs), fibroblastic growth factors (FGF), insulin, insulin-like growth factors (IGF), and transcriptional regulators of adipocyte and osteoblast differentiation including PPARγ (Muruganandan et al., 2009).

It seems that the function of osteoblasts and adipocytes is different in relation to hematopoiesis. Osteoblasts and endothelium constitute functional niches that support haematopoietic stem cells in mammalian bone marrow (Calvi et al., 2003). On the contrary, adipocytes' number correlates inversely with the haematopoietic activity of the marrow as confirmed by clinical and experimental observations. Fatty infiltration of haematopoietic red marrow follows irradiation or chemotherapy. Notably, the number of mature adipocytes increases in aplastic anemia, a representative bone marrow failure syndrome (Young, 1999). In transgenic mice (A-ZIP/F1) without white fat (Moitra et al., 1998), and in mice treated with the PPARγ inhibitor bisphenol A diglycidyl ether, which inhibits adipogenesis (Wright et al., 2000), marrow engraftment after irradiation is accelerated relative to wild-type or untreated mice. These data implicate adipocytes as predominantly negative regulators of the bone-marrow microenvironment, and indicate that antagonizing marrow adipogenesis may enhance haematopoietic recovery in clinical bone-marrow transplantation (Naveiras et al., 2009).

In an *in vitro* study, preadipocytes were shown to support hematopoiesis by direct cell–cell contact and secretion of various hematopoietic cytokines. In contrast, mature adipocytes lose hematopoietic supporting capacity, or rather secrete cytokines that suppress hematopoiesis (Nishikawa et al., 1993).
These data suggest that adipocytes are not simple space fillers, but play an active role in the bone marrow stroma as regulator of hematopoiesis.

Compounds used in the present study

To evaluate the ability of a set of *in vitro* methods to predict immunosuppression, a panel of known immunotoxic and not immunotoxic compounds was chosen (Table 1). From these, the well-known organotin compound tributyltin chloride (TBTC) was chosen to be tested on human bone marrow cells, because its effect in humans is not clear.

Table 1. Compounds tested with a battery of *in vitro* methods to evaluate immunotoxicity.

Compounds	Type	Proprieties	Use	References
Urethane	chemical	Not immunotoxic	Solubilizer and co-solvent in the manufacture of pesticides, fumigants, and cosmetics. Intermediate for pharmaceuticals, and in biochemical research.	HSDB, 2000
Furosemide	drug	Not immunotoxic	Diuretic used in the treatment of oedema with heart failure and with renal and hepatic disorders.	Martindale, 2002
Verapamil	drug	immunotoxic	Calcium-channel blocker, used for hypertension and arrhythmias treatment.	Martindale, 2002
Benzo(a)pyrene	chemical	immunotoxic	Major component of the total content of polynuclear aromatic compounds in the environment. Human exposure occurs primarily through cigarette smoking, inhalation of polluted air and by ingestion of food and water contaminated by combustion effluents.	IARC, 1983
Cyclosporin A	drug	immunotoxic	Immunosuppressant drug with a specific action on T-lymphocytes. Used to prevent organ graft rejection.	Martindale, 2002
Tributyltin chloride	chemical	immunotoxic	It is used as plastic stabiliser, catalytic agent, agricultural pesticide, rodent repellent, and as antifouling agent in paints	Fent, 1996

Organotin compounds

Organotin compounds are defined as being chemicals with at least one covalent bond between an organic moieties and a tin atom (Fig. 4). Depending on the number of organic moieties present, they are classified as mono-, di-, tri- or tetra-organotins. Intriguingly, the possible applications but also toxicity varies between the number, size and complexity of the organic moieties.

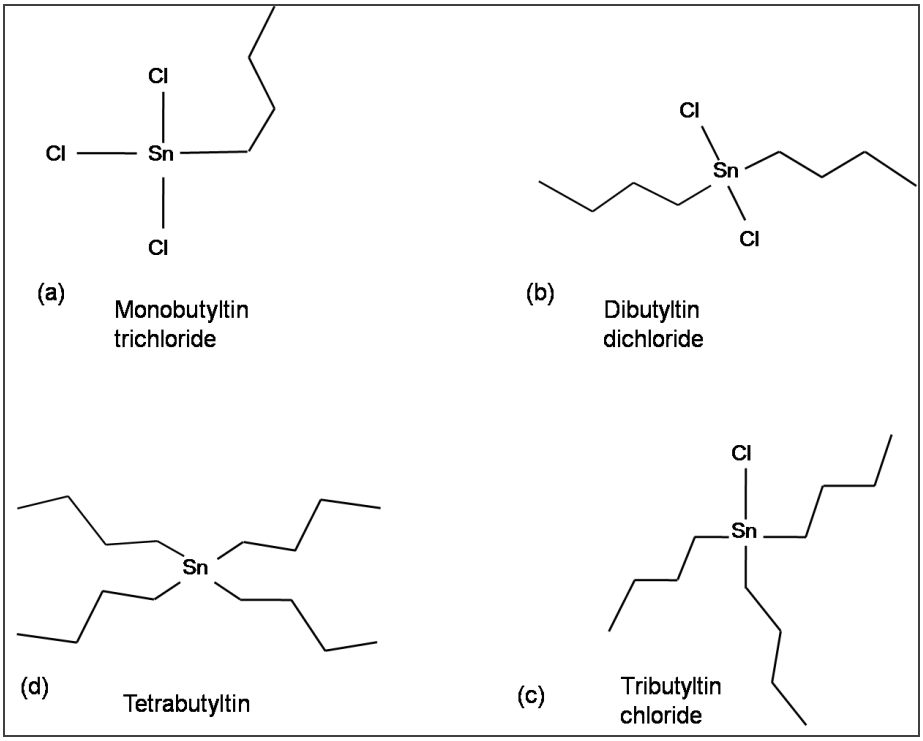

Figure 4. Examples of organotin compounds. In this case the organic moiety R is butyl and X is chloride (Cl). (a) Monobutyltin chloride ($RSnX_3$). (b) Dibutyltin chloride (R_2SnX_2). (c) tributyltin chloride (R_3SnX). (d) tetrabutyltin (R_4Sn).

Organotin applications

Monosubstituted ($RSnX_3$) and disubstituted (R_2SnX_2) organotin compounds are mainly used as stabilizers in polyvinyl chloride (PVC) films to prevent changes upon exposure to light and heat. Disubstituted organotin compounds are also used as catalysts in the production of polyurethane foams and in the vulcanization of silicones (at room-temperature). The tetrasubstituted organotin compounds (R_4Sn) are mainly used as intermediates in the preparation of other organotin compounds (IPCS, 1980).

Trisubstituted organotin compounds (R_3SnX) have biocidal properties that are strongly influenced by the R-groups. The more important of these compounds are the tributyl-, triphenyl-, and tricyclohexyltin compounds, which are used as agricultural and general biocides (against fungi, bacteria, helmintics, herbs, mollusks, nematodes, and insects) but also as rodent repellent (IPCS, 1980).

Tributyltin (TBT) is also widely used as an impregnation material in prints and textiles, slime control in paper mills, and a wood preservative, and is also used as a disinfectant in circulating cooling waters. The most extensive use of TBT is as a de- or anti-fouling agent in paints, for coating structures exposed to the aquatic environment such as ships, oil rigs, and water intake pipes (Cooney, 1994). Due to its widespread use as an antifouling agent in boat paints, organotin is a common contaminant of marine and freshwater ecosystems exceeding acute and chronic toxicity levels. Consequently, organotins are one of the most significant pesticides in marine and freshwaters and its environmental level, fate and toxicity are of current concern (Nakanishi, 2008). The European Community adopted a regulation in 2003, which prohibits the use of organotin-containing anti-fouling paints on all ships entering ports in the Community in order to reduce or eliminate the presence of these compounds in the environment and their adverse effects on environmental and human health (Regulation EC No. 782/2003).

Because of the widespread use of TBT, humans can be exposed to this organotin compound through different ways. TBT enters the human food chain mainly through consumption of contaminated marine and freshwater species (Snoeij et al., 1987; Antizar-Ladislao, 2008). In addition, the presence of TBT in wood preservative for domestic use and in interior latex paint may lead to human exposure by inhalation or absorption through the skin (Wax and Dockstader, 1995). Occupational exposure to TBT occurs primarily during the manufacture and formulation of these compounds. Also the industrial use of TBT as a wood preservative, and the application and removal of TBT-containing paints are considered important means of occupational exposure (Corsini et al., 1997). Levels of organotins, such as dibutyltin (DBT) and TBT, in wildlife and human tissue samples are in the range of 3–100 nM (Takahashi et al., 1999; Kannan et al., 1999). TBT possesses both lipophilic and ionic properties that promote bioaccumulation in lipids and binding to macromolecules upon exposure (Adeeko et al., 2003).

Toxicity of organotin compounds

Toxicity studies indicate that some organotin compounds cause neurotoxicity, producing behavioral and neurological symptoms, (Brown et al., 1979) pancreatic and hepatic toxicities (Merkord et al., 2001). They are also known for their reproductive and developmental toxicity, immunotoxicity and endocrine disrupting activity.

Organotins toxicity depends not only on their chemical features, but also on their distribution in the body. In a study in rats, the content of tributyltin (TBT) and its metabolites, dibutyltin (DBT) and monobutyltin (MBT), was examined in the liver, brain and fat tissues in a two-generation reproductive toxicity study with TBTC. It appeared that irrespective of dietary concentration of TBTC, the highest concentration of metabolite in the liver was consistently MBT, followed by DBT, and then TBT. In contrast, TBT

was consistently present at the highest concentration in the brain, nearly always followed by DBT and MBT. In fat tissues, the concentrations of these three butyltin compounds showed similar relationships to those observed in the brain, although the concentrations were much lower. In the liver, the concentration of TBT was higher in females, and those of DBT and MBT were higher in males, suggesting a gender effect (Omura et al., 2004). The analysis of the same metabolites in human liver of occupationally exposed men showed that the DBT/MBT ratio was significantly higher in young men compared to older ones, suggesting that either younger men were more recently exposed to, or have a lower capacity to debutylate DBT than older men. Young men may therefore be potentially more susceptible to butyltin toxicity (Nielsen and Strand, 2002).

The most prominent TBT effects are related to the immune and endocrine systems, described in the next two paragraphs.

Organotin compounds and immunotoxicity

Some organotin compounds are classical immunotoxic compounds with a selective thymotoxic effect. TBT and DBT induce thymus atrophy in rats due to a selective reduction in the number of rapidly proliferating lymphoblasts (Snoeij et al., 1988). *In vitro* studies indicate that apoptosis is involved in the cytotoxic effects of di-*n*-butyltin dichloride (DBTC) and TBTC, but it seemed to be not involved in thymus atrophy at a dose that selectively inhibits immature thymocyte proliferation. At higher doses apoptosis appears involved in the thymotoxic effects of both organotin compounds (Raffray et al., 1993; Gennari et al., 1997). The subset of thymocytes that is subject to apoptosis by organotins, i.e. the $CD4^{+}CD8^{+}$ subset, differs from the one (immature blastoid $CD4^{-}CD8^{+}$ and early $CD4^{+}CD8^{+}$ subsets) that has been characterized as sensitive to the anti-proliferative effects of organotins (Gennari et al., 2002). It has been shown in human peripheral blood lymphocytes that TBT induces apoptosis in

resting T cells and necrosis in activated T cells (Stridh et al., 2001), which may be due to difference in active molecular pathways in resting and activated T cells.

Butyltin compounds also affect innate immune cells. For instance, both organotin compounds inhibit tumor-killing functions of natural killer cells *in vitro* (Whalen et al., 2002). Exposure to TBT, which decreases cAMP levels in NK cells, caused a decrease in cytotoxic function of these cells by as much as 90% (Bariagaber and Whalen, 2003).

Although TBT presence in human blood has been documented (Kannan et al., 1999), few studies are available on TBT effects on humans and are related to toxicological investigations on human natural killer cells (Whalen and Loganathan, 2001; Alouch et al., 2006).

Organotin compounds as endocrine disruptors

In the early 1980s it became clear that organotins can cause imposex in mollusks (Smith, 1981). Imposex is an abnormal induction of male sex characteristics in female marine invertebrates and represents one of the clearest examples of environmental endocrine disruption.

Subsequent toxicity studies have demonstrated that TBT is capable of inhibiting the activity of P450 aromatase in various cell types, including granulosa cell–like tumor cell line (Cooke, 2002; Heidrich et al., 2001; Saitoh et al., 2001). Generally, effective concentrations of organotins to inhibit aromatase activity are high, but in human ovarian granulosa cells, these compounds suppress aromatase activity at the nanomolar concentrations. Contrary to this, in human choriocarcinoma cells, these compounds enhance estrogen biosynthesis along with the increase of aromatase activity at the same low concentrations (3-100 nM) (Nakanishi et al., 2002). This discrepancy is due to a different transcription regulation of the CYP19 gene coding for aromatase. Since aromatase is the key step in the conversion of androgens to estrogens, its inhibition results in an increase in

testosterone and decrease in estrogen levels and may explain the observed female to male conversion (Matthiessen and Gibbs, 1998).

The endocrine-disrupting potential of TBT is based not only on its direct action on key steroid regulatory enzymes (e.g., aromatase activity), but also on an effect on transcriptional regulation. For example, expression of the aromatase gene was down-regulated by TBT in human ovarian granulosa cells, similar to the effects found with ligands of either peroxisome proliferator receptor γ (PPARγ) or retinoid X receptors (RXRs) (Mu et al., 2001; Saitoh et al., 2001). The potential to modulate sex steroid homeostasis through transcriptional regulation, particularly through a nuclear receptor-mediated signaling pathway, is an intriguing explanation for TBT-mediated endocrine disruption. It has been shown that TBT is an agonistic ligand for PPARγ and RXR nuclear receptors (Kanayama et al., 2005).

RXR plays a central role as the common heterodimeric partner to many other nuclear receptors in different hormonal signaling pathways. RXR-specific ligands can contribute to regulation of gene expression. In particular, RXR-PPARγ heterodimers have been shown to play a key role in adipocyte differentiation and energy storage (Auwerx, 1999; Fig. 5).

PPARγ activation increases the expression of genes that promote fatty acid storage and represses genes that induce lipolysis in adipocytes of the white adipose tissue. Moreover, it has been shown that TBT promotes adipogenesis in mouse 3T3-L1 preadipocytes and *in vivo* in adult C57BL/6J mice (Grun et al., 2006).

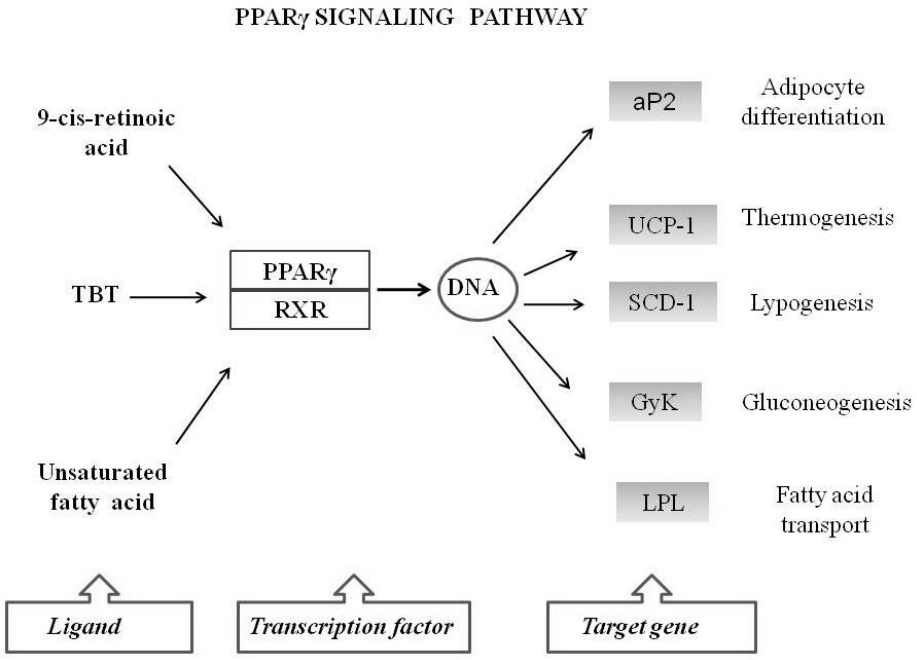

Figure 5. Heterodimers formation between RXR and PPARγ modulates the expression of genes involved in adipocyte differentiation, thermogenesis, gluconeogenesis, lypogenesis, and fatty acid transport. TBT can bind to nuclear receptors RXR and PPARγ, in a similar way to their natural ligands (9-cis-retinoic acid, unsaturated fatty acid) activating the heterodimer RXR/PPARγ transcription factor.

Aim of the thesis

The need to eventually use alternative methods in the assessment of chemical toxicity as determined by EU legislation, combined with the almost complete absence of validated *in vitro* methods to determine immunosuppression (apart from the CFU-GM test) was the starting point for the present research project on *in vitro* immunotoxicity testing.

The aim of the thesis was to evaluate the ability of a set of *in vitro* methods in classifying a panel of chemicals with known immunotoxic proprieties.

A preliminary study (chapter 2) was performed to investigate the ability of a limited number of *in vitro* methods to evaluate the effects of different

chemicals on the immune function of human, mouse and rat cells. Non-cytotoxic concentrations of a panel of compounds known for their immunotoxicity were tested, using the validated CFU-GM test and lymphocytes stimulation assays.

In vitro tests, in human, are normally performed on mature cells, in particular on blood circulating cells (such as leukocyte counts, immunophenotyping, cytokine release assay). These tests do not allow to evaluate deficiency in cell differentiation. Since chemicals can affect not only mature cells but also progenitors, it is important to develop tests that can detect toxic effects also at very early stages of cells differentiation.

Lymphocytes are important actors in the adaptive immune response and a defect in their differentiation process can seriously compromise the immune system activity (Janeway and Travers, 1997). Consequently, the effect on lymphocyte differentiation can be considered an important endpoint in immunotoxicity evaluation.

The *in vitro* method available to maintain and induce lymphocyte differentiation uses murine bone marrow cells cultured with the long term culture (LTC) method (Whitlock et al., 1984; Dorshkind, 1986). This technique was developed with murine cells, because it is difficult to maintain and induce the differentiation of human lymphocytes *in vitro*. In fact, there are very few studies describing the use of human cells; generally co-culture systems with murine cells (for the stroma) and human cells are used (Miller et al., 1999). The possibility to have an *in vitro* method using human cells together with a human stroma layer would bring this system closer to the human *in vivo* situation. An *in vitro* method, which allows the maintenance and the differentiation of lymphocytes from human mononuclear bone marrow cells is described in chapter 3.

This method is a LTC, which can be divided into 2 parts:

- During the first 2 weeks the stroma was formed. To allow a complete stroma formation horse serum and hydrocortisone were added to the medium, which inhibits lymphocyte proliferation (myeloid conditions).
- At the end of the second week, the medium was completely changed and a medium without horse serum and hydrocortisone was used, to favour lymphocyte development (lymphoid conditions).

The chemical classified as the most immunotoxic in chapter 2, TBTC, was tested on primary human bone marrow cells, cultured with the LTC method. The TBTC effect on mesenchimal and lymphoid populations was assessed. Moreover, the potential of the LTC method in maintaining and differentiate human lymphocytes *in vitro* was evaluated.

Using the method described in chapter 3, TBTC was tested during the first 2 weeks (myeloid conditions) to evaluate its activity on the stroma formation (chapter 4) and on the lymphocyte population (chapter 5). Chapter 4 focuses on the TBTC effect on adipocyte differentiation during the bone marrow stroma formation. Using the same culture conditions, TBTC toxicity on B and T cells is analyzed in Chapter 5.

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Chapter two

IN VITRO TESTS TO EVALUATE IMMUNOTOXICITY: A PRELIMINARY STUDY

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ABSTRACT

The implementation of Registration, Evaluation and Authorisation of new and existing Chemicals (REACH) will increase the number of laboratory animals used, if alternative methods will not be available. In the meantime, REACH promotes the use of *in vitro* tests and, therefore, a set of appropriated alternative testing methods and assessment strategies are needed.

The immune system can be a target for many chemicals including environmental contaminants and drugs with potential adverse effects on human health. The aim of this study was to evaluate the predictivity of a set of *in vitro* assays to detect immunosuppression. The tests have been performed on human, rat and murine cells. Different endpoints have been assessed: cytotoxicity, cytokine release, myelotoxicity and mitogen responsiveness. For each of these endpoints IC50s values have been calculated.

Six chemical substances, representative of the full range of *in vivo* responses and for which good human and/or animal data are available either from databases or literature, have been selected: two chemicals classified as not immunotoxic (Urethane and Furosemide), and four (tributyltin chloride (TBTC), Verapamil, Cyclosporin A, Benzo(a)pyrene) with different effect on immune system.

All the tests confirmed the strong immunotoxic effect of TBTC as well as the negative controls. For one chemical (Verapamil) the IC50 is similar through the different tests. The IC50s obtained with the other chemicals depend on the endpoints and on the animal species.

The clonogenic test (CFU-GM) and the mitogen responsiveness showed similar IC50s between human and rodent cells except for Cyclosporin A and TBTC.

All different tests classified the compounds analyzed in the same way.

INTRODUCTION

In October 2003 the European Commission adopted a new EU regulatory framework for chemicals (COM, 2003 644). Under the proposed new system called Registration, Evaluation and Authorization of Chemicals (REACH) enterprises that manufacture or import more than 1 tonne of a chemical substance per year would be required to register it in a central database. REACH would give greater responsibility to industry to manage the risks from chemicals and to provide safety information on the substances. The aim is to improve the protection of human health and environment through the better and earlier identification of the properties of chemical substances.

In vivo studies are very expensive, require a high number of animals and raise important ethical concern. For this reason the European policy is promoting alternative methods to the use of laboratory animals, in order to reduce and whenever possible replace animals employed for scientific studies and costs (Balls et al., 1995). Different institutes are developing *in vitro* tests able to predict compound's effects *in vivo*. *In vitro* methods standardized and validated have replaced or reduced some *in vivo* tests (Genschow et al., 2002, Pessina et al., 2003).

Among important target organs of chemical exposure is the immune system. Immunotoxicity can be defined as the adverse effect of chemicals or agents on the immune system. The effect may be increased immune activity, manifested as either hypersensitivity or autoimmunity, or decreased immune activity, with reduced ability to fight infectious agents and increased incidence of cancer (Snodin, 2004).

A workshop, held at ECVAM in 2003 (Gennari et al., 2005), reports the state of the art of *in vitro* systems for evaluating immunotoxicity. This project is based on workshop recommendations and is focused on immunosuppression.

The purpose of this study was to compare the predictivity of several *in vitro* tests, i.e. proliferation assays and cytokine production using both human and rodent cells. A panel of six substances was selected: four positive and two

negative compounds, chosen among drugs and chemicals. The four with known toxic effects on immune system were: Verapamil, Benzo(a)pyrene, Cyclosporin A, Trybutiltin chloride (TBTC). The two not immunotoxic were Urethane and Furosemide.

Verapamil is a calcium-channel blocker, used for hypertension and arrhythmias treatment. It has been shown that Verapamil inhibits, in a dose-dependent fashion, the proliferation of T-cells after mitogen stimulation (Birx et al., 1984; Chow and Jusko, 2004).

Human exposure to Benzo(a)pyrene occurs primarily through cigarette smoking, inhalation of polluted air and by ingestion of food and water contaminated by combustion effluents. Benzo(a)pyrene is present as a major component of the total content of polynuclear aromatic compounds in the environment (IARC, 1983). In addition to being carcinogenic and mutagenic, Benzo(a)pyrene has been found to be potent immunosuppressant. Effects have been documented on cell-mediated immunity, humoral immunity, and on host resistance.

Cyclosporin A is a powerful immunosuppressant with a specific action on T-lymphocytes. It is widely used to prevent organ graft rejection (Martindale, 2002[b]).

Tributyltin chloride (TBTC) is an organotin compound used as plastic stabilizer, catalitytic agent, agricultural pesticide and as antifouling agent in paints. It causes thymus atrophy in rodents (Snoeij et al., 1987), depletion of lymphocytes in spleen and lymph nodes, lymphopenia and alteration of serum immunoglobulin levels (Kimura et al., 2005).

Urethane is utilized as a solubilizer and co-solvent in the manufacture of pesticides, fumigants, and cosmetics, as an intermediate for pharmaceuticals, and in biochemical research (HSDB, 2000). Furosemide is a potent diuretic. It is used in the treatment of oedema associated with heart failure, including pulmonary oedema, and with renal and hepatic disorders (Martindale, 2002[a]).

MATERIALS AND METHODS

Drugs

Urethane (99%) was supplied by Sigma-Aldrich (St Louis, MO, USA); it was solved in water. Furosemide was supplied by APP (American Pharmaceuticals Partners, Schaumburg, IL), it was solved in water for injection. Verapamil hydrochloride was supplied by ABBOT LABORATORIES (North Chicago, USA) as water solution containing Verapamil hydrochloride 2.5 mg/ml and sodium chloride 8.5 mg/ml. Benzo(a)pyrene (97%) was supplied by Sigma-Aldrich and solved in DMSO. Cyclosporin A was supplied by BEDFORD LABORATORIES (Bedford, OH, USA) as Cyclosporin 50 mg, polyoxyethylated castor oil 650 mg, absolute alcohol 33.2% (v/v) and water for injection. Tributyltin chloride (TBTC, 96%) was supplied by Sigma-Aldrich and dissolved in DMSO.

Drug dilution of test compounds were prepared freshly for each experiment in DMSO (TBTC, Benzo(a)pyrene), water (Urethane) or in culture medium (Verapamil, Cyclosporin A, Furosemide).

The final concentration of DMSO never exceeded 0.5%.

CFU-GM assay

Cord blood cells were seeded in MethoCult-H4001 medium (Methocult, StemCell Technologies, Vancouver, BC, Canada). This medium contains 1% of methylcellulose in IMDM, 30% FBS, 1% of Bovine Serum Albumin (BSA), 2mM L-glutamine and 10 ng/ml granulocyte-macrophage-colony stimulating factor (GM-CSF).

Briefly, 22 µl of 200X drug solutions and 300 µl of cells (1.1×10^6 cells/ml) were added to tubes containing 4 ml of MethoCult. 1 ml of methylcellulose-cell suspension was then seeded into the 35 mm Petri dishes.

The cultures were incubated at 37°C and 5% CO_2 under saturated humidity for 14 days.

The final concentrations of drugs were from 0.1µM to 160000 µM for Urethane, from 0.01µM to 150µM for Furosemide, from 0.1µM to 113µM for Verapamil, from 0.1µM to 200µM for Benzo(a)pyrene, from 0.1µM to 66.6µM for Cyclosporin A, from 0.001µM to 3.3µM for TBTC.

Source of progenitor cells

Human cord blood cells were used as the source of progenitors for the CFU-assays.

Cord blood cells were obtained, frozen, from Biopredic (Rennes, France) and thawed before using, as indicated by supplier. Cells were diluted in 30% FBS-IMDM and used for the clonogenic test at a cell density of 1.1 x 10^6 cells/ml.

Colony scoring

A CFU-GM (colony forming unit-granulocyte macrophage) colony is defined as an aggregate containing 50 or more cells. Morphologically, four classes of CFU-GM colonies can be observed: compact, diffuse and spread multicentric and multifocal colonies. A compact colony has a central dense nucleus with a peripheral halo. Diffuse and spread colonies are without an apparent nucleus. Multicentric colonies appear with two or more dense nuclei nearby and with a common peripheral halo growing at the same depth in the plate. Multifocal colonies are aggregates of several colonies or clusters with or without a peripheral halo (Pessina et al., 2001).

Expression of results

Cell proliferation is expressed as a percentage of growth, with respect to the number of colonies in control dishes (100%).

Although the number of colonies scored in control plates, at a fixed density, varied between experiments performed on different days, differences in absolute colony counts did not affect the IC values. Colony formation

linearity was used as acceptance criteria for colony growth in control dishes or well plates. In each experiment at least two different concentrations of cells were plated as described by Pessina (Pessina et al., 2001).

The concentrations of test compound which inhibit growth by 50 % of CFU-GM (IC50) in comparison with control cultures were calculated using the Hill function analysis.

Results are reported as the mean ± standard error of at least two experiments, each done in triplicate.

Cytokine release

Study protocol

Peripheral blood was obtained from healthy subjects. Subjects were selected according to the guidelines of the Italian Health authorities and to the Declaration of Helsinki principles. Criteria for exclusion were abnormal laboratory values, medication known to affect the immune system, i.e. steroids and nonsteroideal antinflammatory drugs, or patients suffering from malignancies, inflammations and infections. Subjects were enrolled among colleagues and were informed about methods and aims of the study. Blood samples (5 ml) were taken by venous puncture with sodium citrate 0.5 M as anticoagulant. Sodium citrate was chosen instead of heparin or EDTA as anticoagulant, since functional assays were performed using the whole blood assay and heparin may be contaminated with endotoxin, while EDTA interferes with cell activation. Blood was diluted 1:10 with cell culture medium RPMI 1640 (Sigma, St Louis, USA) containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin as previously described (Kirchner et al., 1982).

Cytokine production

For the evaluation of cytokine production, cultures were set up in 24 well plate (Iwaki, Hasai, Japan) containing 0.5 ml of 1:10 diluted whole blood in medium alone or in the presence of increasing concentrations of the selected

chemicals and of lipopolysaccharide (LPS, from *Escherichia coli* serotype 0127:B8, Sigma) at final concentration of 1 µg/ml or phytohemagglutinin (PHA, Invitrogen, Paisley, UK) at final concentration of 1.2 µg/ml. Cells were incubated for 24 h for LPS-induced TNF-α release and 72 h for PHA-induced IFN-γ release at 37 °C in a humidified 5% CO_2 incubator. Cell-free supernatants obtained by centrifugation at 1200 rpm for 5 min were stored at –20 °C until measurement. Cytokine production was assessed by ELISA using commercially available kit (Immunotools, Friesoythe, Germany). Results are expressed in pg/ml. Each chemical was tested on three different donors.

Proliferation with anti-CD3 stimulation

Human lymphocytes proliferation

Peripheral blood was collected by venous puncture on EDTA in human volunteers after filling an agreement form. Mononuclear cells (monocytes and lymphocytes) were isolated from peripheral blood (EDTA) using Ficoll hypaque gradient centrifugation (Pharmacia). Lymphocytes proliferation was induced using anti-CD3 antibody (UCHT-1 clone) directed to the epsilon chain of the human CD3 complex. Anti-CD3 antibodies was precoated on 96 wells plates 24h before adding the cells at room temperature (4 µg/well in 100 µl of PBS)**.** On the day of the experiment, complete media (RPMI 1640, penicillin, streptomycin, glutamine, sodium pyruvate, 10% fetal bovine serum) 50 µl, chemical 50 µl and mononuclear cells ($4x10^5$/well) 100 µl were added in this order. Cells and chemicals were diluted in complete media. Cells were then incubated at 37°C, 5% CO_2 for 48h or 72h. ^{3}H-thymidine (0.5 µCi/well) was added 6h before the end of the experiment. At the end of the experiment cells were harvested and radioactivity was counted using a β counter (Beckman). Results were expressed in cpm.

Mouse lymphocytes proliferation

Splenocytes (mouse C3H strain) were isolated from C3H mice using mechanical dissection and counted using trypan blue.

Lymphocytes proliferation was induced using anti-CD3 antibody (1452C-11 hybridoma) directed to the epsilon chain of the murine CD3 complex. On the day of the experiment, cells ($4x10^5$/well) 50 µl, chemical 50 µl, complete media 50 µl (RPMI 1640, penicillin, streptomycin, glutamine, sodium pyruvate, 2-mercaptoethanol) at $5x10^{-5}$M, (10% fetal bovine serum) and antibody 50 µl (10 µg/ml) were added in this order. Cells, chemicals and antibody were diluted in complete media. Cells were then incubated at 37°C, 5% CO_2 for 48h or 72h. ^{3}H-thymidine (0.5 µCi/well) was added 6h before the end of the experiment. At the end of the experiment cells were harvested and radioactivity was counted using a β counter (Beckman). Results were expressed in cpm.

Proliferation test on rats cells

Animals

SPF-bred female Wistar rats of the strain Hsd cpB:WU were used (Harlan Winkelmann GmbH, Borchen, Germany).

Cell material

Cell suspensions obtained from spleen were tested. In a first step the optimal cell density for stimulation regimes with the T cell mitogen Concanavalin A was determined $1x10^5$ cells/well ($2 x 10^6$cells/ml) were subsequently used. Measurements were done in quadruplicates in each case (50µl per well).

Medium

Cells were cultured in RPMI 1640 additioned with L-glutamin (Invitrogen Life Technologies, Karlsrhue, Germany), sodium pyruvate, MEM (non-essential amino acids), penicillin/streptomycin, ß mercaptoethanol and 5% mitogen free fetal calf serum.

Mitogen stimulation

Concanavalin A (ConA, Sigma) was used to stimulate cell proliferation. Cells were incubated for 24h with ConA at the final concentrations of 2µg/ml.

The application volume was 50µl/well to give a final volume of 100µl per well (50µl cell suspension and 50µl mitogen or medium alone).

BrdU incorporation

Mitogen stimulation was assessed by BrdU (5-bromo-2´deoxyuridine) incorporation using the Cell Proliferation ELISA BrdU Kit (Roche), following Supplier's specifications. The ELISA was purchased from Roche (Roche Diagnostics- Applied Science, Mannheim, Germany).

Briefly, 10µl of the BrdU solution in RPMI medium (1:100 diluent) was added to each well after incubation periods of 24h or 48h. The plates were then incubated for another 24h at 37°C and 5% CO_2. After this final incubation period with BrdU the plates were centrifuged at 1200 rpm for 10min, labeling medium carefully removed and plates dried at 60°C for 1h.

Cells were then incubated with FixDenat Reagent 200µl/well for 30 min at RT, then washed three times with buffer 300µl/well. Anti BrdU labeling was done adding 100µl/well for 60 min at RT and then washed with buffer 200µl/well, three times. Substrate solution 100µl/well was added and after about 8 min of incubation, 1M H_2SO_4 25µl/well was added to stop the reaction. Plates were mixed on a shaker for 1 min (300 rpm) and the absorbance read at 450nm/ 630nm.

MTT assay

The analysis of cell viability was performed by the MTT assay.

MTT solution 5 mg MTT/ml was prepared in sterile PBS (MTT, Sigma), while isopropanole/HCl solution (isopropanole Merck) was prepared mixing 100 ml isopropanole with 0,4 ml HCl 10N.

Cell suspension was distributed on a 96 well plate, 100µl cell /well. Cells were incubated with mitogen for 24h or 48h at 37°C and 5% CO^2. After incubation, 10 µl MTT was added to 100µl cell suspension and cells were shaked (very slowly) for 4h at 37°C, then centrifuged at 1200 rpm for 2 min. Supernatant was carefully removed and 10 µl of 3% SDS, 50 µl isopropanole-HCl were added to each well and slowly shaked for 15 min. Absorbance was evaluated by a photometer at 570 nm, using 630 nm reference.

Proliferation test on mouse and rats cells

Spleen cells from rat (Wistar Rivm:TOX, males) and mice (Balb/c, Rivm, males) were incubated for 24h with increasing concentrations of the selected chemicals in 6 well-plates at 37 °C and 5% CO_2: 2,5 ml of the chemical solution was added to 2,5 ml of a cell suspension at $1x10^7$ cells/ml (RPMI 1640, 1% penicillin and streptomycin, 10% FCS). After incubation, cells were harvested washed twice and resuspended at $4x10^6$ cells/ml. Cell viability was assessed by trypan blue dye exclusion. To evaluate mitogen responsiveness, concanavalinA (ICN-biochemicals), LPS from Escherichia coli serotype O127:B8, (Sigma-Aldrich) and Lectin from Phytolacca Americana (Sigma-Aldrich) were used. In wells of 96 round bottom well plates 0.1 ml of the cell suspension was added together with 0.05 ml of the mitogens (final concentrations in the wells: ConA: 3,33 µg/ml; LPS: 16,5 µg/ml; Lectin: 5 µg/ml) and incubated for 48h. Twenty hours before terminating the incubation, 3H-Thymidine (Amersham, 10 µl/well, 1µCi) was added. The cells were harvested on glass fibres filters (LKB-Wallac, Turku, Finland), using a multiple cell culture harvester (LKB-Wallac). Scintillation liquid (LKB-Wallac) was added to the filters and the radioactivity in the filters was counted in a liquid scintillation counter (LKB-Wallac).

Each test was performed in triplicate; spleen cells from rat were tested on an individual animal basis; spleen cells from mice were pooled and divided in three portions.

LDH

Three solutions were prepared: 50 mM phosphate buffer, 60 mM sodium piruvate, 18 mM NADH. These were used to prepare a reaction mixture: 50 mM phosphate buffer 98 ml, 60 mM sodium piruvate 1 ml, 18 mM NADH 1 ml.

Cells were plated in 96 well plates (15.000/cells per well) and incubated in DMEM with 2mM glutamine, 10% FBS for 24h and then treated with compounds.

After 24h of incubation 1 ml medium was collected from cell plates and kept on ice. The rest of medium was aspirated. Cells were washed twice with PBS without Ca^{2+} and Mg^{2+} and then scraped off after adding 1ml cold PBS without Ca^{2+} and Mg^{2+}, to each well. Samples were collected in 1 ml eppendorf tubes and kept on ice until processing. It is better to analyze samples straight after collecting them, however if this is not possible they can be kept at 4°C for 24h. Cells have to be sonicated just before analysis. 1 ml cuvettes were prepared with 20 µl of sample and 1 ml of reaction mixture. Samples were read one by one at 340 nm (2 min for each one).

Enzyme activity can be expressed as µmol/min or µmol/min/l (U/l):

$$\frac{\Delta Absorb.x\ V_t\ x\ 1000}{\varepsilon\ x\ d\ x\ \Delta t} = \mu mol/min \qquad \frac{\Delta Absorb.x\ V_t\ x\ 1000}{\varepsilon\ x\ V_s\ x\ d\ x\ \Delta t} = \mu mol/min/l$$

V_t is the total volume in the cuvette (1020 µl). V_s is the sample volume, ε is the wave length. d is the distance between the light origin and the cuvette. Δt is the time of reading.

Statistical analysis

The concentrations which inhibit the 50% of growth (IC50) were calculated according to the Reed and Muench formula (Reed and Muench, 1938) and Hill function analysis. Data were expressed as mean ± standard error of the mean.

RESULTS

Viability tests

Before performing functional assays, cytotoxicity was evaluated through different tests: MTT, trypan blue dye exclusion and LDH. The IC50 values (μM) obtained for each compound, after 24h exposure, are reported in Table 1. Not cytotoxic concentrations were used for proliferation and cytokine release analysis.

Table 1. Viability data. This table summarizes cytotoxic test results performed by all laboratories participating on this study.

	Cytotoxicity tests: IC50 (μM)		
COMPOUNDS	**MOUSE**	**RAT**	**HUMAN**
Urethane	>10000	>10000	>10000
Furosemide	>1000	>1000	>1000
Verapamil	>15	>15	>100
Benzo(a)pyrene	>200	>200	>50
Cyclosporin A	15.61 (± 1.9)	>6	>5
TBTC	0.046 (± 0.06)	0.02 (±0.001)	>0.1

CFU-GM test

In figure1 dose-response curves of the selected compounds tested are reported. Urethane treatment decreased the colony number only at very high concentrations (1000 μM), while for Furosemide there was no decrease in the colony number at the concentrations tested. Verapamil, Benzopyrene, Cyclosporin A and TBTC induced a dose-related decrease in the colony number after 14 days exposure.

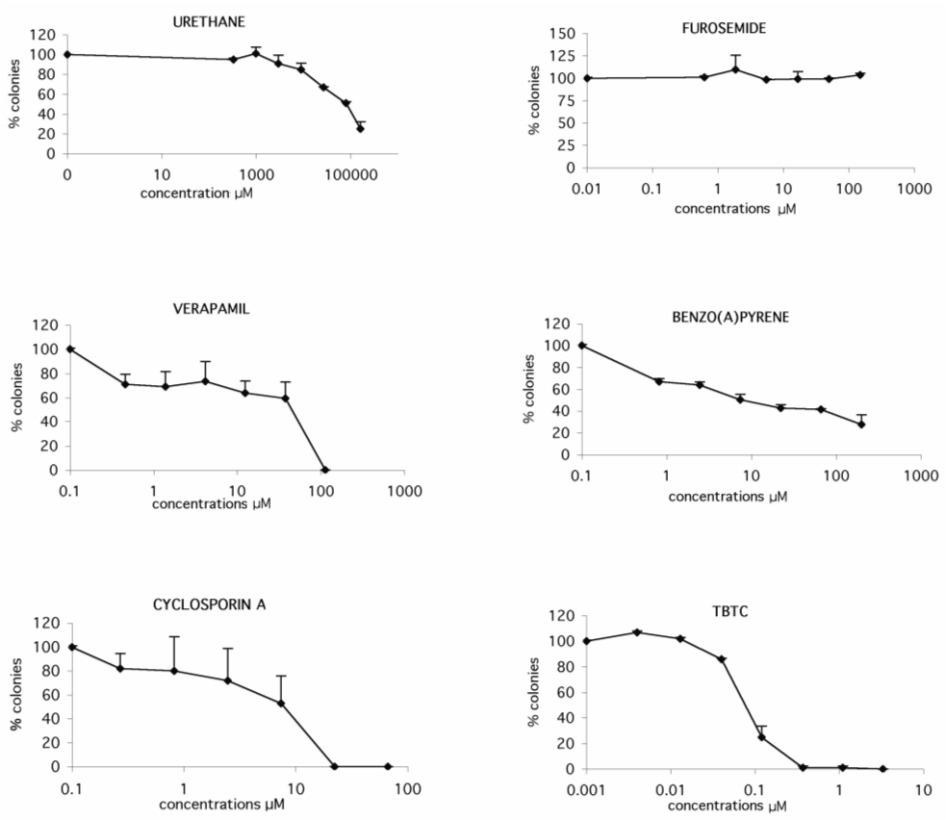
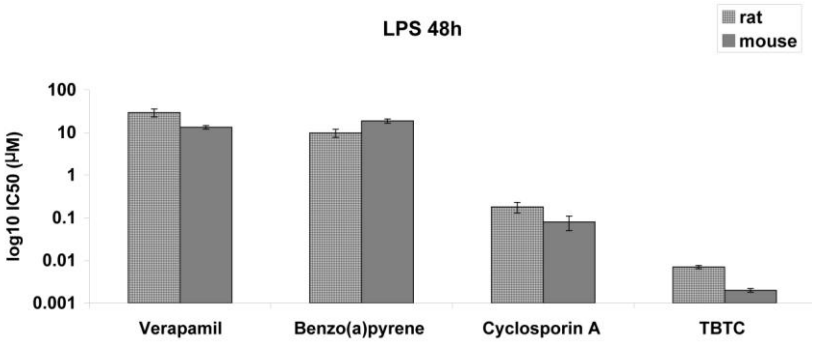


Figure 1. CFU-GM test-graphs show the colonies number counted at different compound concentrations (µM). Colonies were counted after 14 days exposure.

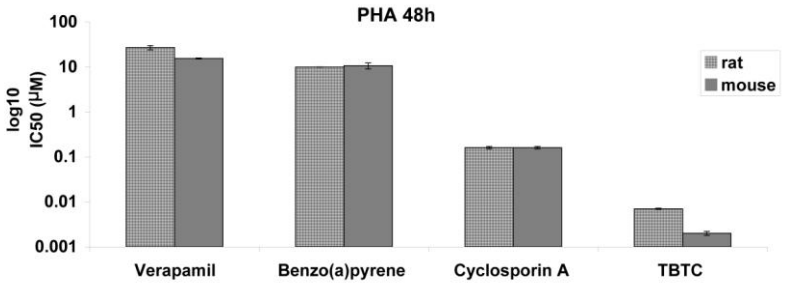
Mitogen stimulation on rodent spleen cells

Rat and mouse spleen cells were treated with chemicals for 24h and then with LPS or PHA for 48h to assess mitogen responsiveness. In figure 2, the IC50 values calculated for each compound in both species are reported. All positive compounds inhibited cell proliferation. The IC50 values were in the same range for both rat and mouse for all substances tested, a part from TBTC for which rat IC50 was higher than mouse IC50 (0.007 ± 0.0002 versus 0.002 ± 0.0002 with PHA, 0.007 ± 0.0006 versus 0.0025 ± 0.0002 with LPS). The negative compounds failed to modulate mitogen response (data not shown).

Rat spleen cells were also stimulated with ConA at 48h or 24h (figure 3). IC50 values were higher after 24h stimulation than after 48h for all compounds tested.



(a)



(b)

Figure 2. Comparison between rat and mouse IC50 values. (a) LPS stimulation at 48h. (b) PHA stimulation at 48h. ^{3}H-thymidine was added to assess mitogen responsiveness.

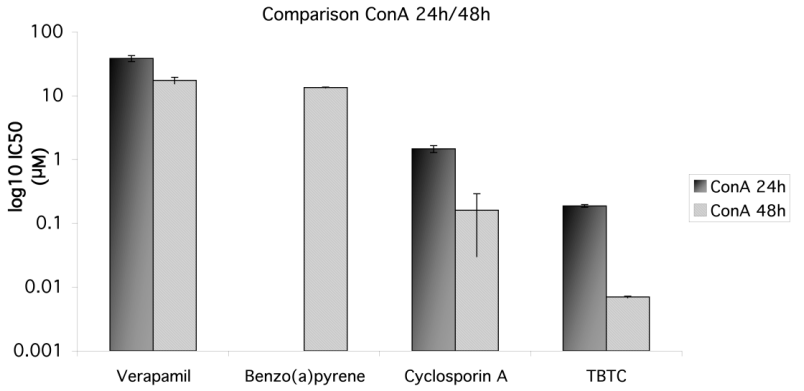


Figure 3. Comparison between ConA treatment at 24h and 48h. Rat spleen cells were treated with compounds and then stimulated with ConA for 24h. In the second case cells were treated with compound and mitogen for 48h.

Anti-CD3 antibody stimulation on mouse spleen cells and human lymphocytes

Mouse and human cells were treated with anti-CD3 antibody to stimulate T lymphocyte proliferation. In table 2 the IC50 values calculated after treatment are reported. Also in this case, both Urethane and Furosemide were confirmed negative in both the two species.

The effect of Benzo(a)pyrene among mouse and human cells was very different: the IC50 values was 12.82 (± 1.11) µM for human lymphocytes, while the compound did not inhibit murine cells proliferation even at the highest dose tested.

The IC50 value for Cyclosporin A was ten time higher in human than in mouse lymphocytes, while the IC50 values for Verapamil were similar between mouse and human lymphocytes.

Table 2. Anti- CD3 antibody stimulation. Cells from mouse lymphocytes and from human peripheral blood have been stimulated with anti-CD3 antibody and treated with compounds. IC50 values (µM) have been calculated.

	Anti- CD3 antibody stimulation	
	IC50 (µM)	
COMPOUNDS	**MOUSE LYMPHOCYTES**	**HUMAN LYMPHOCYTES**
Urethane	>10000	>17000
Furosemide	>1000	>100
Verapamil	30.27 (± 3.5)	20.95 (± 1.16)
Benzo(a)pyrene	>160	12.82 (± 1.11)
Cyclosporin A	0.13 (± 0.05)	1.00 (± 0.13)
TBTC	>0.1	Not tested

Cytokine release from human lymphocytes

The effect of the selected chemicals on cytokine production was assessed using the whole blood assay. Each chemical was tested in at least three donors. Since the amount of cytokines produced varies among donors, it was not possible to combine values, and therefore it was more meaningful to compare the trend of modulation. In fact, despite the different cytokine produced the effects of the different chemicals were consistent among donors.

In figure 4 are reported the effect of the selected chemicals on a representative donor, while in table 3 the data are summarized. The "+" symbol means that cytokine release is modulated (both as increase or decrease), while the "-" symbol means that there is no modulation in respect to vehicle treated cells. Compounds tested were classified as positive if one or more parameters were altered at, at least, one concentration or only one parameter in a dose-related fashion in more than one donor.

All four positive compounds modulated PHA-induced IFN release, and with the exception of Cyclosporin A, they also modulated LPS-induced TNF-α release. The negative coumpound Urethane was overall unable to affect TNF-α and IFN-γ production, while Furosemide at high concentrations, which resulted to be cytotoxic, decreased PHA-induced IFN-γ release, while TNF-α release was not affected.

Table 3. Cytokine release analysis. INF-γ was measured after 72h PHA stimulation, TNF-α after 72h LPS stimulation. "+" symbol means that there is a variation in cytokine releasing which can be an increase or a decrease; "-" symbol means invariability. P stays for positive (immunotoxic), while N negative. Furosemide cannot be classified.

	DONOR #1		*DONOR #2*		*DONOR #3*		*DONOR #4*		
COMPOUNDS	**TNF**	**IFN**	**TNF**	**IFN**	**TNF**	**IFN**	**TNF**	**IFN**	**CLASSIFICATION**
Urethane	-	-	-	+	-		-	-	**N**
Furosemide	-	+	-	+	-		-	+	**(cytotoxicity)**
Verapamil	+	+	+	+	+		+	+	**P**
Benzo[a]pyrene	+	+	-	+	+		-	+	**P**
Cyclosporin A	-	+	-	+	-		-	+	**P**
TBTC	+	+	+	+	+				**P**

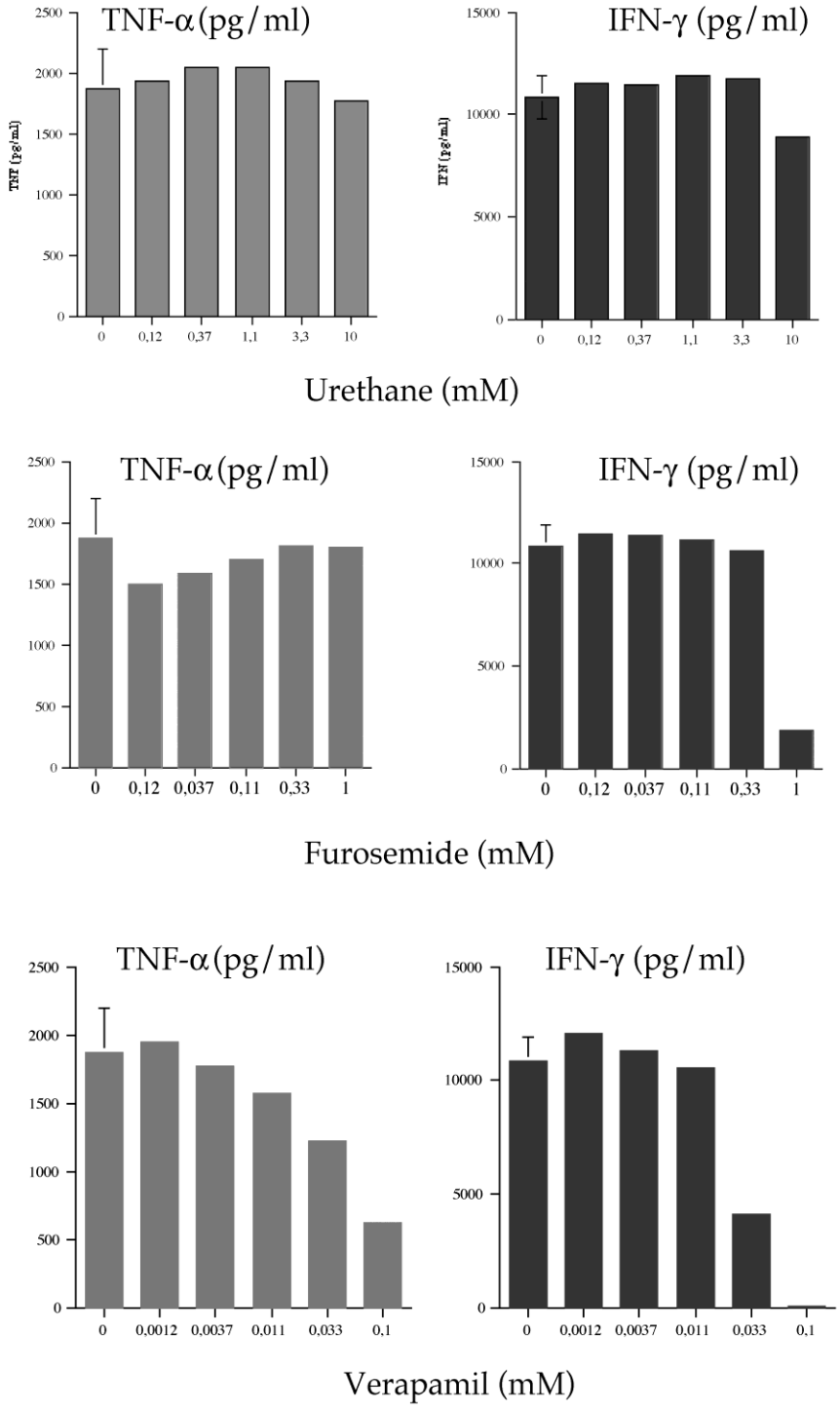


Figure 4. (*Continued*)

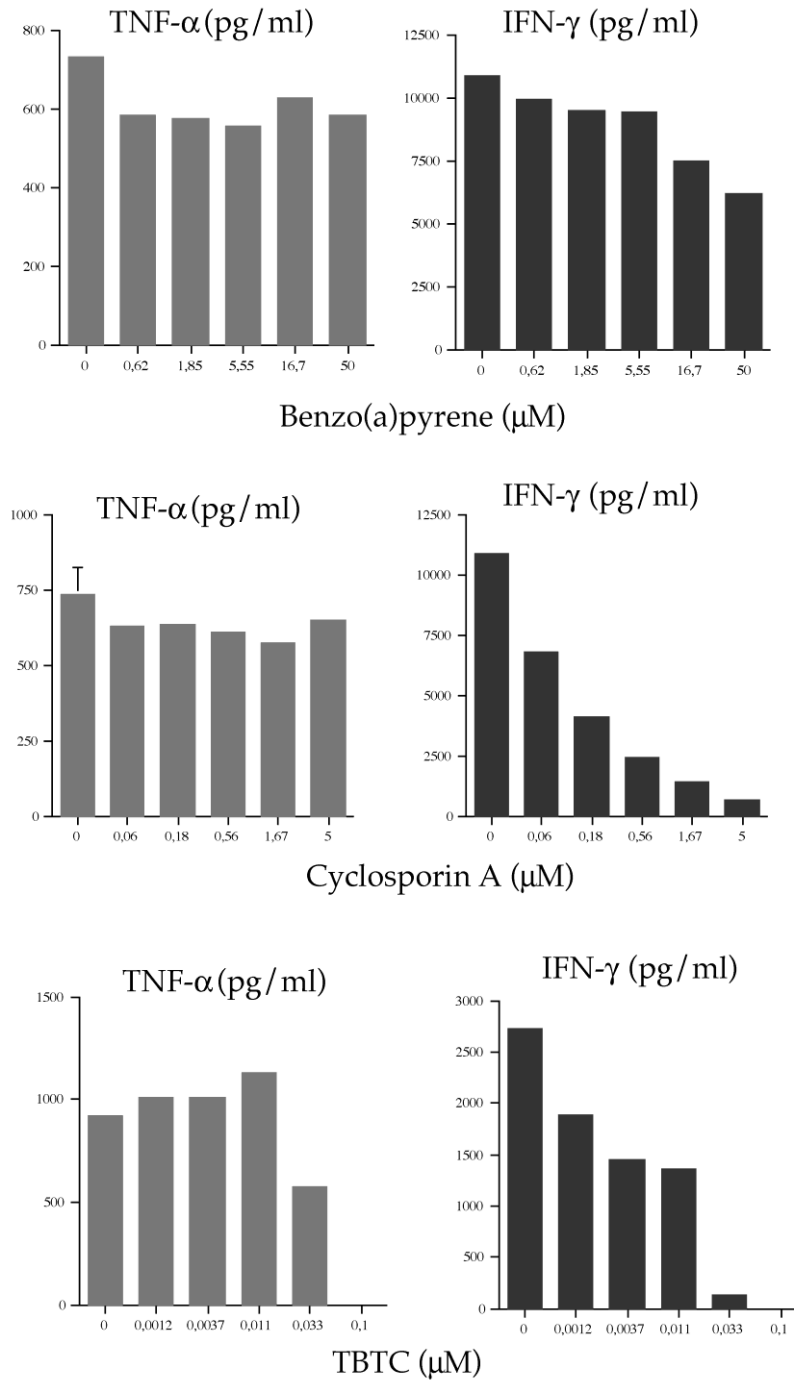


Figure 4. Cytokine modulation on a representative donor. Graphs on the left show the TNF-α (pg/ml) release upon LPS and compound stimulation. Graphs on the right show INF-γ (pg/ml) release upon PHA and compound stimulation.

IC50 comparison through different specie

Where applicable, for each compound tested the IC50 was calculated and results compared by species. Table 4 shows that Urethane and Furosemide were confirmed as not immunotoxic. Verapamil and Benzo(a)pyrene IC50 values was in the same range for both rodents and human. Cyclosporin A and TBTC IC50 is higher in human than in rodents. Mouse seemed, in particular, the most sensitive to Cyclosporin A and TBTC treatment.

All tests performed showed that from Verapamil to TBTC toxicity increase, with the same trend in all species.

Table 4. IC50 Comparison. The IC50 values (μM) obtained from each different test are reported as range. "no effect" means that the compound is not immunotoxic.

COMPOUNDS	MOUSE	RAT	HUMAN
Urethane	No effect	No effect	No effect
Furosemide	No effect	No effect	No effect
Verapamil	**13**<IC50<**30**	**17**<IC50<**30**	**20**<IC50<**22**
Benzo(a)pyrene	**10**<IC50<**18**	**9**<IC50<**13**	**11**<IC50<**12**
Cyclosporin A	**0.08**<IC50<**0.3**	**0.16**<IC50<**0.18**	**1**<IC50<**8**
TBTC	**0.002**<IC50<**0.003**	**0.007**	**0.07**

DISCUSSION

At present, no validated *in vitro* tests are available that can replace *in vivo* methods to evaluate immunotoxicity (Gennari *et al*., 2005). Significant progresses have been made in the last years to promote the establishment of new sensitive methods to assess immunotoxicity (Wagner et al., 2006; Dean, 1997).

The purpose of this study was to assess and compare the potentiality of different in *vitro* tests to correctly identify known immunotoxic compounds. Overall, all assays were able to correctly classify the selected chemicals. At present, *in vivo* immunotoxicity assessment relays on weights of lymphoid organs, histopathology of primary and secondary lymphoid organs, total white blood cell counts, immunophenotyping of peripheral blood leukocytes and quantification of total serum immunoglobulin levels. *In vitro* models could be used for pre-screening of immunotoxic potential, as a strategy. One could start from evaluation of myelotoxicity; compounds that damage or destroy the bone marrow are immunotoxic, since all progenitors derive from the pluripotent stem cells present in the bone marrow (Luster et al., 1985; Kim et al., 2001). Thus, if a compound is myelotoxic it would not be necessary to evaluate other endpoints. The methodology for evaluating myelotoxicity, *in vitro*, the CFU-GM test, has been recently validated (Pessina et al., 2003). In addition, compounds that are not myelotoxic may affect lymphocytes which are the primary effectors and regulators of acquired immunity. If the cells are viable (80% or greater) basic functionality can be determined assessing cell proliferation using mitogens such as plant lectin (PHA, ConA) or anti-CD3 and anti-CD28 antibody (especially for T cells). In parallel, proper immune functionality depends on cytokine production and their quantitative alterations can also be considered as a measure of immunomodulation (Gennari et al., 2005).

There is also the need to use more than one end-point due to the complexity of the immune system and to the different mechanism of action of the compounds (Lebrec et al., 1995).

In the present study, both cell proliferation and cytokine production were used to assess the immunotoxic potential of xenobiotics. Both proliferation and cytokine release are considered to be very relevant to investigate the toxicity towards the immune system (Langezaal et al., 2001). Proliferation was evaluated with CFU-GM test, with anti-CD3 antibody stimulation and with different mitogens (LPS and some lectins). Despite differences between tests used and the different species analyzed, all tests agreed in classifying compounds through all species. Urethane and Furosemide were confirmed not immunotoxic. Other compounds revealed immunotoxic with an increasing toxicity degree from Verapamil to TBTC, in all species. Verapamil and Benzo(a)pyrene imunotoxicity, generally, was similar in all species, while Cyclosporin A and TBTC seemed to have a stronger toxic effect on mouse than on rat cells. Human cells were more resistant than rodents cells to Cyclosporin A and TBTC treatment, in fact IC50 values where 10 times higher in human than in rat cells. The comparison between IC50 values from CFU-GM test and anti-CD3 stimulation on human cells showed an almost 10-fold different sensitivity to Cyclosporin A. The CFU-GM assay measured the clonogenicity of myeloid progenitors, whereas anti-CD3 antibody stimulates specifically T lymphocytes which are the primary target of Cyclosporin A toxicity (Sigal et al., 1991). This put in evidence the mechanism of action of the drug and the ability of the two tests to discriminate the proliferation of different cell populations.

Regarding cytokines release, IFN-γ and TNF-α were used as markers. TNF-α is a pleiotropic inflammatory cytokine, produced by several types of cells, but especially by macrophage. TNF-α plays an important role in the immune response to bacterial, and certain fungal, viral, and parasitic invasions as well as in the necrosis of specific tumors (Janeway et al., 1999). IFN-γ is

produced by lymphocytes activated by specific antigens or mitogens and is a potent activator of macrophages.

The amount of cytokine produced depends not only on the treatment but also on the donor. It is, however, possible to observe the same trend among the different donors. At least three donors should be used to establish consistency and more than one end point should be considered: INF-γ resulted more modulated than TNF-α, probably because the compounds tested had mainly lymphocytes as target cells.

Cytokine release analysis agreed with proliferation tests results, in compounds classification, as discussed before.

CONCLUSION

This preliminary study shows that, *in vitro* tests performed well in classifying the selected compounds (both chemicals and drugs). Each test analyzes a particular aspect of immunotoxicity, for this reason it is important to develop an integrated system able to detect the most relevant endpoints.

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Chapter three

MAINTENANCE AND CHARACTERIZATION OF LYMPHOCYTES IN HUMAN LONG TERM BONE MARROW CULTURES TO STUDY IMMUNOTOXICITY

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ABSTRACT

Immunotoxicity of xenobiotics is of growing concern for various levels in society, including industry and regulatory authorities. Despite that EU legislation aims at reducing the number of laboratory animals by promoting the development of alternative validated methods, at present, immunotoxicity is generally evaluated through standard *in vivo* toxicity studies. The lack of alternative methods is due, in particular, to the complexity of the immune system and its responses, but possibly alternative methods for immunosuppressive chemicals are most achievable.

The present study describes a long term culture (LTC) method capable of inducing the formation of lymphocyte subsets from human mononuclear bone marrow cells that may allow evaluation of lymphotoxicity. The LTC consisted of a two stages: a myeloid stage to allow the formation of a stromal layer and a lymphoid stage to allow expansion of lymphocytes. Results show that the use of IL-7 in LTC inhibits precursor and mature B cells, while it supports the proliferation of $CD3^{+}CD8^{+}$ and $CD3^{+}CD4^{+}$ T-cells.

The bone marrow LTC model may in future be used to test the effect of xenobiotics on stromal dependent lymphocyte formation.

INTRODUCTION

The immune system is an important and vulnerable target of xenobiotics, as a consequence, immunotoxicity evaluation is becoming a growing concern for regulatory authorities. At present, immunotoxicity is evaluated mainly *in vivo* through standard toxicity studies (STS) as indicated in various guidelines. The Organization for Economic Co-operation and Development (OECD) Test Guideline No. 407 includes parameters of immunotoxicological relevance as part of a repeated dose 28 day oral standard toxicity study (STS) in rodents (OECD, 2008). This guideline indicates that information about the toxicity on the immune system can be obtained through the analysis of total and absolute differential leukocyte counts, detection of globulin serum level, gross pathology and histology of lymphoid organs, organ weight (of thymus and spleen), and histology of bone marrow, BALT (bronchus-associated lymphoid tissues), NALT (nasal-associated lymphoid tissues). The International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) guideline S8 (ICH, 2006) indicates that all new human pharmaceuticals should be evaluated for the potential to produce immunotoxicity using STS. If a cause for concern is identified from STS, additional immunotoxicity studies should be performed to verify the immunotoxic potential of the compound.

The European Union legislation aiming at reducing the number of animals used for experiments (Council Directive 86/609/EEC, 1986) encourages the development of alternative testing methods. The general thought is that effects on the immune system are very difficult to reproduce *in vitro*, because of the requirement of complex cellular interactions. However, some isolated processes may be studied *in vitro* (e.g., T lymphocytes proliferation and cytokines release).

The CFU-GM (colony forming unit-granulocyte macrophage) test, used to predict myelotoxic effects in humans, is the only validated immunotoxicity

in vitro method (Pessina et al., 2003). At present no validated alternative methods are available to study the toxicity on lymphocyte differentiation, as it is difficult to maintain them *in vitro*, especially starting from human progenitors. Methods based on long term culture (LTC) techniques to study murine lymphocyte formation *in vitro* are already available for many years (Dexter and Lajtha, 1974; Dexter et al., 1977; Whitlock and Witte, 1982). These LTC from murine bone marrow cells allow the formation of an adherent feeder layer of phagocytic cells, endothelial cells, and adipocytes, which release cytokines and growth factors mimicking the *in vivo* stromal feeder layer. These adherent populations are essential for long-term support of lymphocyte formation. Different modifications of LTC were used to permit the maturation of murine B lymphocytes, such as the use of a stromal cell line from myeloid long term bone marrow cultures (Collins and Dorshkind, 1987) or the use of 2-mercaptoethanol instead of high concentrations of horse serum (HoS), use of a higher incubation temperature (37 °C instead of 33 °C) or low concentrations of fetal bovine serum (FBS) (Whitlock et al., 1984). Gartner and Kaplan (1980) described a method for LTC of human bone marrow cells, based on experience with the murine test system. They showed that the medium supplemented with FBS and HoS allowed the growth of monocytoid and myeloid cells more than lymphoid cells.

Despite all these attempts showing that it is possible to induce myelopoiesis or lymphopoiesis in murine LTC (Collins and Dorshkind, 1987; Whilock and Witte, 1982), human cell systems were less explored. Efforts available showed the necessity of specific cytokine cocktail to induce mono-lineage cell differentiation (Canque et al., 2000), or the requirement of a murine fetal liver-derived stromal cell line together with defined cytokines to obtain human myeloid and lymphoid lineages, starting from human bone marrow progenitors (Miller et al., 1999). The latter system allowed formation of

natural killer cells (NK), myeloid lineages and immature B lymphocytes, but not mature B cells.

The present study aims to set up an *in vitro* lymphocyte formation system using human cells only, as this would be more relevant than using murine systems to translate *in vitro* findings and to predict human health risks.

This study describes the establishment of a LTC starting from human haematopoietic stem cells, as partly described by Dorshkind (1986) who obtained mouse B lymphocytes from hematopoietic precursors. In our study, the LTC was divided into two stages. During the first stage the differentiation of the myeloid lineage was induced and the stromal feeder layer was formed. During the second stage the lymphoid lineage was stimulated by the preformed stromal feeder layer in combination with specific medium components. IL-7, which has been shown to be important on murine lymphopoiesis (Dias et al., 2005), was tested for its relevance to human lymphopoiesis *in vitro*.

Combinations of specific antibodies were used to identify the phenotype of lymphocytes during their differentiation. During early B cells development TdT is present in the cytoplasm together with CD79α and CD79β (early pro-B cells). When TdT is no more expressed, μ chain appears in the cytoplasm (late pro-B cells). CD79α and CD79β are still present intracellulary and only after the expression of μ-chain they migrate to the cell surface (pre-B cells). The μ-chain in large pre-B cells is expressed transiently at the cell surface in combination with a surrogate light chain as part of a pre-B cell receptor. Once a light chain gene is assembled and a complete IgM molecule is expressed on the cell surface, cells are considered mature B lymphocytes (Janeway and Travers, 1997).

T cells differentiation in the bone marrow is poorly characterized, because T cells precursors complete their maturation in the thymus. $CD34^{+}CD2^{+}$ T cell precursors migrate from bone marrow to the thymus. They are located in the outer cortical areas of the thymus, and are $CD4^{-}CD8^{-}$. Productive

rearrangement of the T cell receptor β-chain (TCR β) is followed by its expression on the T cell membrane together with CD3 and the surrogate α-chain. Signaling through the pre-T cell receptor causes the cells to stop rearranging β chain undergo a period of proliferation, and begin expressing both CD4 and CD8, becoming double positive ($CD4^+CD8^+$) T cells. Late thymic T-cell differentiation is characterized by high levels of surface CD3 and by a loss of the co-expression of the CD4 and CD8 antigen. T cells $CD3^+CD4^+$ or $CD3^+CD8^+$ are defined as mature T lymphocytes (Janeway and Travers, 1997).

MATERIALS AND METHODS

Source of progenitor cells

Frozen human mononuclear bone marrow cells (BMC) were obtained from Lonza (Verviers, Belgium) and thawed before use, following the procedure indicated by the supplier. Each LTC was prepared with BMC from the same donor. Different donors were used from one experiment to another one.

Cytokine and enzyme solutions

IL-7 (Sigma-Aldrich Steinheim, Germany) was diluted in sterile bidistilled water, obtaining a 100 μg/ml stock solution. This cytokine was added to the medium to a final concentration of 10 ng/ml.

A collagenase/dispase solution was prepared to detach adherent cells. Collagenase type I (Invitrogen) stock solution of 1576 U/ml and dispase (Invitrogen) stock solution of 307 U/ml were prepared in HBSS (Invitrogen). Collagenase and dispase were mixed 1:1 and the solution diluted 1:10 in HBSS just before use.

Long term cells culture (LTC)

For LTC, BMC were thawed and cultured using McCoy's 5A medium with GlutaMAX (Gibco, Grand Island USA). This basal medium was supplemented with 12% FBS (South American Origin, Cambrex), 12% HoS (heat-inactivated New Zealand Origin, Gibco), 1% sodium pyruvate solution 100 mM, 1% MEM non essential amino acids 100X, 1% penicillin and streptomycin, 1% hydrocortisone (HyC) solution 10^{-4} M (Sigma-Aldrich, Steinheim, Germany), 1% MEM vitamin solution 100X, 0.6% MEM essential amino acids solution 50X (Gibco). BMC $7.5x10^6$ were seeded in 75 cm^2 flasks (Corning, USA) with a final volume of 15 ml and incubated at 37 ^oC.

After 14 days LTC, culture conditions were changed from myeloid (MC) to lymphoid (LC) by using medium without HoS and HyC, since these factors

inhibit the development of lymphocytes (Phillips, 1980 and see Fig. 1). At that time (designated time 0) $1.5x10^6$ fresh bone marrow mononuclear cells were added to each flask in order to increase the number of stem cells that could become lymphocytes under the new culture conditions. If indicated IL-7 was added during LC-culture. Half of the medium was changed twice a week. Cells were analysed using a flow cytometry once a week.

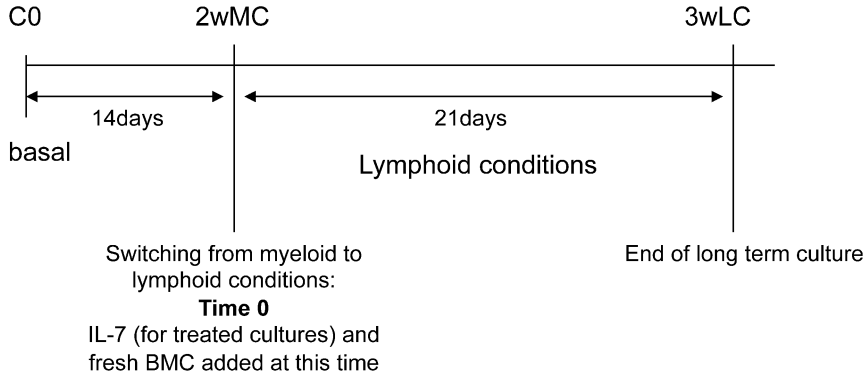

Figure 1. LTC procedure. Bone marrow mononuclear cells were cultured from time C0 under myeloid conditions. After 14 days the medium was replaced, and fresh BMC and IL-7 were added to cultures. From that time on (time 0) cells were cultured using conditions to further support lymphoid differentiation.

Flow cytometry analysis

BMC were analysed after 2 weeks of LTC in myeloid conditions (2wMC, equal to time 0), and from time 0 once a week under lymphoid conditions (1wLC, 2wLC, 3wLC). The analysis on BMC 24h after thawing was also included as being indicative of basal conditions (C0).

Suspended and adherent cells were analysed separately. Cell viability was assessed by trypan blue (Sigma-Aldrich) dye exclusion. After removal of suspended cells, the adherent layer was washed twice with HBSS without Ca^{2+} and Mg^{2+} for 2-3 min. Cells were then incubated at 37 ^{o}C with collagenase/dispase solution for 20 min. Both suspended and adherent cells were washed three times in PBS. Cell suspension was diluted to $1x10^7$ cells/ml in PBS and 100 µl was used for staining. The following antibodies or staining kits were used for the analysis: CD19 R-PE, CD8 FITC (Sigma-

Aldrich), CD45 Pacific Blue (Dako, Glostrup Denmark), CD3 APC (BD Pharmingen), CD4 R-PE (Biosource Invitrogen, CA, USA), MultiMix kit containing CD79α APC, IgM precursor (μ) PE, TdT FITC (Dako), and a CD19/IgM kit (Invitrogen). Absolute cell counts were carried out using CountBright (Invitrogen) according to the manufacturer's protocol.

The CD45 antibody was always used in combination with the other antibodies (except for MultiMix kit) for a better identification of the lymphocyte sub-population. A gate was drawn around the cells on the forward scatter (FSC) versus side scatter (SSC) graph so as to exclude debris and absolute count beads (CountBright). A new graph (CD45 versus side scatter) was drawn using this population and the lymphocyte percentage was calculated from the $CD45^+$ and low SSC population (Fig. 4a).

CD19 and CD3 antibodies were paired to discriminate B and T cells, and the antibody combination CD3/CD4/CD8 was used to identify T lymphocytes. To characterise mature B lymphocytes the CD19/IgM kit was used, and to identify pro- and pre-B cells, cells were permeabilized and fixed using Intrastain kit (Dako), then stained with the MultiMix CD79/TdT/μ kit.

Samples were incubated for 30 min with antibodies. After one wash in PBS, cell staining were detected using FACSAria cytometer (Becton Dickinson, Franklin Lakes, USA) and subset percentages were analysed with FlowJo software (TreeStar, Ashland OR, USA).

Statistical analysis

The GraphPad Prism 4.0 (GraphPad software, San Diego, USA) program was used for statistical analysis. Data were expressed as means of at least three independent experiments ± standard error of the mean (SE). Statistical analysis was performed on raw data using two-way ANOVA followed by a Bonferroni post-test to assess the effect of time and IL-7 treatment on cell samples. Values of $p<0.05$, $p<0.01$ and $p<0.001$ were considered statistically significant.

RESULTS

IL-7 increases the total amount of lymphocytes in LTC

After 2 weeks of culture under MC, cell numbers decreased slightly from starting number of $7.5x10^6$ to $4.5\pm1.1x10^6$ (Fig. 2b). All cell numbers represent the total number obtained from each culture flask. Upon the switch from myeloid (MC) to lymphoid conditions (LC) half of the cultures were supplemented with IL-7. At the same time fresh BMC ($1.5x10^6$) were added to each flask to increase the number of stem cells able to differentiate into lymphocytes under the new culture conditions.

During the first two weeks of LTC, i.e under MC, stroma formation was favoured, whereas lymphocytes were less prominent. Under lymphoid conditions, IL-7 induced an increase in the total cell number (Fig. 2), as well as in the proportion of ($CD45^+SSC^{low}$) lymphocytes, in particular in the suspended population (Fig. 3).

Under LC, only those cultures supplemented with IL-7 showed a significant increase in cell numbers up to $10.5\pm2.0x10^6$, whereas without IL-7 cell number remained at the same level (around $6.0\pm0.6x10^6$ at 3wLC). The increased proportion of lymphocytes was reflected in an increase in the absolute number of lymphocytes, from around $0.284x10^6$ up to $1.388\pm0.430x10^6$ in the suspended population and up to $1.717\pm0.451x10^6$ in the adherent population (Table 1). Notably, the cell viability of weekly samples of the cultures was always over 90%.

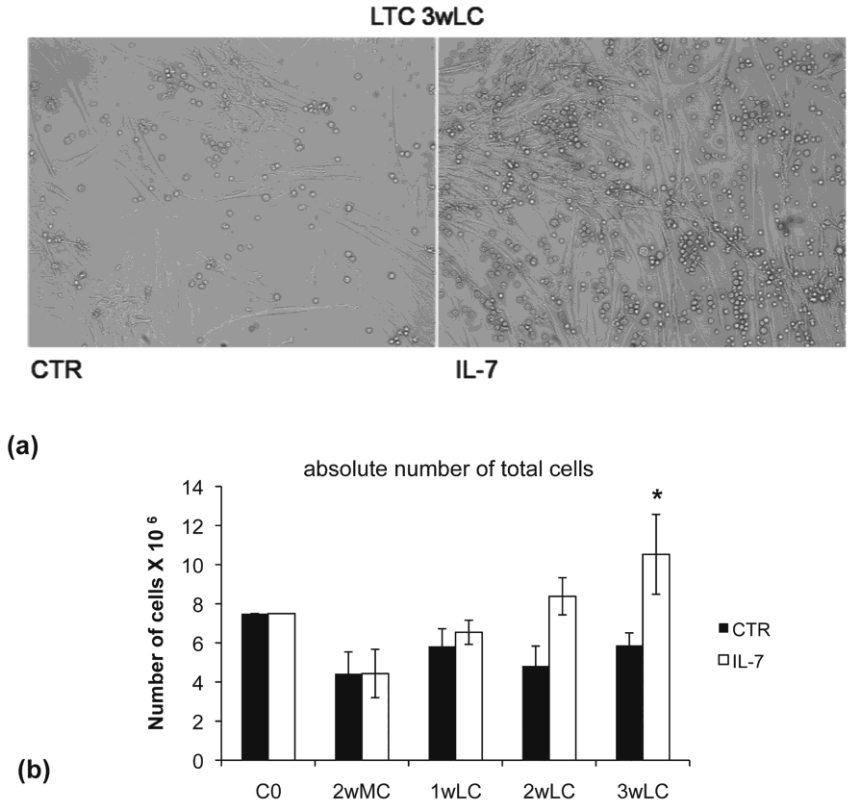

Figure 2. Bone marrow long term culture. (a) Images of bone marrow LTC were captured after 3 weeks under lymphoid conditions. Image on the left is an example of the control culture. Image on the right is an example of the culture treated with IL-7. Magnification 10X. (b) Absolute number of the entire cell population (suspended fraction + adherent fraction) in each culture condition, from C0 (basal condition) to 3wLC. Statistical analysis was performed on six independent experiments (* $p<0.05$), comparing each time point with C0, and IL-7 treated samples with the respective control.

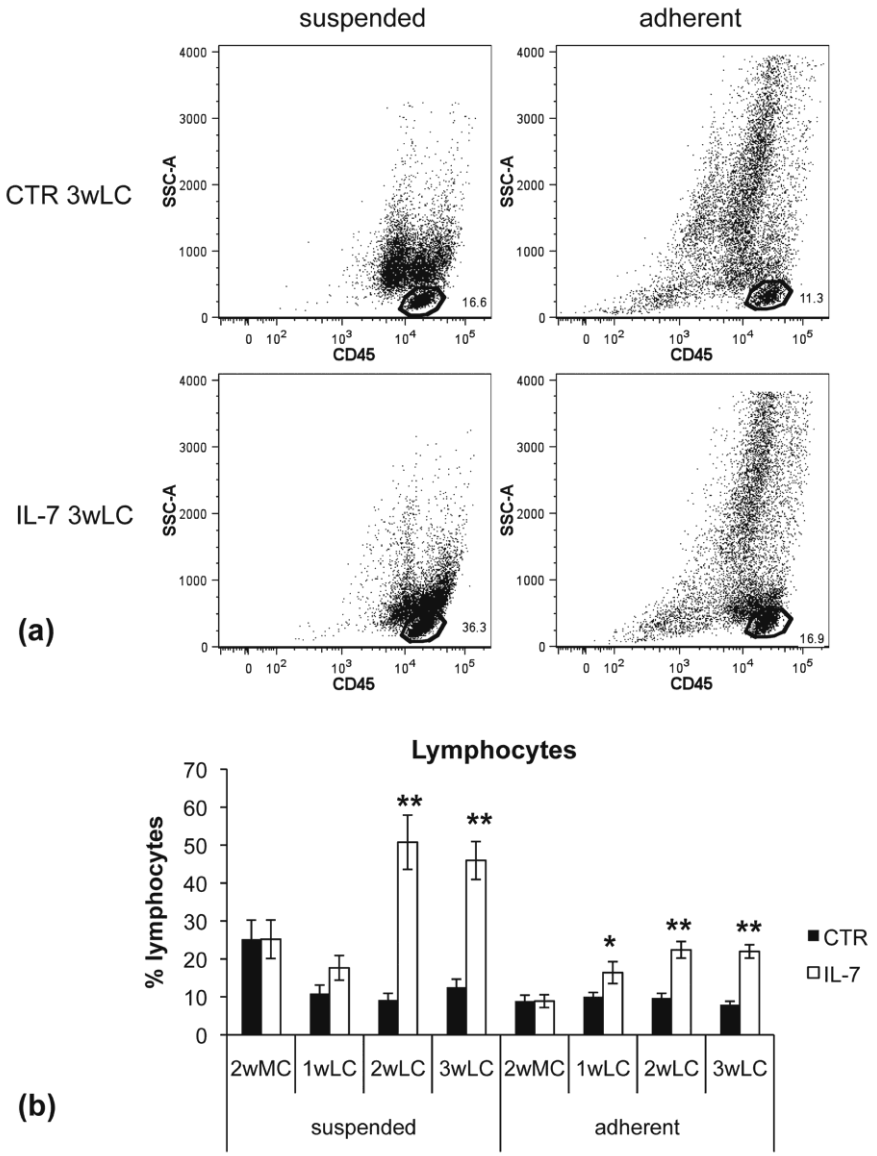

Figure 3. Total lymphocyte population. The lymphocyte population was determined through FACS analysis. The percentage of lymphocytes was calculated as the total $CD45^{+}$ low SSC population. (a) Images of the flow cytometry analysis of BMC after 3wLC (representative of one experiment). The circle is the gate on the lymphocyte population, selected on cells positive for CD45 staining. (b) The graph summarises FACS analysis results from 2wMC, until 3wLC. Statistical analysis was performed on six independent experiments (* $p<0.05$; ** $p<0.01$), comparing each time point with 2wMC, and IL-7 treated samples with the respective control.

IL-7 supports the expansion of $CD3^+CD19^-$ lymphocytes

It was next evaluated which lymphocyte subset was responsible for the observed IL-7-induced increase. The percentage of B lymphocytes, characterized as $CD3^-CD19^+$ cells within the $CD45^+SSC^{low}$ population, continued to decrease in the suspended fraction until 3wLC, both in the control (0.9±0.0%) and in IL-7-treated (0.4±0.1%) samples (Fig. 4b). In the adherent population, the $CD3^-CD19^+$ percentage was generally higher than in the suspended fraction and significantly increased over time in the IL-7-deprived conditions up to 3wLC (Fig. 4b). IL-7 did clearly not favour B cell development. The proportional changes are reflected in absolute cell count (Table 1). $CD3^+CD19^-$ T cell percentage did not significantly differ between CTR and IL-7-treated cultures (Fig. 4c), but the absolute cell count, revealed that IL-7 treatment increased $CD3^+CD19^-$ cell number up to 7-fold in the suspended fraction, and up to 6-fold in the adherent fraction compared to CTR samples (Fig. 4c, Table 1).

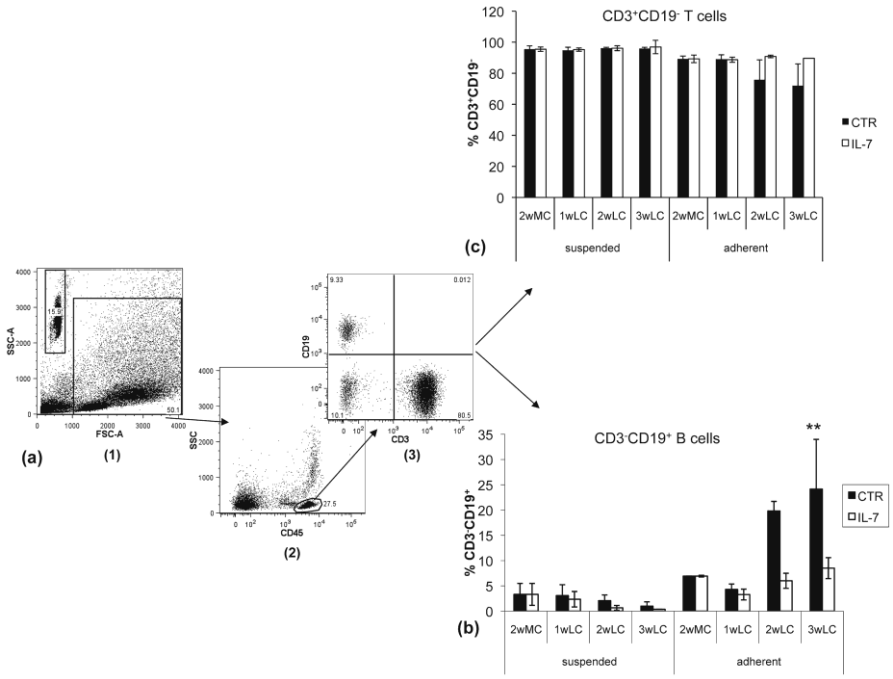

Figure 4. Flow cytometry analysis of $CD3^+CD19^-$ T cells and $CD3^-CD19^+$ B cells. (a) The lymphocyte population was determined through FACS analysis with the use of CD45 staining. A gate was drawn around cells on the forward scatter (FSC) versus side scatter (SSC) graph so as to exclude debris and CountBright beads (gated in the upper left side of graph1) (graph 1). A new graph (CD45 versus side scatter) was drawn using this population and the lymphocyte percentage was determined from the $CD45^+$ and low SSC population (graph 2). CD3/CD19 dot plot was obtained after gating lymphocytes population from the $CD45^+$ and low SSC population. The $CD3^+CD19^-$ and $CD3^-CD19^+$ percentages were calculated based on the total lymphocyte population (graph 3). (b) Percentage of $CD3^-CD19^+$ B cells in suspended and adherent phase. (c) Percentage of $CD3^+CD19^-$ T cells in suspended and adherent phase. Statistical analysis was performed on three independent experiments (** $p<0.01$), comparing each time point with 2wMC, and IL-7- treated samples with the respective control.

IL-7 treatment stimulates both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets

In suspended cell fraction (Fig. 5), the percentage of $CD3^+CD4^+$ T cells was higher than $CD3^+CD8^+$ T cells from 2wMC to 3wLC, both in CTR (respectively 65.2±7.0% to 53.1±9.0% $CD3^+CD4^+$ cells, and 29.7±4.5% to 22.7±8.5% $CD3^+CD8^+$ cells) and IL-7-treated samples (65.2±7.0% to 70.1±7.6% $CD3^+CD4^+$ cells and 29.7±4.5% to 36.0±7.8% $CD3^+CD8^+$ cells).

Subset percentages obtained by flow cytometry analysis did not reveal any significant difference between CTR and IL-7-treated cultures, but the absolute cell count showed a significant increase of both $CD3^+CD4^+$ and $CD3^+CD8^+$ cell number (Table 1) induced by IL-7 at 2wLC and 3wLC. $CD3^+CD4^+$ cell number increased in IL-7-treated samples up to 7-fold compared to CTR; in parallel, $CD3^+CD8^+$ cell number increased up to 10-fold compared to CTR (Table 1).

The $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were rarely detectable in the adherent fraction (data not shown). On the contrary, $CD3^+CD4^-CD8^-$ T cell percentage was higher in the adherent fraction compared to the suspended one as shown in table 1.

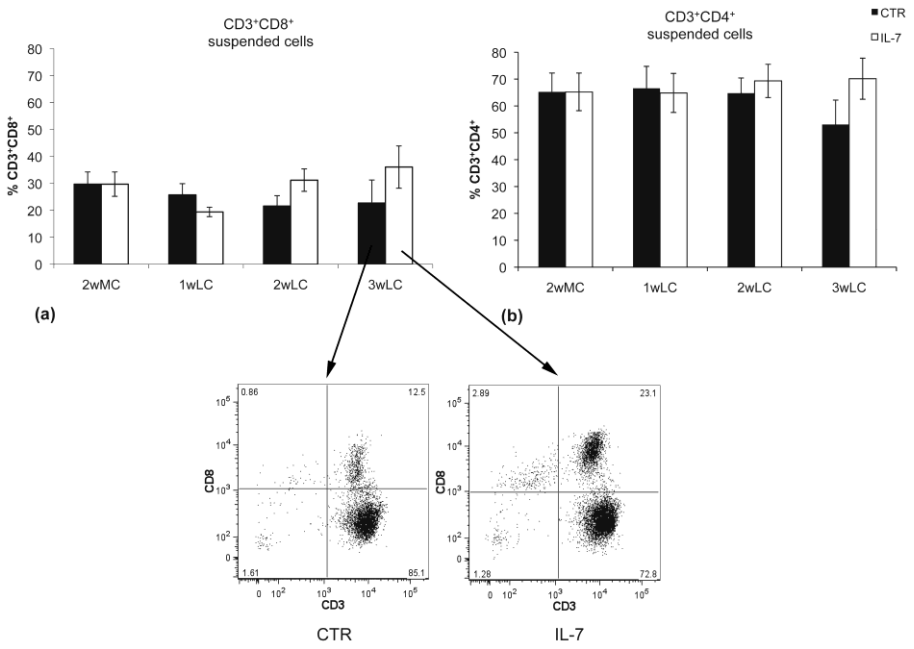

Figure 5. Flow cytometry analysis of T lymphocytes using CD3, CD4, CD8 antibodies. Only results for suspended cells are reported, with and without IL-7. (a) The graph summarizes results for the $CD3^+CD8^+$ cell percentage. Images of the flow cytometric analysis of BMC (representative of one experiment) are reported for control and IL-7 treated cultures after 3wLC. (b) The graph reports $CD3^+CD4^+$ cell percentage

IL-7 treatment inhibits B-cell precursors and B cell maturation

Since IL-7 had such a strong inhibiting effect on B cell development we analysed various ontogenetic sub-fractions by staining cells from LTC with a mix of three antibodies targeting intracellular proteins present in immature B cells: TdT, CD79α and μ. TdT is an early differentiation marker and was expressed only in some C0 samples at very low percentage (0.5%, data not shown). Since TdT^+ cells were not present in our cultures, only the following phenotypes were considered: $CD79\alpha^+\mu^+$, $TdT^-CD79\alpha^+$, $TdT^-\mu^+$.

In CTR cultures, the $CD79\alpha^+\mu^+$ percentage was maintained at the same level from 2wMC (2.8±1.0%) up to 3wLC (3.6±0.8%), in the suspended fraction; on the contrary, the $CD79\alpha^+\mu^+$ percentage was significantly reduced by IL-7 at 3wLC (0.5±0.1%).

In the absence of IL-7 the percentage of $CD79\alpha^+\mu^+$ significantly increased from 2wMC (5.5±1.6%) to 3wLC (12.6±4.4%) in the adherent fraction. At the same time, IL-7 significantly reduced $CD79\alpha^+\mu^+$ percentage in the adherent fraction (from 5.5±1.6% at 2wMC to 1.9±0.3% at 3wLC) (Fig. 6a). The same changes were observed for $TdT^-CD79\alpha^+$ and $TdT^-\mu^+$ B cell populations (data not shown).

The percentage of mature B cells ($CD19^+IgM^+$) increased significantly from 2wMC (2.0±1.1%) up to 2wLC (13.9±0.1%) in the adherent fraction, although from 2wLC to the end of the culture, the $CD19^+IgM^+$ percentage was reduced (7.6±2.1% at 3wLC); a similar change was observed for suspended cells (Fig. 6b). The inhibitory effect of IL-7 on $CD3^-CD19^+$ B cells was also apparent using $CD19^+IgM^+$ phenotyping, both in the adherent (from 2.0±1.1% at 2wMC to 2.3±1.3% at 3wLC) as well as the suspended population (from 1.0±0.2% at 2wMC to 0.3±0.0% at 3wLC) (Fig. 6b).

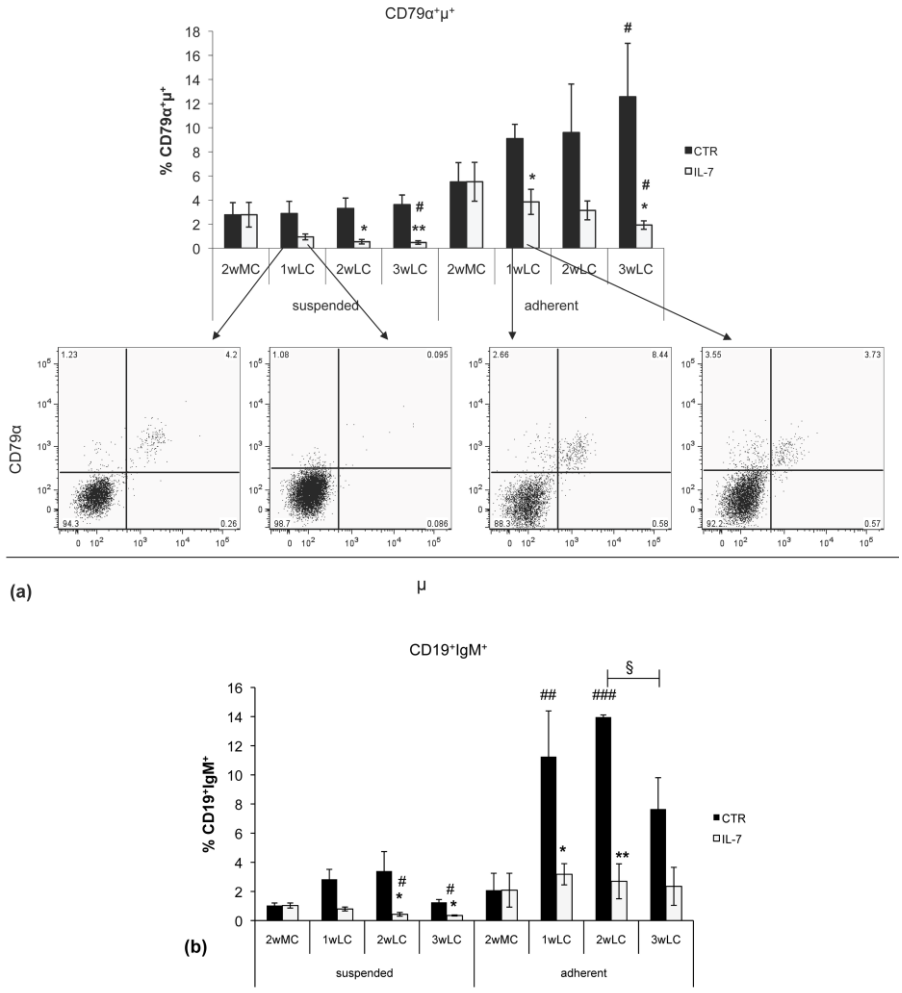

Figure 6. Flow cytometry analysis of B cell precursors ($CD79\alpha^+\mu^+$ subset) and mature B cells ($CD19^+IgM^+$). Results for suspended and adherent cells, with or without IL-7 are reported. (a) The graph summarizes results related to the $CD79\alpha^+\mu^+$ subset. The following dot plots are also reported as examples of one experiment: CTR 1wLC and IL-7 1wLC suspended cells, CTR 1wLC and IL-7 1wLC adherent cells. (b) The graph shows the percentage of $CD19^+IgM^+$ B lymphocytes. Statistical analysis was performed on three independent experiments comparing each IL-7 treated samples with the respective control (* $p<0.05$; ** $p<0.01$), and comparing each time point with time 0 (2wMC) (# $p<0.05$; ## $p<0.01$; ### $p<0.001$). The comparison between CTR samples at 2wLC and 3wLC is also reported (§ $p<0.05$).

Table 1. The table reports the absolute cell count for each culture flask (number x 10^6), obtained from FACS analysis. The following populations were analysed: the total lymphocyte population, $CD3^-CD19^+$ B cells, $CD3^+CD19^-$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^-CD8^-$ T cells. Statistical analysis was performed on three independent experiments (for the total lymphocyte population six independent experiments were analysed) comparing each time point with time 0 (2wMC) and IL-7 treated samples with the respective control (***p<0.05***). ND: not detected.

fraction	**time**	**total lymphocytes**		**$CD3^-CD19^+$**		**$CD3^+CD19^-$**		**$CD3^+CD4^+$**		**$CD3^+CD8^+$**		**$CD3^+CD4^-CD8^-$**	
		CTR	**IL-7**	**CTR**	**IL-7**	**CTR**	**IL7**	**CTR**	**IL-7**	**CTR**	**IL-7**	**CTR**	**IL-7**
suspended	2wMC	0.284±0.076	0.284±0.076	0.008±0.002	0.008±0.002	0.284±0.093	0.284±0.093	0.185±0.049	0.185±0.049	0.079±0.016	0.079±0.016	0.008±0.002	0.008±0.002
	1wLC	0.177±0.018	0.484±0.167	0.007±0.001	0.005±0.001	0.215±0.006	0.324±0.008	0.112±0.025	0.131±0.061	0.044±0.010	0.035±0.012	0.008±0.005	0.006±0.002
	2wLC	0.157±0.056	***1.388±0.430***	0.002±0.000	0.001±0.000	0.098±0.029	***0.692±0.062***	0.056±0.003	***0.441±0.039***	0.018±0.004	***0.215±0.039***	0.005±0.001	0.015±0.004
	3wLC	0.217±0.041	***1.221±0.560***	0.002±0.000	0.001+0.000	0.235±0.035	***0.852±0.008***	0.098±0.046	***0.455±0.079***	0.029±0.013	***0.325±0.097***	0.006±0.003	0.011±0.004
adherent	2wMC	0.216±0.081	0.216±0.081	0.027±0.009	0.027±0.009	0.313±0.195	0.313±0.195	ND	ND	ND	ND	0.212±0.096	0.212±0.096
	1wLC	0.400±0.071	0.658±0.162	0.020±0.001	0.009+0.001	0.501±0.179	0.523±0.009	ND	ND	ND	ND	0.240±0.081	0.379±0.154
	2wLC	0.336±0.104	***1.245±0.165***	0.058+0.012	0.018+0.006	0.200±0.012	***1.131±0.251***	ND	ND	ND	ND	0.207±0.075	***1.093±0.090***
	3wLC	0.320±0.054	***1.717±0.451***	***0.122±0.021***	0.045±0.014	0.381±0.066	***2.252±0.550***	ND	ND	ND	ND	0.185±0.021	***1.705±0.581***

DISCUSSION

The aim of the present work was to obtain a human *in vitro* model to assess the immunotoxic potential of chemicals. To that end, we used a two-stage LTC procedure to allow *in vitro* development of lymphoid cells from precursor cells. The first stage allowed the formation of bone marrow stroma-like microenvironment required for subsequent lymphopoiesis (Nagasawa, 2006) For the second stage, culture conditions were adjusted (elimination of HoS and HyC) to stimulate lymphocyte development and inhibit further stroma formation (Phillips, 1980).

Both in the suspended as well as in the adherent fractions, the percentage of lymphocytes was higher in the presence of IL-7. Further analyses demonstrated that IL-7 inhibited B cell development and stimulated T cell development. More detailed analysis of the B cell compartment showed that IL-7 inhibited B cell precursors ($CD79\alpha^{+}\mu^{+}$) and their development, since under these conditions the percentages of mature B cell ($IgM^{+}CD19^{+}$) were lower than CTR. IL-7 showed a stimulatory effect on the T cell compartment increasing $CD4^{+}$, $CD8^{+}$ and $CD4^{-}CD8^{-}$ cell numbers.

It has been shown that (CD19-negative) pro-B cells express CD79α (Dworzak et al., 1998) and that the loss of CD34 and TdT, and the co-expression of μ–chain and CD79α within the cytoplasm, indicate the progression to the pre-B cell stage (Koyama et al., 1997). In our hands, TdT was detected at a very low level (0.5%) only in BMC after thawing, after which this marker completely disappeared during culture. It is not clear if TdT^{+} cells were not detected because B cell precursors differentiate or because the culture conditions did not support their maintenance. Since the immature B cells present in our model were negative for TdT, and positive for CD79α and μ-chain, they can be considered as pre-B cells.

The LTC procedure used in this study was a modification of *in vitro* methods developed using mouse cells that similarly allowed the maintenance and development of $CD45^{+}$ lymphocytes, in particular in the presence of IL-7

(Dorshkind, 1986; Tushinski et al., 1991). Studies in mouse have shown that IL-7 is involved in maintaining the B cell potential in common lymphoid progenitors (CLP) (Dias et al., 2005). In humans, the IL-7 receptor is expressed mainly in CLP and in pro-B cells, and it is shown to be essential for the differentiation of human $CD34^+$ BMC into pro-B cells (Taguchi et al., 2007). The IL-7 receptor is not present in pre-B cells that arise from pro-B cells (Taguchi et al., 2007), which can be the reason why pre-B cells ($CD79\alpha^+\mu^+$) were not stimulated by IL-7. So in humans, in contrast to mouse, IL-7 does not induce the growth of pre-B cells from adult human bone marrow. Remarkably, in our human *in vitro* system B cell development was not insensitive to the presence of IL-7 since IL-7 caused a reduction of all B cell subsets, i.e. immature ($CD79\alpha^+\mu^+$) and mature (IgM^+CD19^+ or $CD3^-CD19^+$) B cells, both adherent and suspended fraction. Since data in literature suggest that B cells in our culture should not display the IL-7 receptor (Taguchi et al., 2007), the effects observed could be due to an indirect effect of IL-7 via stromal cells. Possibly, because B cells survival and differentiation is strictly dependent by the contact with the stroma feeder layer (Wilson and Trumpp, 2006), IL-7 interferes with the production of cytokines or growth factors important for B lymphopoiesis; in fact, the higher B cells reduction, especially in the adherent fraction, was observed in immature B cell subsets. The inhibition of immature B cells causes a reduced release of more mature B cells in the suspended fraction. Maybe IL-7 interferes with $CD19^+IgM^+$ B cell differentiation but not with their proliferation, because the cell percentage was maintained at the same level from 2wMC to 3wLC, in the adherent fraction, on the contrary, cell percentage of the suspended fraction was reduced during the time. It should be investigated if, in this model, B cells express IL-7 receptor, in this case IL-7 would have a direct inhibitory activity on B lymphocytes. Therefore, in our model IL-7 inhibits the B cell lineage through a mechanism induced in pre-B and mature B lymphocytes that has yet to be explained.

So, our findings demonstrate that IL-7 preferentially promotes the growth of various T cell subsets from human bone marrow, and are thus in line with human data (Tushinski et al., 1991; Dias et al., 2005) indicating that our model may indeed be relevant for the human situation.

The presence of mature T lymphocytes in LTC bone marrow culture is probably due to the maintenance of T cells already present in basal conditions, because fresh mononuclear bone marrow cells contain not only stem cells, but also mature lymphocytes. Mature $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes were found only in the suspended fraction, probably because they do not need the contact with cells of the adherent layer. In contrast, $CD4^-CD8^-$ T cells, considered immature T lymphocytes and representing the majority of $CD3^+CD19^-$ lymphocytes in the adherent fraction, apparently need the contact with cells of the feeder layer. Important to note is that committed "extrathymic" T cell progenitors, at various stages of T cell differentiation including pre-T cells, exist in human bone marrow (Klein et al., 2003). In addition, it has been demonstrated that murine bone marrow cultures, depleted of mature T lymphocytes, give rise to T cells with the mature $CD4^+CD8^-$ and $CD4^-CD8^+$ surface phenotype via a $CD4^+CD8^+$ intermediary stage that recapitulates the principal features of thymic maturation. The presence of mature T cells strongly inhibits this process (Garcia-Ojeda et al., 1998). In our study, $CD4^+CD8^+$T cells were not detected. However, the finding that our model supports the formation of immature T cells suggests that the two-stage LTC model presented here may be relevant to study immature T cell development as well.

The proportion of $CD3^+CD4^+$ cells (between 60% and 50% in the control samples and between 60% and 70% in the IL-7 treated samples), was always about twice as high as the proportion of $CD3^+CD8^+$ subset (between 30% and 20% in the control samples, and between 30% and 40% in the IL-7-treated samples). This ratio of about two to one is consistent with findings observed for normal T cells in peripheral blood (Armitage et al., 1990) and

with results obtained by Tushinski (1991). In conclusion, the LTC described here allows the maintenance of human $CD45^+$ lymphocytes. By using specific culture conditions, i.e. use of IL-7, the LTC can be conditioned to favour one or the other lymphocyte subset. Clearly, more investigations are necessary to further optimise and evaluate if this method can be used to assess particular immunotoxic effects of xenobiotics on B and T cell development.

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Chapter four

TBTC INDUCES ADIPOCYTE DIFFERENTIATION IN HUMAN BONE MARROW LONG TERM CULTURE.

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ABSTRACT

Organotins are widely used in agriculture and chemical industry, causing persistent and widespread pollution. Organotins may affect brain, liver and immune system and eventually human health. Recently, it has been shown that tributyltin (TBT) interacts with nuclear receptors PPARγ (peroxisome proliferator-activated receptor γ) and RXR (retinoid x receptor) leading to adipocyte differentiation in the 3T3 cell line.

Since adipocytes are known to influence haematopoiesis, for instance through the expression of cytokines and adhesion molecules, it was considered of interest to further study the adipocyte-stimulating effect of TBTC in human bone marrow cultures.

Nile Red spectrofluorimetric analysis showed a significant increase of adipocytes in TBTC-treated cultures after 14 days of long term culture. Real-time PCR and Western blot analysis confirmed the high expression of the specific adipocyte differentiation marker aP2 (adipocyte-specific fatty acid binding protein). PPARγ, but not RXR, mRNA was increased after 24h and 48h exposure. TBTC also induced a decrease in a number of chemokines, interleukins, and growth factors. Also the expression of leptin, a hormone involved in haematopoiesis, was down-regulated by TBTC treatment. It therefore appears that TBTC induced adipocyte differentiation, whilst reducing a number of haematopoietic factors. This study indicates that TBTC may interfere in the haematopoietic process through an alteration of the stromal layer and cytokine homeostasis.

INTRODUCTION

Organotin compounds are widely used as plastic stabilisers, catalytic agents, agricultural pesticides, rodent repellents, and as antifouling agents in paints (Fent, 1996). This widespread use causes a ubiquitous and persistent environmental contamination with direct effects on animal health. Organotin compounds affect different organs including the liver (Ueno et al., 2003), brain, immune organs, and in particular the thymus (Bressa et al., 1991, Snoeij et al., 1988). Some of the most toxic organotins are tri-butyltin (TBT) compounds (Maguire, 1987). The biocide bis(tri-n-butyltin)oxide (TBTO) inhibits the transcription of some genes involved in lipid metabolism in primary rat thymocytes. This effect is considered the first indication of disruption of cellular function (Baken et al., 2007). TBT has been shown to induce the conversion of fibroblasts (mouse 3T3-L1 cell line) into adipocytes (Inadera and Shimomura, 2005; Kanayama et al., 2004). The underlying mechanism of this TBT effect is not known, but it has been demonstrated that TBT compounds are high affinity ligands of the retinoid x receptor-α (RXRα) and peroxisome proliferator-activated receptor-γ (PPARγ), activating PPARγ pathway (Kanayama et al., 2004).

The heterodimer RXRα/PPARγ plays a key role in the differentiation and energy storage capacity of adipocytes by activating the transcription of a number of target genes such as the one coding for the adipocyte differentiation marker aP2 (adipocytes-specific fatty acid binding protein-2). Importantly, the TBT serum concentration in humans (27 nM) would be sufficient to activate high affinity receptors like RXRα and PPARγ (Grun et al., 2006). Consequently, the effect of TBT on adipocytes may be an environmental risk factor in the development of metabolic disorders like obesity and type-2 diabetes in which adipocytes play a crucial role (Spiegelman et al., 1993).

A thoroughly studied organotin compound is tri-butyltin chloride (TBTC) which also causes reproductive abnormalities (Ogata et al., 2001) and like

some other tri-organotin compounds acts as an endocrine disruptor (Grun and Blumberg, 2006).

The activity of TBTC on adipocyte conversion in human bone marrow has, however, not yet been investigated. Also the role of adipocyte-conversion in TBTC-mediated immunotoxic effects is unknown.

Adipocytes represent the most abundant stromal cell in human adult marrow, but their function is not completely clear. They are, however, no longer considered as simple space fillers; in fact they play an active role in the bone marrow stroma. It has been hypothesized that adipocytes participate in the overall lipidic metabolism and provide a local energy reservoir in the bone marrow. They are also involved in thermogenesis: studies in rodents have shown the presence of uncoupling protein 1 (UCP-1) which is typical of brown adipose tissue (Marko et al., 1995). This type of adipocyte is most abundant in long bones where the temperature is lowest (Gimble et al., 1996).

Adipocytes influence haematopoiesis through mechanisms which have not yet been elucidated. It is known that in humans, the number and size of adipocytes increase with advancing age when haematopoiesis decreases; on the contrary, when haematopoiesis is stimulated, by a peripheral cytopenia, the number of adipocytes decreases (Laharrague et al., 1998). Possibly, adipocytes exert their effect by expressing adhesion molecules or by producing cytokines, one of which is leptin. Leptin is a regulating hormone for food intake and energy homeostasis, and its deficiency can cause obesity, diabetes, and infertility in humans. Leptin has also been shown to be involved in angiogenesis, reproduction, lymphoid organ homeostasis, and haematopoiesis (Zhang et al., 2005). With regard to the latter, leptin stimulates lymphopoiesis and erythropoiesis, whereas its role on myelopoiesis appears redundant (Bennett et al., 1996).

The aim of this study was to investigate the effect of TBTC on human bone marrow cells. The first step was to verify if TBTC induces adipocyte

differentiation. Secondly, we studied the effect of TBTC on the production of cytokines in bone marrow cultures.

MATERIALS AND METHODS

Chemical compounds and solutions

Tributyltin chloride (TBTC) (Merck, Schuchardt, Germany) stock solution (10µM) was prepared using DMSO (Sigma-Aldrich) and was then diluted in the medium to a final concentration as indicated.

Oil Red O (Sigma-Aldrich) stock solution was dissolved in isopropanol 3.5mg/ml and filtered through Whatman 3MM (Whatman International, Maidstone, England). The working solution was prepared by diluting Oil Red O stock solution in distilled water (3:2).

Ripa-like buffer was prepared using: Tris (pH 7.4) 50mM, NaCl 250mM, SDS 0.1%, DTT 2mM, NP40 0.5%. Just before protein extraction, protease inhibitors were added to Ripa–like buffer: PMSF (500mM) 1:1000, Leupeptin (0.5mg/ml) 1/1000, Apoprotinin (2mg/ml) 1/1000, Pepstatin A (1.4mg/ml) 1/2000.

Source of progenitor cells

Human bone marrow cells (BMC) were used as the source of progenitors for the CFU-assays and long term cultures. BMC were obtained, frozen, from Cambrex Bio Science (Walkersville, U.S.A) and thawed before usage, as indicated by supplier.

Long term cells culture (LTC)

For long term cultures, BMC were thawed and cultured using McCoy's 5A medium with GlutaMAX (Gibco, Grand Island USA). To this basal medium, the following were added: 12% FBS (South American Origin, Cambrex), 12% horse serum (heat-inactivated New Zeland Origin, Gibco), 1% sodium pyruvate solution 100mM, 1% MEM non essential amino acids 100X, 1% penicillin and streptomycin, 1% hydrocortisone solution 10^{-4}M, (Sigma-Aldrich, Steinheim, Germany), 1% MEM vitamin solution 100X, 0.6% MEM essential amino acids solution 50X (Gibco). BMC were seeded

$5x10^5$/ml in $25cm^2$ and $75cm^2$ flasks (Corning, USA) in a final volume of 5ml and 15ml respectively and incubated at 37°C. Half the medium was changed twice a week. Cells can be maintained in these conditions for a number of weeks. In this study, BMC were cultured in LTC conditions for 24h, 48h (short term exposure) or for 14 days (long term exposure).

CFU-GM assay

For the CFU-GM (colony forming unit-granulocyte macrophage) assay BMC were seeded in MethoCult-H4001 medium (Methocult, StemCell Technologies, Vancouver, BC, Canada). This medium contains 1% of methylcellulose in IMDM, 30% FBS, 1% of Bovine Serum Albumin (BSA), 2mM L-glutamine and 10ng/ml granulocyte-macrophage-colony stimulating factor (GM-CSF). Briefly, 22µl of 200X drug solutions and 300µl of cells ($1.1x10^6$ cells/ml) were added to tubes containing 4ml of MethoCult. 1ml of methylcellulose-cell suspension was then seeded into 35mm Petri dishes. The cultures were incubated at 37°C and 5% CO_2 under saturated humidity for 14 days. The final TBTC concentrations used were from 0.0007µM to 0.07µM.

Colony scoring

A CFU-GM colony is defined as an aggregate containing 50 or more cells. Morphologically, four classes of CFU-GM colonies can be observed: compact, diffuse and spread multicentric and multifocal colonies. A compact colony has a central dense nucleus with a peripheral halo. Diffuse and spread colonies are without an apparent nucleus. Multicentric colonies appear with two or more dense nuclei nearby and with a common peripheral halo growing at the same depth in the plate. Multifocal colonies are aggregates of several colonies or clusters with or without a peripheral halo (Pessina et al., 2001).

Cell proliferation is expressed as a percentage of growth, with respect to the number of colonies in control dishes (100%).

The concentrations of test compound which inhibit growth by 50% of CFU-GM (IC50) in comparison to the control cultures were calculated using the Reed and Muench formula (Reed and Muench, 1938). Results are reported as the mean ± standard error of at least two experiments, each done in triplicate.

Morphological analysis

BMC were seeded at $5x10^5$ cells/well, in 12 well plates (Corning) and treated with 0.001μM and 0.01μM TBTC in DMSO (0.1%), a control with medium only and a vehicle control were also included. After 14 days, the medium was removed and cells were washed twice with D-PBS (Gibco) with Ca^{2+} and Mg^{2+}. Cells were fixed with 10% formaldehyde solution (Sigma-Aldrich, diluted in D-PBS) for 30 min, then stained with Oil Red O solution for 10 min. After washing under tap water, cells were stained with Mayer's Hematoxylin (Sigma-Aldrich) for 2-3 min and washed under tap water for about 10 min. Cells were observed under the microscope (20X) and images captured using Image-Pro Plus software.

Quantification of intracellular lipid droplets

BMC were seeded as mentioned above, in 12-well plates (Corning), at $5x10^5$ cells/ well and cultured in the presence of TBTC. After 14 days cells were analysed with AdipoRed assay kit (Cambrex Bio Science). AdipoRed is a Nile Red solution which becomes fluorescent when partitioned in a hydrophobic environment. The assay was performed following the supplier's instructions. Briefly, the culture supernatant was removed and each well rinsed with 1ml PBS. Then 2ml PBS and 60μl AdipoRed were added for each well. After 10 min incubation at room temperature, fluorescence was measured using a Fluoroskan Ascent (Thermo scientific, USA) microplate fluorometer using an excitation wavelength of 485nm and emission

wavelength of 535nm. The blank value was subtracted from each sample and results were expressed as relative fluorescent units (RFU).

RNA extraction and real- time PCR

BMC were seeded at $2.5x10^6$ cells in $25cm^2$ cell culture flasks and treated with 0.001μM or 0.01μM TBTC in DMSO (0.1%), including also a control with medium only and a vehicle control. Cells were collected after 24h, 48h, or 14 days of exposure, and total RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Italy). The RNA samples were treated with DNAse (DNA-free kit, Qiagen).

Reverse-transcription of RNA (500ng) was carried out using Moloney murine leukaemia virus reverse transcriptase (M-MLV, Promega, Madison USA), with random primers and oligo-dT (Promega) at the same concentration (500μg/ml), and 50U of ribonuclease inhibitor (RNase-Out, Promega).

A quantitative real time PCR (RT-PCR) was then performed on cDNA samples to evaluate the mRNA levels of PPARγ, RXRα, aP2, UCP-1, leptin and beta-2-microglobulin. All of them were analysed using TaqMan gene expression assays ("Assay on demand", Applied Biosystems, U.S.A.). Primers for these genes were designed and labelled at the 5'-end with a reporter dye (FAM) and at the 3'-end with a quencher dye (TAMRA). The primers used were: PPARG-Hs00234592_m1, RXRA-Hs00172565_m1, FABP4-Hs00609791_m1, UCP1-Hs00222453_m1, LEP-Hs00174877_m1, B2M-Hs00187842_m1. All experiments were performed in duplicate in 96-well plates using TAQ-Man Universal Master Mix (Applied Biosystems). RT-PCR amplification was performed using a Gene Amp 7000 Sequence Detection System according to the manufacturer's protocol. PCR conditions were 50°C for 2 min, 95°C for 10 min, then 40 cycles at 95°C for 15 seconds, and 60°C for 1 min. For quantitative evaluation of gene expression, all genes levels were normalised to the beta2-microglobulin mRNA

reference genes (B2M-Hs00187842_m1). Fluorescence data were processed and analysed with ABI PRISM Sequence Detection software (version 1.6 software, Applied Biosystems). The quantification of the PCR assay was based on C_T values. Briefly, the C_T values indicate the fractional cycle number for which the amount of amplified target reaches a fixed threshold. The difference (ΔC_T) between the C_T of the target gene (C_T t) and the reference gene (C_T r) depends on the RNA relative copy number between the target and the reference gene. The difference between the ΔCt value of each sample and the control was then calculated ($\Delta\Delta C_T$). The values obtained from this power: $2^{(-\Delta\Delta Ct)}$ are the relative RNA amounts, which are graphically reported.

Western blot

BMC were seeded at $7.5x10^6$ cells in 75 cm^2 cell culture flasks and grown under the same conditions as described above for long term culture. Cells were treated with 0.001μM or 0.01μM TBTC, medium only or DMSO (0.1%) for 14 days. After treatment, cells were washed twice in PBS and collected as a dried pellet. To lyse the cells and to extract the proteins, the pellet was incubated in Ripa–like buffer (with the addition of protease inhibitors) for 30 min on ice. After centrifugation (15 min at 13000 rpm, 4ºC) the supernatant was collected and the protein quantified using the Lowry test (Bio-Rad protein assay kit). 50μg of proteins for each sample were resolved on a 10% NuPAGE Bis-Tris Gel (Invitrogen, LifeTechnologies, Italy) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Massachussets, USA). The primary antibodies used were: aP2 1μg/ml (Abnova Corporation, Taiwan); GAPDH 1μg/ml (Santa Cruz Biotechnologies). GAPDH expression was simultaneously estimated in each sample as the internal control by Western blotting technique. The secondary antibodies were diluted 1/10000 (Chemicon, USA). Membranes were incubated with Immobilon western,

chemiluminescent HRP substrate (Millipore) for 5 min. Images were then captured using Gel Logic 2200 Imaging System and analysed using Kodak Molecular Imaging Software (Eastman Kodak Company, Rochester, USA).

Cytokine array

Human cytokine antibody array 6 (RayBiotech, Norcross, GA) was used to determine relative changes in protein expression. Proteins from cell lysate and conditioned media from both control and treated cells were hybridised on cytokine array membranes. Each cytokine antibody is present as 2 spots on the same membrane. Protein extraction and quantification was performed as described for Western blotting. Membranes were used following the supplier's protocol. Briefly, membranes were incubated with 1.2ml conditioned media or with 60µg protein, diluted in 1.2 ml blocking buffer for 2h. After washing, the membranes were incubated with biotin-conjugated antibody and then with HRP-conjugated streptavidin for 2h respectively. Membranes were incubated for 2 min with a specific detection buffer provided with the kit. Images were captured with Gel Logic 2200 Imaging System and analysed with a specific Kodak Molecular Imaging Software array tool (Eastman Kodak Company). For each spot the net density grey level was determined by subtracting the background grey level. After background subtraction, the spot net density was normalised on the positive control, according to the formula: normalised signal intensity of particular spot = signal intensity of particular spot x (1/ positive signal intensity). Relative fold difference in cytokine amount was then determined in reference to the amount present on the control culture membrane: average treated culture spot/average control culture spot.

Statistical analysis

Data obtained from AdipoRed assay were the results of five independent experiments, performed in duplicate. These data were graphically reported as means ± standard error of the mean (SE).

Data obtained by real time PCR, were expressed as means of two independent experiments performed in duplicate ± standard error of the mean (SE). The statistical analysis was performed using Student t-test calculated on the raw data. Values of $p<0.05$ (*) and $p<0.01$ (**) were considered statistically significant.

RESULTS

Low concentrations of TBTC stimulate CFU-GM colony formation

To determine the effect of TBTC on granulocyte-macrophage progenitor cell proliferation CFU-GM tests were performed. It appeared that at low concentrations TBTC stimulated granulocyte-macrophage colony formation, whereas at concentrations higher than 0.007µM, TBTC decreased the colony number (figure 1). The mean of the absolute GM-CFU colonies number taken as 100% was $74^{\pm}13$ (SE), while the IC50 was $0.03^{\pm}0.004$µM. From these results two concentrations of TBTC were chosen for further experiments: 0.001µM, which was in the ascending part of the curve and 0.01µM, which was in the descending part of the curve. Both concentrations were within the range of TBTC concentrations measured in human tissue samples: 0.003-0.1µM (Grun et al., 2006).

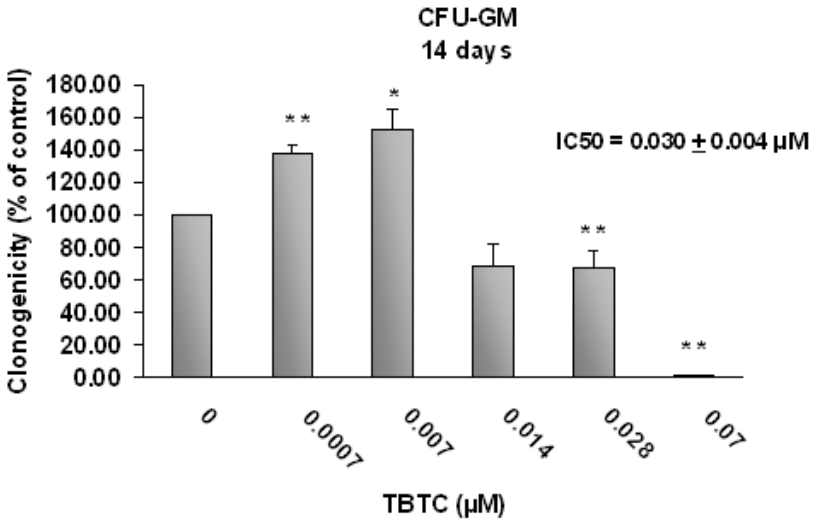


Figure 1. CFU-GM test. Colony number was counted after 14 days exposure to different TBTC concentrations (µM). Results were reported as a percentage of the control. Statistical analysis was performed on 3 independent experiments (* $p<0.05$; ** $p<0.01$).

TBTC stimulates adipocyte formation from BMC

LTC conditions allowed myeloid and mesenchymal cell differentiation from BMC with the formation of an adherent layer (stroma) together with cells growing in suspension, which are mostly lymphocyte-like as well as undifferentiated cells. In the adherent layer, we observed the presence of adipocytes, whose number increased after treatment with TBTC (figure 2). Differentiated adipocytes appeared more round-shaped and full of lipid droplets. The quantitative analysis of these droplets with Nile Red showed a significant increase in lipid upon exposure to TBTC (figure 3). For cultures treated with TBTC 0.001µM and with TBTC 0.01µM, the RFU values were 2 fold and almost 3 fold higher than the control cultures, respectively.

Because white and brown adipocytes have different influences on hematopoiesis, it is important to know which of these adipocyte types is stimulated by TBTC (Tsai et al., 2005). In cultured cells, mRNA UCP-1, which is considered a specific marker for brown adipocytes, was negative for both control and treated samples, indicating that the culture contained no brown adipocytes (data not shown).

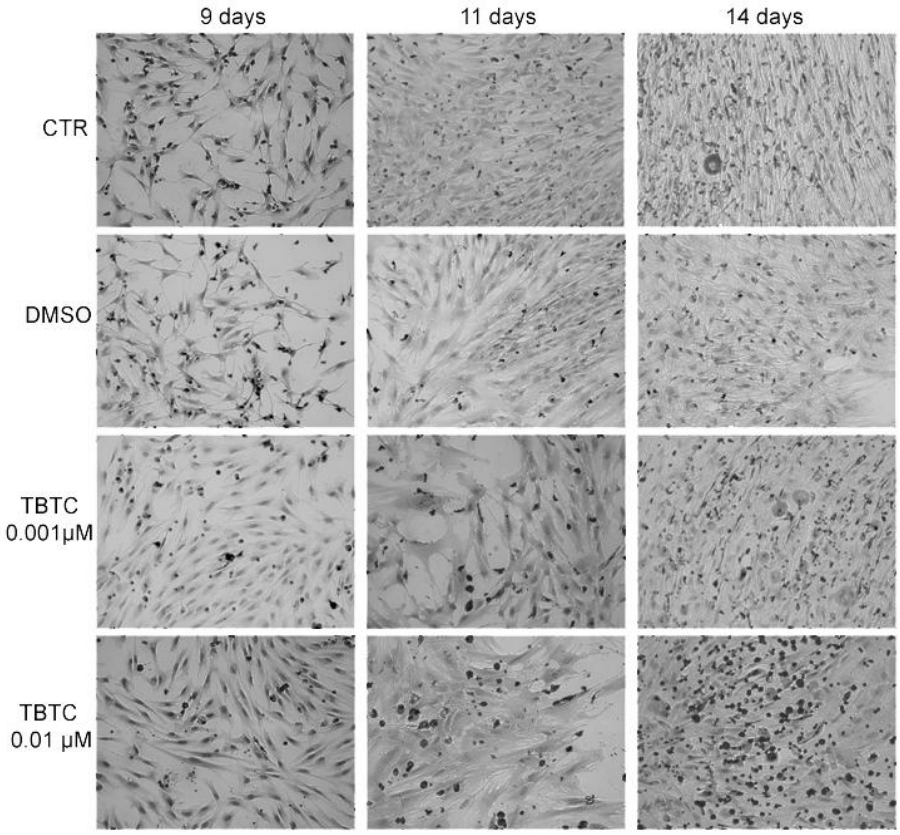


Figure 2. Oil Red O staining. Images were captured after 9, 11 and 14 days LTC. Magnification 20X.

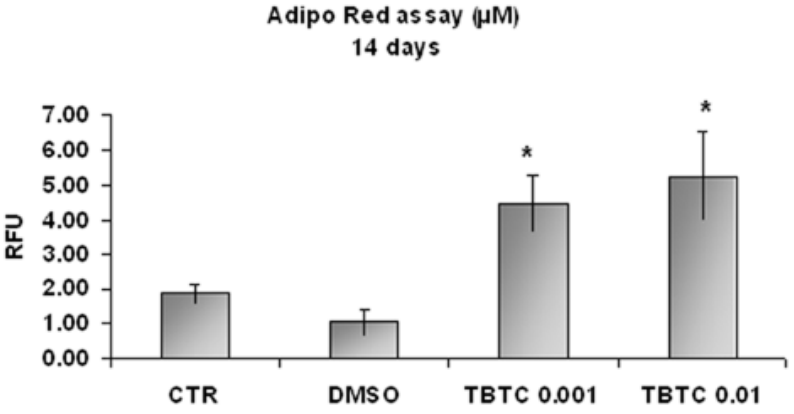


Figure 3. Adipo Red analysis after 14 days culture of BMC. Statistical significance: * $p<0.05$. TBTC concentrations are expressed in µM.

TBTC modulates genes involved in the PPARγ pathway

Since PPARγ pathway plays a key role in adipocyte differentiation, we analysed the expression of molecules directly involved: PPARγ, RXRα and aP2. mRNA expression was investigated for short term (24h and 48h) and for long term exposure (14 days).

Short term exposure

BMC were cultured in LTC conditions for 24h and 48h. RNA extracted from untreated cells 24h after thawing, was also analysed as a control for the basal conditions (C0).

Figures 4A and 4B show that TBTC induced a concentration- and time-dependent increase in aP2 (from 3 to 3.8 fold after TBTC 0.001μM treatment for 24h and 48h respectively; from 5.6 to 7.4 fold after TBTC 0.01μM treatment for 24h and 48h respectively) and PPARγ mRNA levels (from 1.7 to 2.7 fold after TBTC 0.001μM treatment for 24h and 48h respectively; and from 2.8 to 4.7 fold after TBTC 0.01μM treatment for 24h and 48h respectively), compared to controls. On the other hand, RXRα mRNA-levels decreased after 24h exposure at both 0.001 and 0.01μM TBTC (0.6 fold lower than control), but after 48h exposure a decrease was only observed at the lowest TBTC concentration (0.8 fold lower than control), whereas at the highest TBTC concentration mRNA levels of RXRα were similar to the control (figure 4C).

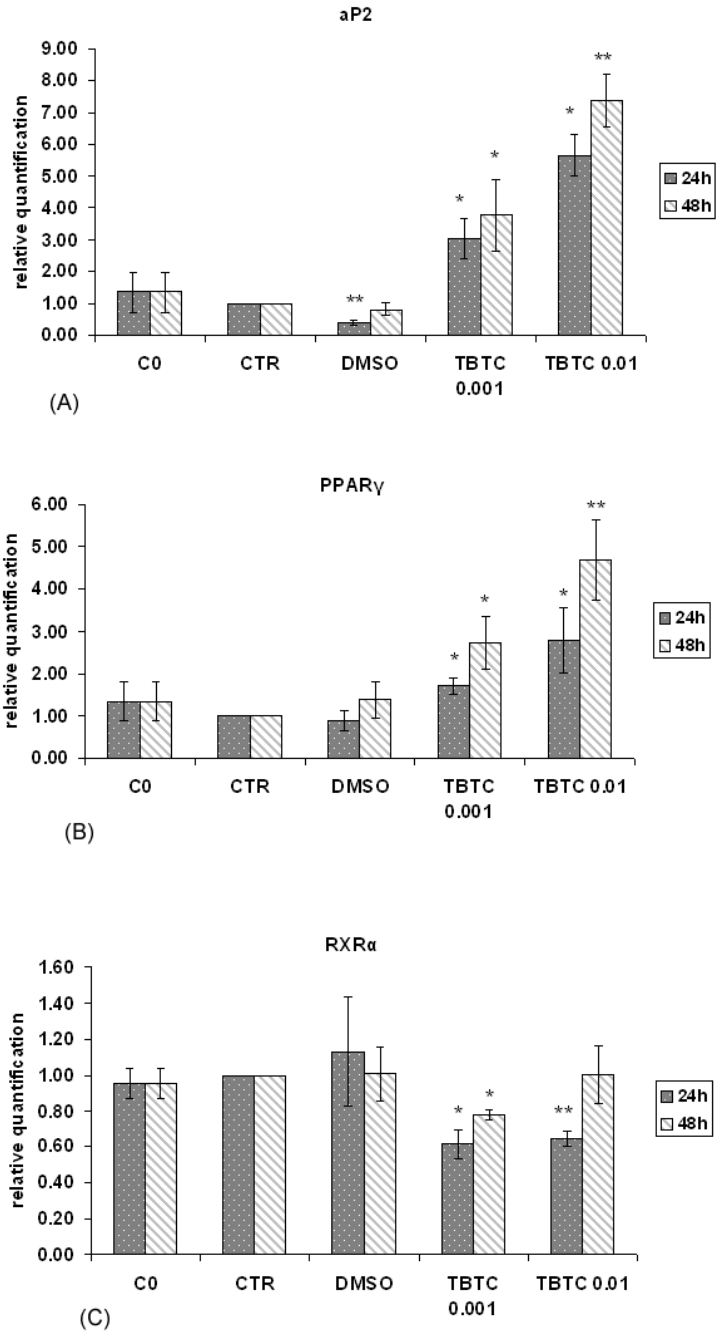


Figure 4. Real time PCR analysis at time 0h, 24h and 48h. (A) aP2 mRNA analysis. (B) PPARγ mRNA analysis. (C) RXRα mRNA analysis. Statistical significance: * $p<0.05$, ** $p<0.01$. TBTC concentrations are expressed in µM.

Long term exposure

BMC were cultured in LTC conditions. After 14 days, protein and RNA were extracted and used for Western Blotting and RT-PCR experiments, respectively. PPARγ expression was significantly lower in cells at 0.001µM TBTC than in untreated cells (0.7 fold lower than control, figure 5A). This expression profile is opposite to that observed at 24h-48h, displaying an increase in PPARγ expression.

RXRα expression only decreased at 0.01µM TBTC (0.7 fold lower than control), and not at 0.001µM TBTC (figure 5B). The aP2 mRNA expression significantly increased compared to controls at both TBTC concentrations tested (4 fold increase after TBTC 0.001µM treatment and 6.6 fold after TBTC 0.01µM treatment, figure 6A). The aP2 protein expression was confirmed by Western blotting analysis (figure 6B). At the protein level, aP2 expression appeared to be increased by TBTC in a concentration-dependent fashion.

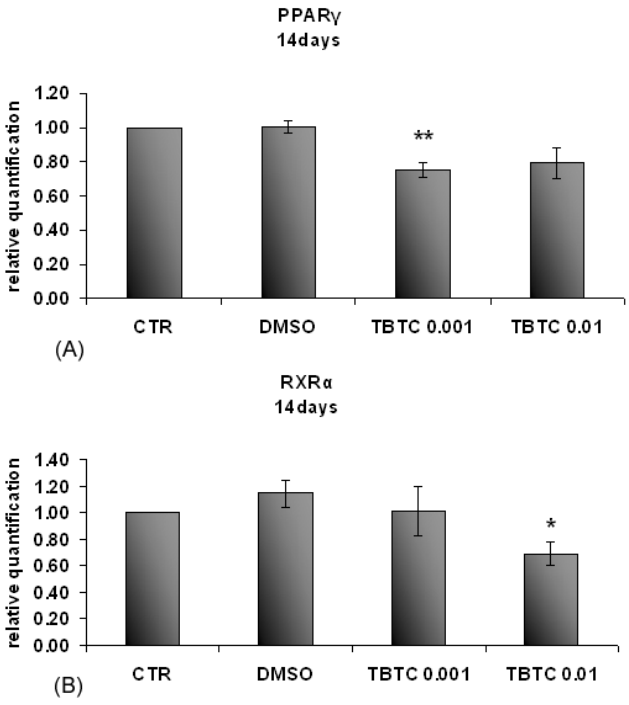


Figure 5. Real time PCR analysis after 14 days LTC.
(A) PPARγ mRNA expression.
(B) RXRα mRNA expression.
Statistical significance: * $p<0.05$, ** $p<0.01$.
TBTC concentrations are expressed in µM.

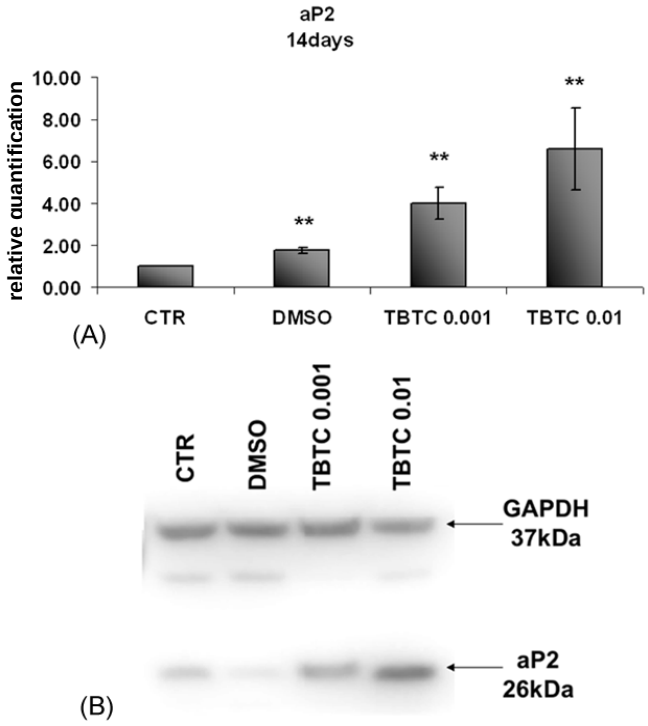


Figure 6. aP2 expression analysis after 14 days culture. (A) Relative quantification of aP2 mRNA. (B) Western Blot analysis shows that aP2 was more expressed in treated cells than in the control. The protein level seemed to increase with TBTC concentration. Statistical significance: ** $p < 0.01$. TBTC concentrations are expressed in µM.

Formation of leptin is decreased by TBTC

Leptin expression level increased in all samples from 24h-48h to 14 days of culture, whereas it was almost undetectable at 24h and 48h of culture under any of the conditions. At day 14 (figure 7), the level of leptin mRNA was significantly lower (0.5 fold) in cells exposed to 0.01µM TBTC compared to untreated control cells.

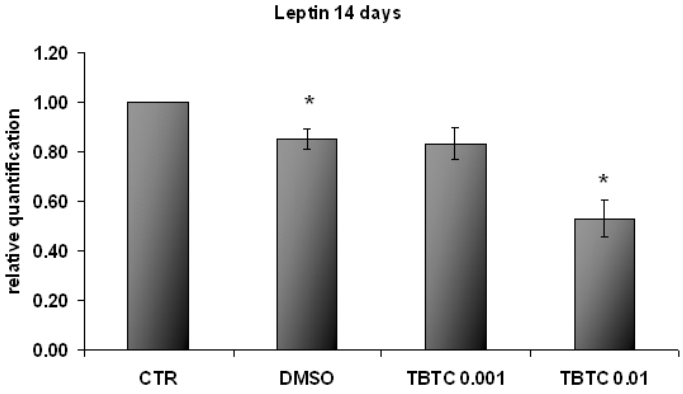
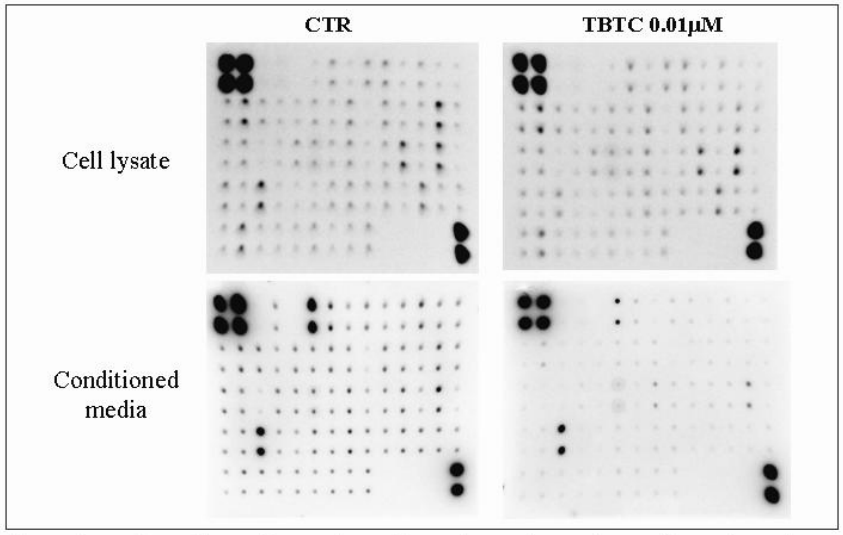


Figure 7. Relative quantification of leptin mRNA. Leptin expression results after 14 days LTC. Statistical significance: * $p<0.05$. TBTC concentrations are expressed in µM.

TBTC reduces cytokine expression

Results revealed that 0.01µM TBTC induced a general decrease in cytokine expression compared to control samples (figure 8). Cytokines that were down-regulated more than 2 fold are reported in Table 1. Different cytokine families were down-modulated by TBTC treatment: an angiogenic factor, various chemokines, and a number of growth factors and interleukins. Among these, CKβ8-1 and CNTF (ciliary neurotrofic factor) protein level decreased both inside and outside cells. The same effect was observed for leptin, i.e. the decrease of protein expression followed the decrease of leptin-coding mRNA levels (figure 7).



(A)

(B)

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	POS	POS	NEG	NEG	Blank	Angiogenin	BDNF	BLC	BMP-4	BMP-6	CK β 8-1	CNTF	EGF	Eotaxin
2	POS	POS	NEG	NEG	Blank	Angiogenin	BDNF	BLC	BMP-4	BMP-6	CK β 8-1	CNTF	EGF	Eotaxin
3	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	I-309	IFN-γ	IGFBP-1	IGFBP-2	IGFBP-4
4	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	I-309	IFN-γ	IGFBP-1	IGFBP-2	IGFBP-4
5	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	PARC
8	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	PARC
9	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β 3	TNF-α	TNF-β	Blank	Blank	Blank	Blank	POS
10	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β 3	TNF-α	TNF-β	Blank	Blank	Blank	Blank	POS

Figure 8. Cytokine array membranes. (A) The figure shows four membranes hybridised with cell lysate or with conditioned media from control culture (CTR) and from treated culture (TBTC 0.01µM). Images are representative of two independent experiments. Membranes are oriented as the table in figure 8B. (B) The table shows each antibody position in the membrane.

Table 1. Overview of factors, produced by bone marrow cells that were affected by TBTC. Proteins whose expression was reduced more than 2 fold (control/treated sample) are reported in two columns. The first refers to protein from cell lysate, the second refers to protein from conditioned media. Results reported are representative of 2 independent experiments.

Cytokines		Fold decrease (Control/treated sample)	
		Cell lysate	**Medium**
Angiogenic factor	Angiogenina	4.6	
CC chemokines	CKβ 8-1 (CCL23)	2.5	3.2
	MCP-1 (CCL2)	2.4	
	PARC (CCL18)		2
	RANTES (CCL5)		2.5
	MDC (CCL22)		4.7
	MIP-3α (CCL20)		2.4
	MIP-1δ (CCL15)		2
CX3C chemokines	Fractalkine (CX3CL1)		3.5
CXC chemokines	MIG (CXCL9)		2
	NAP-2 (CXCL7)		3
Neurotrofic factor	CNTF	2	2.2
	NT-3		4
Growth factors	SCF		2.3
	PDGF-BB		4.3
Growth factor binding protein	IGFBP-2	2.4	
	IGFBP-4		2.4
Interleukines	IL-2	2	
	IL-10		4.5
	IL-7		2.4
	IL-13	3.3	
	IL-3	2.4	
Hormones	Leptin	3.2	2
Pro-inflammatory	TNF-α		4
	TNF-β		2.4
Anti-inflammatory	TGF-β3		3

DISCUSSION

TBTC induces a decrease in granulocyte-macrophage proliferation, as already shown in a previous work on human cord blood cells (Carfi' et al., 2007). These data confirm the myelotoxic effect of TBTC. However, at concentrations lower than 0.007 µM TBTC appears to stimulate the formation of granulocytes and macrophages indicating a hormetic effect of TBTC. In addition, these low concentrations also stimulate the formation of adipocytes as evidenced by Oil Red O staining, droplet formation and synthesis of aP2 mRNA.

Our data support the findings by others (Inadera and Shimomura, 2005; Kanayama et al., 2005) that TBTC stimulates adipocyte differentiation in murine 3T3-L1 preadipocytes. Notably, present results extend these findings by showing that TBTC initiates formation of (UCP-1-negative) white adipocytes also in human bone marrow cultures.

Kanayama showed that TBT may induce adipocyte differentiation via stimulation of the PPARγ/RXR heterodimer. On the contrary, Inadera found that the combined treatment with TBT and a selective PPARγ inhibitor (GW9662) did not prevent aP2 expression, suggesting that the effect of TBT may not be PPARγ-dependent. Possibly, the incubation time may have influenced the outcomes and conclusions of these previous reports as our results show that PPARγ transcription was increased by TBTC at 24h and 48h, but decreased again after two weeks of exposure. Previous reports used incubation times of 6 (Kanayama et al., 2005) to 8 days (Inadera et al., 2005). Based on these kinetic differences it is suggested that TBTC may indeed promote adipocyte induction through activation of PPARγ expression, but that as soon as stroma is formed and adipocytes have matured, PPARγ is down-regulated again.

TBTC is also known to have an effect on immune system development, specifically on thymocytes. Bone marrow cells are however also sensitive to TBTC, albeit to a lesser extend than thymocytes (Snoeij et al., 1986[b]). We

therefore wondered whether TBTC, whilst stimulating the formation of adipocytes, affected the production of cytokines and other factors relevant to hematopoiesis. In general, TBTC induced a general decrease in cytokine protein expression. Among the proteins whose expression was reduced by TBTC, different groups were distinguished: cytokines stimulating (SCF, IL-3, IL-7, PARC or CCL18, PDGF-BB, NT-3, CNTF), or with inhibitory action (CKβ8-1, MIP, NAP-2, TGF-β3) on hematopoiesis, chemokines (RANTES or CCL5, MDC or CCL22, Fractalkine or CXC3CL1, MCP-1 or CCL2), molecules involved in inflammation (IL-10, IL-2, IL-13, TNF-α, MIG or CXCL9), growth factor-binding proteins (IGFBP-2, IGFBP-4) and the hormone leptin.

IL-10, IL-2 and IL-13 regulate inflammatory and immune responses (Minty et al., 1993), but also influence development of lymphocytes (IL10 and IL2 for T cells and IL13 for B cells). The inhibitory effect of TBTC on IL-2, IL3 and also on IL-7 expression may disturb early prothymocyte development (Chervenak et al., 1991; Haks et al., 1999; Wiranowska et al., 1987) and thus contribute to the thymocyte depletion observed *in vivo* (Snoeij et al., 1988).

The inhibition of IL-2 and TNF-α production is consistent with studies which proved that PPARγ ligands are capable of reducing the gene expression of these and other (IFN-γ) cytokines (Chinetti et al., 2000; Cunard et al., 2002).

Chemokines may have multiple effects and affect many cell types but in general they are important in the recruitment, differentation and activation of inflammatory cells such as T cells, dendritic cells, mast cells, eosinophils and neutrophils (Smit and Lukacs, 2006). They may also influence each other's production and activation; hence it is difficult to conclude what the specific effect of TBTC would be. Based on what is known from the *in vivo* effects of this compound, the inhibition of chemokine production may explain part of the thymotoxic effect, but may also indicate that potentially, organotins have an effect beyond T cell differentiation.

Intriguingly, some cytokines which have an inhibitory effect on specific progenitor's differentiation were also down modulated by TBTC treatment. CKβ8-1 and MIP-3a (CCL20) and MIP1d (CCL15) (macrophage inflammatory protein) inhibit colony formation from myeloid progenitors (Forssmann et al., 1997; Han et al., 2003; Noh et al., 2005), whereas NAP-2 (neutrophil activating peptide-2) inhibits megakaryocytopoiesis (Gewirtz et al., 1995). Apparently, despite the finding that the present effects of TBTC are not factor-specific but probably relate to a more general biochemical effect, like ATP synthase or macromolecular synthesis inhibition, (Raffray et al., 1993; Von Ballmoos et al., 2004; Snoeij et al. 1986[a]**),** the net result is an inhibition of differentiation of early lymphocytes and possibly other leukocytes**.**

Of potential relevance to the adipocyte stimulating effect is that TBTC treatment decreased the expression of the insulin-like growth factor binding proteins IGFBP-2 and IGFBP-4. These belong to a family of 6 different IGFBPs that control IGF-I (Insulin like growth factor-I) activity and bioavailability (Boney et al., 1994). Bone marrow stromal cells synthesise and secrete IGF-I and IGFBPs, thereby influencing haematopoiesis (Grellier et al., 1995). In our model IGFBP-2 protein in cell lysate and IGFBP-4 secreted protein decreased after treatment with TBTC, in accordance with a study on mouse primary stromal cells (Lecka-Czernik et al., 2007). This work described that rosiglitazone, which is a PPARγ agonist, down regulates components of the IGF regulatory system. Notably**,** IGF is known to stimulate adipocyte formation (Jia and Heersche, 2000).

The inhibitory effect of TBTC on leptin production is of particular importance as this may link the pro-adipocyte with the immunotoxic effects of TBTC. Leptin is expressed in bone marrow stromal cells, and has a proliferative effect at the level of a multilineage progenitor (Bennet et al., 1996). It is also known (Gerhardt et al., 2001; Umemoto et al., 1997) that a reciprocal regulation exists between leptin and many of the chemokines,

cytokines (TNF-α (Simons et al., 2005), IL-6, IL-8 and MCP-1 (Wong et al., 2007)) and growth factors (SCF, (Umemoto et al., 1997), CNTF, (Ott et al., 2004; Zvonic et al., 2003)) influenced by TBTC.

Experiments with bone marrow cells from *db/db* mice, in which the leptin receptor is truncated, revealed that the myeloid and lymphoid colony-forming potential was significantly reduced compared to the wild-type control marrow. Similar analysis of bone marrow from *ob/ob* mice, which are also deficient in leptin production, indicated that the proliferative capacity of lymphoid progenitors was compromised in the absence of a functional leptin signaling pathway (Zhang et al., 2005). The analysis of *db/db* mice peripheral blood showed that the steady-state levels of B cells and CD-4 expressing T cell were dramatically reduced, demonstrating that the leptin pathway plays an essential role in lymphopoiesis (Bennet et al., 1996). It has been shown that leptin administration increases thymic cellularity in *ob/ob* mice and protects from starvation-induced lymphoid atrophy (Howard et al., 1999). Since leptin seems to be an important regulator of hematopoiesis (Lai Kwan Lam and Lu, 2007; Umemoto et al., 1997), its down-modulation could represent one mechanism of TBTC's toxic effect on the immune system.

In all, our data show that TBTC stimulates the formation of adipocytes in bone marrow stromal cells, but at the same time inhibits the formation of crucial hematopoietic factors, most notably of leptin. Our data may provide an addition to the earlier proposed mechanism by which organotins induce thymus atrophy. Apart from directly stimulating apoptosis and inhibiting immature thymocyte proliferation (Gennari et al., 2000), TBTC may disturb the development of leukocyte precursors by stimulating adipocyte formation. Further *in vitro* and *in vivo* studies are needed to examine the inverse relation between stimulation of adipocytes and inhibition of leukocyte differentiation.

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Chapter five

SELECTIVE INHIBITION OF B LYMPHOCYTES IN TBTC TREATED HUMAN BONE MARROW LONG TERM CULTURES.

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ABSTRACT

Tri-butyltin-chloride (TBTC) is well known for its immunotoxic effect, in particular towards immature thymocytes. TBTC is also known to induce adipocyte differentiation in primary human bone marrow cultures, which is reflected in the decrease in a number of adipocyte-derived cytokines, chemokines and the adipocyte-linked hormone leptin. Since adipocytes influence haematopoiesis and lymphopoiesis for instance by these cytokines and hormones, we here investigated whether TBTC has an effect on specific lymphocyte subsets in human bone marrow primary cultures.

FACS analysis showed a reduction of CD19/CD22-positive B cells by TBTC, both in the presence or absence of cytokines. The treatment did not cause a toxic effect on mature $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, suggesting selective TBTC toxicity on B lymphocytes in the presently used *in vitro* system.

INTRODUCTION

Tri-butyltin-chloride (TBTC) is one of the most toxic organotin compound (Maguire, 1987). Several studies showed widespread organotin pollution, especially in water and seafood, indicating that human exposure may occur through contaminated dietary sources (Fent 1996; Cao et al., 2009). Although TBT is detectable in the liver and in human blood (Kannan et al., 1999; Antizar-Ladislao, 2008), little knowledge is available on the effects of this organotin on human health.

Animal studies suggest that the spectrum of potential adverse systemic effects of TBTC in humans could be quite broad, including immunosuppression and endocrine disruption. The effects of TBTC are extensively studied in rodents and it appears that the thymus is one of the main immunological targets of TBTC. It has been shown that at low doses (5-20mg/kg), TBTC inhibits immature thymocytes proliferation whereas at higher doses (30mg/kg or higher) it causes a depletion of thymocytes by apoptosis (Raffray and Cohen, 1993; Gennari et al., 1997). Differences between responses to low and high concentrations of TBTC are evident *in vitro* as well. At low concentrations (0.1µM) TBTC inhibits DNA synthesis (Snoeij et al., 1986[a]), affects macromolecular synthesis (Snoeij et al., 1986[a]; Gennari et al., 2000), and disrupts mitochondrial energy metabolism (Snoeij et al., 1986[a]). At higher concentrations (1–5 µM), TBTC induces apoptosis (Raffray and Cohen, 1991; Gennari et al., 1997). Comparing different rat cell types, Snoeij et al. (1986[b]) showed that bone marrow (BM) cells are slightly less sensitive than thymocytes, while red cells are relatively resistant.

TBTC is also known as endocrine disrupting chemical in some gastropods (Horiguchi et al., 1997), where it acts as a specific inhibitor of the aromatase enzyme, which converts androgen to estrogen. Recently, it has been demonstrated that TBTC can act as PPARγ (peroxisome proliferator-activated receptor γ) and RXR (retinoid x receptor) agonists and that the interaction with these receptors can mediate the observed adverse endocrine

effects, for instance by affecting aromatase gene expression (Kanayama et al., 2005). Kanayama found that TBT stimulates the differentiation of mouse preadipocyte 3T3-L1 cells into adipocytes (Kanayama et al., 2005). Previously, we have shown that TBTC induces adipocyte differentiation in human mononuclear bone marrow cultures, by modulating PPARγ and RXRα gene expression. TBTC treatment caused a change in the stromal cellularity and a concomitant decreased production of various cytokines and of the adipocyte hormone leptin (Carfi'et al., 2008).

Because adipocyte-derived cytokines and leptin are known to regulate lymphopoiesis (Bennett et al., 1996), we decided to investigate, under similar culture condition as used in our previous study (Carfi' et al., 2008), the effect of TBTC on T and B cells to determine if the modification of BM stroma induced by TBTC can affect lymphocytes. We investigated the effects of TBTC on the survival of $CD19^{+}CD22^{+}$ B cells and of $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cell subsets. Moreover, we evaluated the effect of the addition of some cytokines, which were found previously to be down-modulated by TBTC (Carfi' et al., 2008). The expression of PPARγ and c-myc mRNA was also investigated.

MATERIALS AND METHODS

Chemical compounds and solutions

Tributyltin chloride (TBTC) (Merck, Schuchardt, Germany) stock solution (10 µM) was prepared in DMSO (Sigma-Aldrich, Steinheim, Germany) and diluted in the medium to a final concentration of 0.001 µM. The final DMSO concentration was always 0.1%.

A stock solution of each cytokine was prepared using sterile bidistilled water (IL-3, IL-7, GM-CSF, Flt-3) or a PBS solution supplemented with 0.1% BSA (SCF, IL-6). The IL-3, IL-7, GM-CSF, SCF and IL-6 stock solutions were prepared at 100 µg/ml, while the Flt-3 stock solution was 10 µg/ml. All the cytokines were added to the medium to a final concentration of 10 ng/ml. GM-CSF was purchased from Gentaur (Brussels, Belgium), and all other cytokines were purchased from Sigma-Aldrich.

A collagenase/dispase solution was used to detach adherent cells. Collagenase type I (Invitrogen) stock solution of 1576 U/ml and dispase (Invitrogen) stock solution of 307 U/ml were prepared in HBSS (Invitrogen). Collagenase and dispase were mixed 1:1 and the solution diluted 1:10 in HBSS just before use.

Source of progenitor cells

Human bone marrow cells (BMC) were obtained, frozen, from Lonza (Verviers, Belgium) and thawed before usage, following the procedure indicated by supplier.

Long term culture

The long term cultures (LTC) method used is published in a previous study (Carfi' et al., 2008). Briefly, BMC were thawed and cultured in McCoy's 5A medium with GlutaMAX (Gibco, Grand Island USA) containing 12% FBS (South American Origin, Lonza), 12% horse serum (heat-inactivated New Zeland Origin, Gibco), 1% sodium pyruvate solution 100 mM, 1% MEM

non- essential amino acids 100X, 1% penicillin and streptomycin, 1% hydrocortisone solution 10^{-4}M (Sigma-Aldrich), 1% MEM vitamin solution 100X, and 0.6% MEM essential amino acids solution 50X (Gibco). Cytokine cocktail (IL-3, IL-7, GM-CSF, Flt-3, SCF, IL-6) was added to half of BMC cultures (indicated as CK). The remaining cultures were not supplemented with cytokines. TBTC (0.001 µM) was tested on both cultures, i.e. with (TBTC+CK) or without cytokines (TBTC). A control sample for each condition was prepared, i.e. a control supplemented with cytokines (CTR+CK) and a control without supplementation (CTR).

BMC were seeded at $5x10^5$ cells/ml in $25cm^2$ and $75cm^2$ flasks (Corning, USA) in a final volume of 5 ml and 15 ml, respectively and incubated at 37^oC. Half the medium was changed twice a week. In this study, BMC were cultured in LTC conditions for 7 or 14 days. Some cultures were only maintained for 24h or 48h (short term exposure).

Flow cytometry analysis

BMC were analysed 24h after thawing (basal conditions) and after 7 and 14 days LTC using the following antibodies: CD19 R-PE, CD22 FITC, CD8 FITC (Sigma-Aldrich), CD45 Pacific Blue (Dako, Glostrup Denmark), CD3 APC (BD Pharmingen), CD4 R-PE (Biosurce Invitrogen, CA, USA). Both suspended and adherent cells were analysed together to study TBTC effect on the entire cell culture. After removal of suspended cells, the adherent cells were washed twice with HBSS without Ca^{2+} and Mg^{2+} (Gibco) for 2-3 min. Then cells were incubated at 37^oC with collagenase/dispase solution for at least 20 min. Once cells were detached, they were washed three times in PBS together with the suspended cells. Cells were counted with trypan blue (Sigma-Aldrich), to determine the viability. Cells solution was then diluted to $1x10^7$ cells/ml in PBS of which 100µl was stained with CD19, CD22, CD45 (to analyse B cells) or CD3, CD4, CD8, CD45 (to analyse T cells). Samples were incubated for 30 min with antibodies. After one wash in PBS,

cells were analysed using FACSAria (Becton Dickinson, Franklin Lakes, USA) and results were analysed using FlowJo software (TreeStar, Ashland OR, USA). To determine the lymphocytes percentage, a gate was drawn around the cells on the forward scatter (FSC) versus side scatter (SSC) graph to exclude debris and CountBright (used to enumerate cells). A new graph (CD45 versus side scatter) was drawn using this population and the lymphocyte percentage was calculated from the $CD45^+$ and SSC^{low} population (Fig. 2).

Cell death detection

BMC were analysed 24h after thawing (basal conditions), and after 24h, 48h, or 7 days culture using CD19 R-PE and AnnexinV FITC antibodies (Sigma-Aldrich). Suspended and adherent cells were collected from the cultures and analysed together as described in the previous paragraph. Cells were diluted to $1x10^7$ cells/ml in PBS of which 100 µl BMC solution was stained with CD19 antibody for 30 min and washed in PBS, then BMC were suspended in binding buffer 10X (which is provided with the AnnexinV-PI kit, Sigma-Aldrich) diluted 1:10 in D-PBS. Cell suspension was incubated with AnnexinV for 10 min and analysed using FACSAria (Becton Dickinson). Results were analysed through FlowJo (TreeStar, Ashland OR, USA).

$CD19^+$ cells separation

$CD19^+$ cells in culture were separated, after 24h and 48h cell cultures, using Dynabeads CD19 pan B (Invitrogen Dynal, Oslo Norway). $CD19^+$ cells were isolated through positive isolation, then cells were detached from the beads using DETACHaBEAD CD19 (Invitrogen Dynal, Denmark).

RNA extraction and real-time PCR

For RT-PCR, BMC were seeded at $2.5x10^6$ in $25cm^2$ cell culture flasks and treated as mentioned with TBTC. Controls with medium only (CTR) or with

cytokines added (CTR+CK) were also prepared. Cells were collected after 24h and 48h. After $CD19^+$ cells separation, total RNA was extracted using Cell to Ct kit (Applied Biosystems, USA). Cells were lysed and reverse-transcription of RNA was carried out following supplier's protocol.

A quantitative real time PCR (RT-PCR) was then performed on the cDNA samples to evaluate the mRNA levels of PPARγ, c-myc and beta-actin. All of the samples were analysed using TaqMan gene expression assays ("Assay on demand", Applied Biosystems). Primers for these genes were designed and labelled at the 5'-end with a reporter dye (FAM) and at the 3'-end with a quencher dye (TAMRA). The primers used were: PPARG-Hs00234592_m1, c-Myc-Hs00153408_m1, ACTB-Hs99999903_m1. All experiments were performed in duplicate in 96-well plates using TAQ-Man Universal Master Mix (Applied Biosystems). RT-PCR amplification was performed using a Gene Amp 7000 Sequence Detection System according to the manufacturer's protocol. PCR conditions were 50°C for 2 min, 95°C for 10 min, then 40 cycles at 95°C for 15 seconds, and 60°C for 1 min. For quantitative evaluation of gene expression, all gene levels were normalised to the beta-actin mRNA reference genes (ACTB-Hs99999903_m1). Fluorescence data were processed and analysed with ABI PRISM Sequence Detection software (version 1.6 software, Applied Biosystems). The quantification of the PCR assay was based on C_T values. Briefly, the C_T values indicate the fractional cycle number for which the amount of amplified target reaches a fixed threshold. The difference (ΔC_T) between the C_T of the target gene (C_T t) and the reference gene (C_T r) depends on the RNA relative copy number between the target and the reference gene. The difference between the ΔCt value of each sample and the control was then calculated ($\Delta\Delta C_T$). The values obtained from this power: $2^{(-\Delta\Delta Ct)}$ are the relative RNA amounts, which are graphically reported.

Statistical analysis

The GraphPad Prism 4.0 (GraphPad software, San Diego, USA) program was used for statistical analysis. Data were expressed as the means of three independent experiments ± standard error of the mean (SE). Statistical analysis was performed on raw data, using two-way ANOVA followed by a Bonferroni post-test to assess the effect of TBTC and cytokine treatment. Values of $p<0.05$, $p<0.01$ were considered statistically significant.

RESULTS

TBTC affected general morphology of bone marrow cultures

BMC were cultured under LTC conditions for 7 or 14 days and treated with TBTC 0.001 µM, with (TBTC+CK) or without (TBTC) a cocktail of cytokines (IL-3, IL-7, GM-CSF, Flt-3, SCF, IL-6). Control cultures were incubated also with (CTR+CK) or without the cytokine addition (CTR). At 14 days LTC, TBTC incubation caused a decrease in cell numbers (Fig. 1) compared to CTR, and stromal cells appeared more evident. Cytokines clearly stimulated the development of suspended and adherent cells. Cell viability assessed prior to flow cytometry, by trypan blue exclusion, was always higher than 90%.

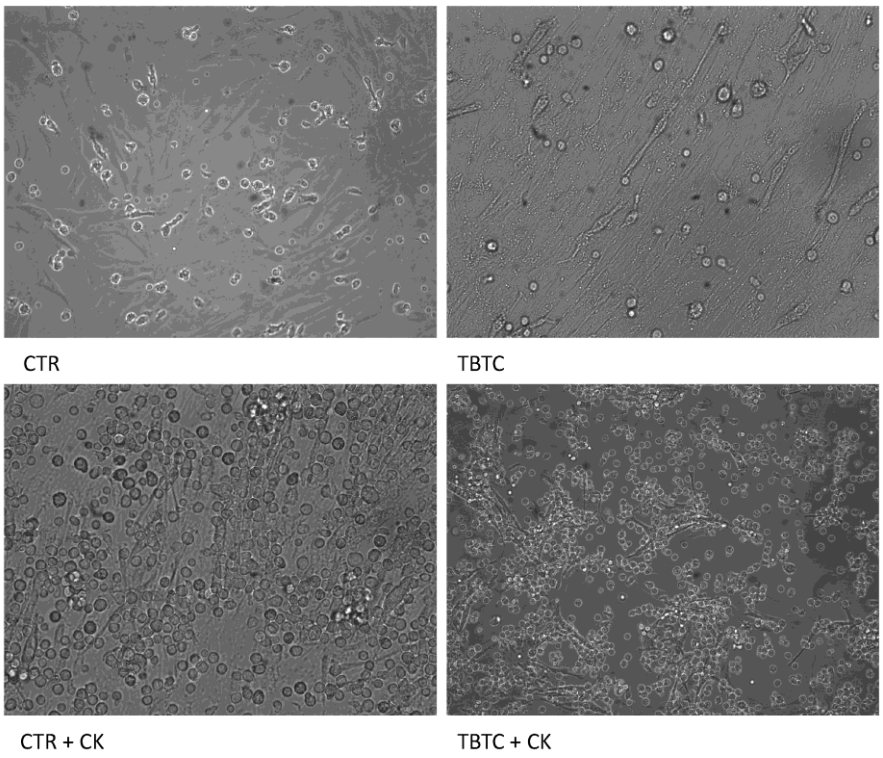

Figure 1. Images of bone marrow long term cultures, captured after 2 weeks of culture. Pictures on the left are examples of control culture with (CTR+CK) and without cytokines (CTR). Pictures on the right are examples of TBTC-treated cultures with (TBTC+CK) or without cytokines (TBTC). Magnification 10X.

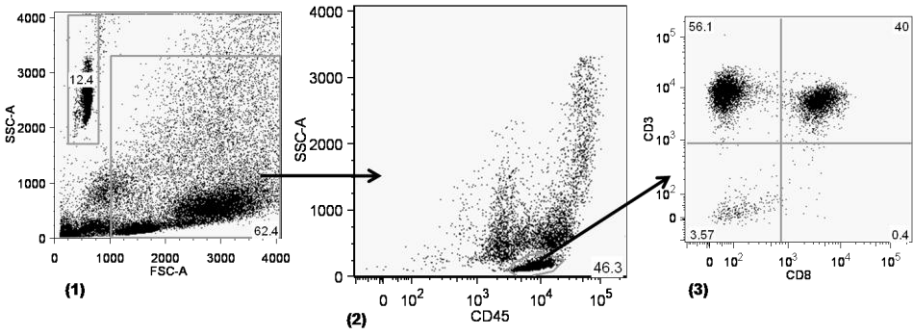

Figure 2. Example of the procedure used for the analysis of FACS data. To determine the lymphocyte percentage, a gate was drawn around the cells on the forward scatter (FSC) versus side scatter (SSC) graph (graph 1) to exclude debris and CountBright (gated on the upper left corner of graph 1). A new graph (CD45 versus side scatter) was drawn using this population and the lymphocyte percentage was calculated from the $CD45^+$ and SSC^{low} population (graph 2). The lymphocyte population is expressed as percentage of all cells (excluding debris and CountBright) CD45 positive. The percentage of the different subsets analysed was calculated within this lymphocyte population, as shown for CD3/CD8 dot plot (graph 3).

TBTC specifically reduced the percentage of $CD19^+CD22^+$ cells, irrespective of the presence of cytokines

The lymphocyte population was determined using CD45 staining and the percentage of the $CD45^+SSC^{low}$ population was calculated (Fig. 2). Within this $CD45^+SSC^{low}$, being the total lymphocyte population, the presence of B cells ($CD19^+CD22^+$) and T cell subsets ($CD3^+CD4^+$; $CD3^+CD8^+$) were analysed (Fig. 3). The lymphocyte percentage was significantly reduced at 14 days LTC both in CTR and TBTC treated cultures, compared to basal conditions, C0 (Fig. 3a). After 7 days LTC, however, the $CD3^+CD4^+$ T cell percentage significantly decreased compared to the basal conditions, both in the TBTC-treated and control samples, while after two weeks the percentage increased again (Fig. 3c). The $CD3^+CD8^+$ subset percentage was at the same level of the basal conditions after both 7 and 14 days LTC (Fig. 3d).

Although the total lymphocyte population analysis did not reveal any effect of TBTC compared to CTR (Fig. 3a), further analysis on specific T and B cell subsets revealed that TBTC significantly reduced the $CD19^+CD22^+$

percentage (Fig. 3b), but did not affect percentages of T cell subsets (Fig. 3c-d).

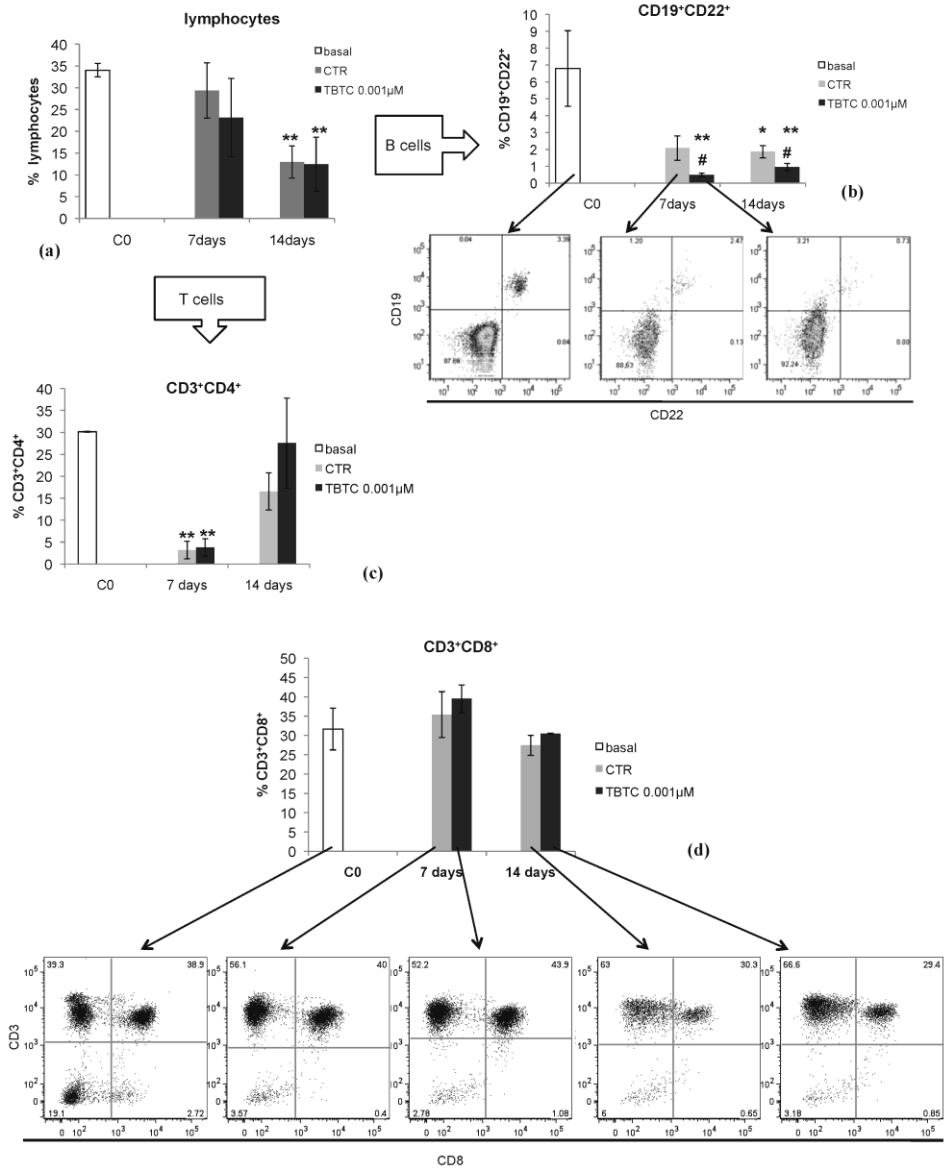

Figure 3. Flow cytometric analysis of BMC 24h after thawing (C0) or after 7 and 14 days LTC, without CK. The figure shows the B ($CD19^+CD22^+$) and T ($CD3^+CD4^+$; $CD3^+CD8^+$) cell subsets, starting from the analysis of the total lymphocyte population. (a) The percentage of lymphocyte population was calculated as shown in Fig. 2. B and T subset percentages within this population were calculated. (b) The histogram shows $CD19^+CD22^+$ percentage. Dot plots are representative of one experiment. (c) Analysis of $CD3^+CD4^+$ subset. (d) Analysis of $CD3^+CD8^+$ subset.

Dot plots are representative of one experiment. The statistical analysis was performed on three independent experiments, comparing CTR and TBTC 0.001µM treated samples with the basal conditions (C0) (* $p<0.05$; ** $p<0.01$), and comparing TBTC 0.001µM with the CTR (# $p<0.05$).

The cytokine treatment (CK) caused a significant decrease in total lymphocyte percentage at 14 days LTC (Fig. 4a), but at the same time, the $CD19^+CD22^+$ percentage was significantly higher in CTR+CK compared to CTR (Fig. 4b). TBTC did not affect the percentage of total lymphocytes under these conditions (Fig. 4a) but it did again significantly reduce the percentage of $CD19^+CD22^+$ B cells. This reduction appeared more pronounced in the presence of CK (Fig. 4b). In fact, CTR+CK samples contained a higher $CD19^+CD22^+$ percentage compared to CTR without CK samples, but actually the percentage of $CD19^+CD22^+$ cells in the TBTC-treated cultures was equally low.

Notably, in control cultures, CK prevented the profound reduction in the percentage of $CD19^+CD22^+$ cells after 14 days LTC.

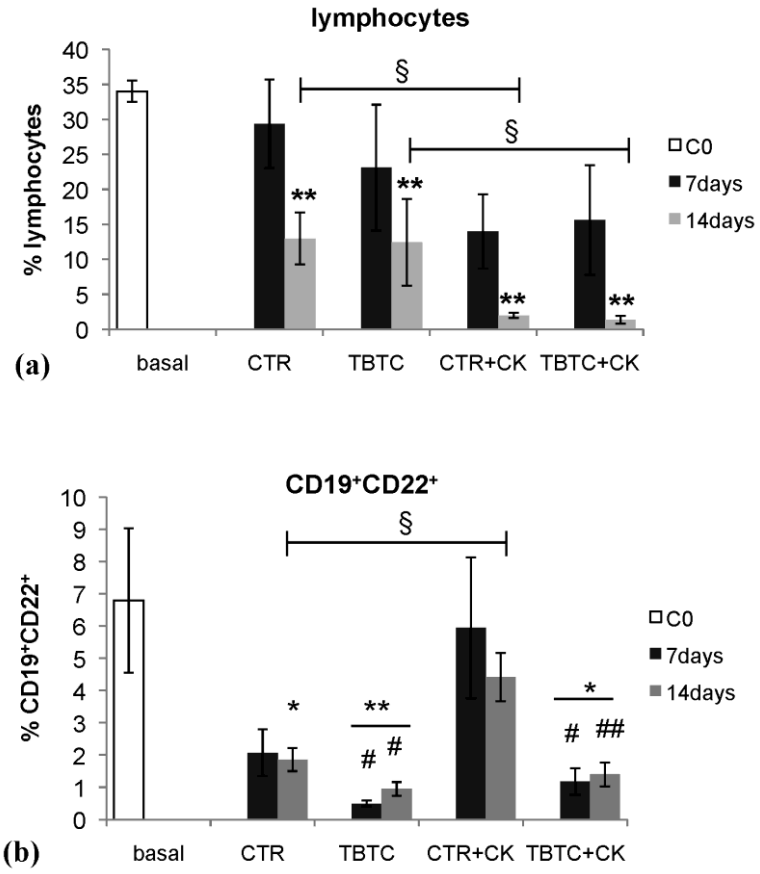

Figure 4. Flow cytometric analysis of BMC at basal conditions (C0) or at 7 and 14 days LTC, in the presence (CTR+CK; TBTC+CK) or absence of cytokines (CTR; TBTC). (a) The percentage of lymphocytes was calculated from the $CD45^+$ and SSC^{low} population as explained in Fig. 2. (b) The graph summarizes results related to $CD19^+CD22^+$ B cells analysis. Statistical analysis was performed on three independent experiments, comparing each TBTC- treated sample with the respective control (# $p<0.05$; ## $p<0.01$) and comparing each sample with basal conditions (* $p<0.05$; ** $p<0.01$). For each time, the comparison between samples with and without cytokines was also performed (§ $p<0.05$).

TBTC induced cell death in $CD19^+$ lymphocytes

The analysis of TBTC toxicity was extended to the total $CD19^+$ lymphocyte population, to verify if it affects only the $CD19^+CD22^+$ subset or the entire $CD19^+$ population. Cell death was investigated by staining with CD19- and AnnexinV-specific antibodies at 24h, 48h and 7 days.

Results are expressed as ratio (TBTC/CTR) of the $CD19^-Annexin^+$ cell percentages (Fig. 5b) or of the $CD19^+AnnexinV^+$ cell percentages (Fig. 5c) calculated from TBTC-treated samples and the respective controls. For both $CD19^-Annexin^+$ (Fig. 5b) and $CD19^+AnnexinV^+$ (Fig. 5c) cell populations, the TBTC/CTR ratio is reported for cultures with (+CK) and without (-CK) cytokines. Figure 5c shows that the TBTC/CTR ratio of the $CD19^+AnnexinV^+$ cell percentage increased in a time-dependent manner in the absence of cytokines. Notably, at 7 days of LTC, the TBTC/CTR ratio was significantly higher in samples without cytokines (2.08 ± 0.08) compared to samples supplemented with cytokines (1.12 ± 0.06). At 24h and 48h no significant differences were observed between samples with and without cytokines.

To verify if TBTC induces cell death not only in $CD19^+$ cells, but also in $CD19^-$ cells, the $CD19^-Annexin^+$ phenotype was analysed. Figure 5b shows that TBTC significantly increased the $CD19^-Annexin^+$ cell percentage at 48h of treatment, in non-cytokines-supplemented cultures. At 24h and 7 days LTC there was no difference between samples with and without cytokines.

TBTC increased both $CD19^-Annexin^+$ and $CD19^+AnnexinV^+$ cell percentages with different kinetics. However, the highest TBTC/CTR ratio was observed for $CD19^+AnnexinV^+$ cell population (2.08 ± 0.08) compared to $CD19^-Annexin^+$ cell population (1.66 ± 0.14). For both $CD19^-Annexin^+$ and $CD19^+AnnexinV^+$ cell populations, it seems that when TBTC was most cytotoxic, cytokines reduced the TBTC/CTR ratio and appeared protective.

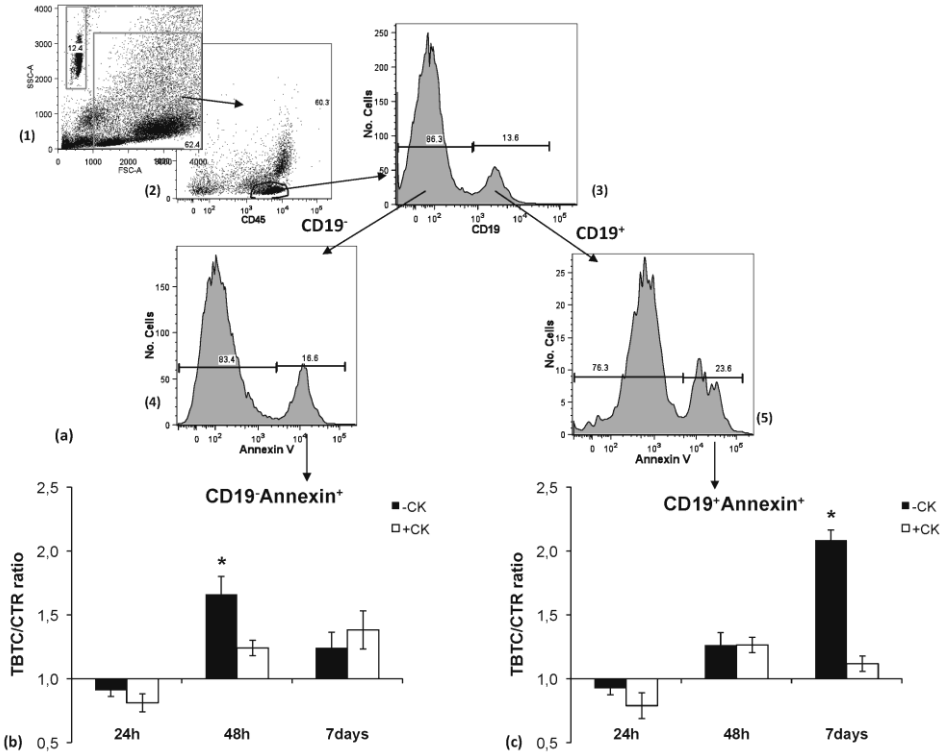

Figure 5. Flow cytometric analysis of cells at 24h, 48h and 7 days LTC. (a) The figure is a representative example of the procedure used to identify $CD19^+$Annexin V^+ cells and $CD19^-$Annexin V^+ cells. A gate was drawn around the cells on the forward scatter (FSC) versus side scatter (SSC) graph to exclude debris and CountBright (graph 1). A new graph (CD45 versus side scatter) was drawn using this population and the lymphocyte percentage was calculated from the $CD45^+$ and SSC^{low} population (graph 2). The lymphocyte population was analyzed to identify $CD19^+$ and $CD19^-$ lymphocyte (graph 3). The $CD19^-$ cells (graph 4) and $CD19^+$ cells (graph 5) were analysed for their positivity to AnnexinV.
The comparison between cultures treated with TBTC 0.001µM (TBTC) and the respective control (CTR) is expressed as ratio, i.e. TBTC/CTR, of the $CD19^-$ AnnexinV^+ cell percentages (b) or of the $CD19^+$AnnexinV^+ cell percentages (c). Each TBTC/CTR ratio was calculated for cultures with (+CK) and without (-CK) cytokines. Statistical analysis was performed on three independent experiments comparing each TBTC/CTR ratio between samples with and without cytokines (* $p<0.05$).

PPARγ – c-myc pathway was not involved in TBTC toxicity on CD19$^+$ cells

TBTC appeared to induce cell death particularly in CD19$^+$ cells. Some chemicals, such as the PPARγ agonist ciglitazone, have been shown to induce apoptosis in mouse B cells through a pathway involving PPARγ, NF-kB (Nuclear Factor- kappa B), and c-myc (Piva et al., 2005). Since in a previous work (Carfi' et al., 2008) TBTC was shown to modulate PPARγ (in a dose-dependent manner), we considered this a possible pathway in TBTC toxicity.

The analysis of PPARγ and c-myc gene expression was done on the total CD19$^+$ B lymphocytes. CD19$^+$ B cells were sorted using magnetic beads and collected from all samples (CTR, TBTC, CTR+CK, TBTC+CK) after 24h, 48h of LTC and from basal conditions (C0). The gene expression analysis on CD19$^+$ purified mRNA showed that the PPARγ gene was not expressed in any of the samples analyzed. Moreover, c-myc mRNA expression was lower than basal conditions in all samples analyzed (Fig. 6).

After 24h of treatment, TBTC increased the expression of c-myc both in samples with or without cytokines, but after 48h, c-myc gene expression was at the same level of CTR in TBTC-treated samples without cytokines. At 48h cytokines continued to stimulate c-myc gene expression, in particular in the non-TBTC-treated cultures. The significant reduction of c-myc mRNA induced by TBTC only in cytokines-supplemented cultures, suggests that cytokines interfere with the mechanism of c-myc modulation TBTC-induced.

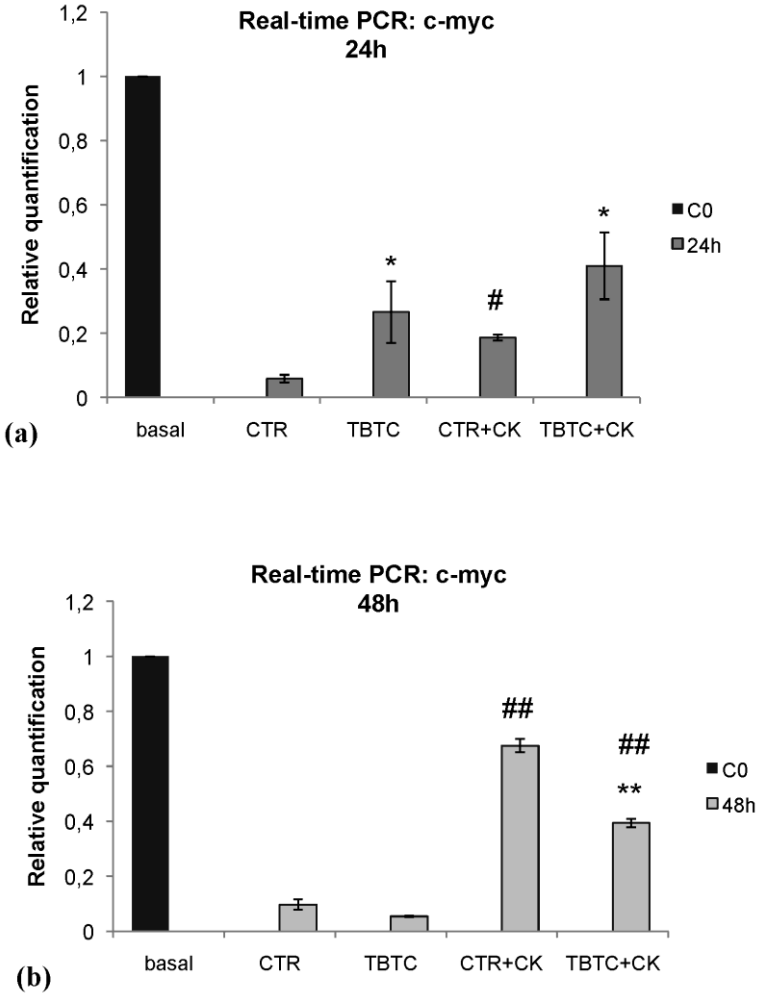

Figure 6. c-myc mRNA expression. Real-time PCR analysis was performed only on $CD19^{+}$ cells. These cells were separated from cell culture at C0, 24h and 48h LTC. (a) Real-time PCR analysis at C0 and 24h. (b) Real-time PCR analysis at C0 and 48h. Statistical analysis was performed on three independent experiments comparing each TBTC treated sample to control (* $p<0.05$; ** $p<0.01$), and comparing samples with and without cytokines (# $p<0.05$; ## $p<0.01$).

DISCUSSION

In this study, we investigated the effects of TBTC on bone marrow cell cultures using the same culture conditions described in our previous work (Carfi' et al., 2008). Although this method, favours myeloid lineage, in the first two weeks, our data show that it is possible to study specific lymphocyte subsets. As expected, the total number of lymphocytes decreased during 14 days LTC, as described earlier (Carfi' et al., 2010). The cytokine cocktail we used does not support lymphocyte maintenance (Fig. 4).

The concentration of 0.001 µM was used to investigate the TBTC toxicity on lymphocyte population, because the higher concentration (TBTC 0.01 µM) used in our previous study (Carfi' et al., 2008) induced toxicity. TBTC at the concentration of 0.001 µM did not reduce the total percentage of lymphocytes nor T lymphocyte subsets $CD3^+CD4^+$ and $CD3^+CD8^+$, but decreased the $CD19^+CD22^+$ subset of B cells. The reduction of $CD3^+CD4^+$ T cells during the first 7 days, irrespective of the presence of TBTC, could be due to cell culture conditions, allowing an initial increase in apoptosis within this cell subset. Later on, after the establishment of the stromal layer, an increased production of cytokines and growth factors could be responsible for an increase of the $CD3^+CD4^+$ cell proliferation. Probably, the $CD3^+CD8^+$ subset expresses a different set of cytokine/growth factor receptors compared to the $CD3^+CD4^+$ subset, facilitating the survival and proliferation of $CD3^+CD8^+$ subset during the 14 days LTC.

The possibility that TBTC has a different effect on different cell subsets has been described earlier. In fact, it is known that TBTC toxicity is not only concentration-dependent, but also varies as a function of cell maturation and lymphocyte subset distribution. In rodents, organotins induce thymus atrophy by selective inhibition of immature thymoblasts proliferation resulting in a marked depletion of small cortical $CD4^+CD8^+$ thymocytes (Snoeij et al., 1988; Pieters et al., 1992). Gennari and co-workers, confirmed

that organotin-induced apoptosis seems to affect $CD4^+CD8^+$ thymocytes in particular (Gennari et al., 2002). Also at molecular level, differences between effects of TBTC on T cell subsets are reported. In fact, studies on human peripheral T lymphocytes showed that TBTC induces a different activation of caspases in $CD8^+$ T cells, rendering these cells more resistant to TBTC than $CD4^+$ cells (Stridh et al., 2001).

In vivo studies on rodents (Boyer, 1989) and fish (Harford et al., 2007) show that organotins decrease numbers and mitogenesis of total lymphocytes. *In vitro*, it has been shown that organotin compounds decrease survival, proliferation and differentiation of human isolated B lymphocytes (De Santiago and Aguilar-Santelises, 1999), but a selective toxicity on this population was not described until now. In fact, studies on rodent thymus and spleen or on human peripheral cells report a specific toxicity on T cells (Stridh et al., 2001; Snoeij et al., 1988). It might be that in our study, B cells are more sensitive to TBTC toxicity because their maturation and survival is strictly dependent on the bone marrow stroma, another important target for TBTC (Carfì et al., 2008). For this reason, our *in vitro* model, which mimicks the physiological environment, may be a good tool to pick up this selective toxicity. On the contrary, T cells complete their maturation in the thymus, and for this reason, we were not able to examine in the present *in vitro* model the effect of TBTC on the full process of differentiation and maturation of these cells.

TBTC immunotoxicity may result from interference with more than one process in immune cell development. Three important candidate targets of relevance to the current study are membrane homeostasis (Bertoli et al., 2001), stromal cellularity and function (Carfi' et al., 2008), and nuclear receptors like PPARγ (Padilla et al., 2002; Ray et al., 2005).

PPARγ agonists, i.e. prostaglandin 15d-PGJ2, a natural PPARγ agonist, and the synthetic agonist ciglitazone, induce apoptosis in human lymphocytes and B lymphomas (Padilla et al., 2002). In a mouse model, it has been

shown that apoptosis induced by PPARγ agonists may involve the NF-kB pathway (Ray et al., 2005), through the inhibition of pro-survival transcription factors, like c-myc. Since it has been demonstrated that organotins are PPARγ agonists and modulate PPARγ gene expression in human bone marrow LTC (Carfi' et al., 2008), we hypothesized that B lymphocytes reduction, induced by TBTC, may be mediated by PPARγ. However, real-time PCR results showed that PPARγ mRNA was not expressed in $CD19^+$ cells in the present system, indicating that the nuclear receptor is not directly involved in TBTC toxicity on B cells.

The comparison between cultures with and without addition of cytokines showed that the percentage of $CD19^+CD22^+$ cells was significantly higher in CTR+CK than in CTR without CK. These data suggest that cytokines may exert a specific stimulating effect on B lymphocyte development, as confirmed by the up-modulation of c-myc mRNA in $CD19^+$ B cells. In these cells, c-myc transcription was induced also by TBTC both in samples with or without CK. This high level of c-myc transcription was maintained at 48h only in samples with CK.

CK were shown to protect lymphocytes against TBTC-induced cell death. The comparison between samples with and without CK shows similar level of TBTC/CTR ratio at 24h, whereas at 48h ($CD19^-AnnexinV^+$) and 7 days ($CD19^+AnnexinV^+$) when TBTC induced the highest cell death ratio (TBTC/CTR), cytokines appeared to be protective against cell death. The different time-course observed in cell death-induction between $CD19^-$ and $CD19^+$ cells, suggests a different sensitivity towards TBTC toxicity.

In conclusion, our results show that TBTC decreased the percentage of $CD19^+CD22^+$ B cell subset within the total population of lymphocytes. In addition, TBTC induced death of lymphocytes through a PPARγ-independent mechanism. Cytokines did not interfere with the reduction of $CD19^+CD22^+$ B cell subset, but they protected both $CD19^+$ and $CD19^-$ lymphocytes against cell death. Since the alteration of BM stromal

cellularity induced by TBTC caused a reduction in the production of stromal derived cytokines (Carfi' et al., 2008), this effect could contribute to TBTC toxicity towards B cells. Clearly, the mechanism of TBTC toxicity on human B cells should be further investigated.

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Chapter six

GENERAL DISCUSSION

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The aim of the present thesis was to design in vitro models to study the immunosuppressive effect of immunotoxic compounds in particular of the classic immunotoxic compound, TBTC.

The first step was to assess the ability of in vitro methods (like CFU-GM assay, ^{3}H-thymidine incorporation or BrdU incorporation, and ELISA to dose cytokine release) to classify a panel of chemical compounds for their immunotoxicity. The second step was to develop a cell culture technique to allow human lymphocyte maintenance and differentiation in vitro. The third step was to test the TBTC on the bone marrow culture developed during the second step.

The first chapter describes the use of a battery of *in vitro* methods (CFU-GM assay, ^{3}H-thymidine incorporation or BrdU incorporation to evaluate mitogen responsiveness to different stimuli, ELISA to dose the cytokine release from stimulated lymphocytes and monocytes) to predict the immunotoxicity of a panel of immunotoxic (TBTC, Verapamil, Cyclosporin A, Benzo(a)pyrene) and not immunotoxic (Urethane and Furosemide) compounds tested on both human and rodent cells.

To identify the dose range to be used in the functional assays, cytotoxicity was evaluated through different tests (MTT, trypan blue dye exclusion and LDH). Then, non-cytotoxic concentrations were used for analysis of proliferation and cytokine release. CFU-GM assay was the first proliferation test used, because damage to the myeloid lineage compromises the bone marrow functionality and hematopoiesis and may results in immunotoxicity (Luster et al., 1985; Kim et al., 2001). The CFU-GM method was validated for predicting acute neutropenia by anticancer drug administration (Pessina et al., 2003). If a compound is myelotoxic, there may be no need to proceed with additional evaluation since the material will be a de facto immunotoxicant (Gennari et al., 2005). However, compounds that are not myelotoxic may still affect lymphocytes.

Proliferation was evaluated using cells from various sources induced by different stimuli: anti-CD3 antibody stimulation in case of mouse spleen cells and human peripheral lymphocytes, and mitogen stimulation like PHA, ConA, LPS in case of rat and mouse spleen cells. If mitogen responsiveness is not affected by the chemical of concern, it does not mean that it is not lymphotoxic. In fact, it could compromise cytokine production, as another important lymphocyte activity (Fig. 1).

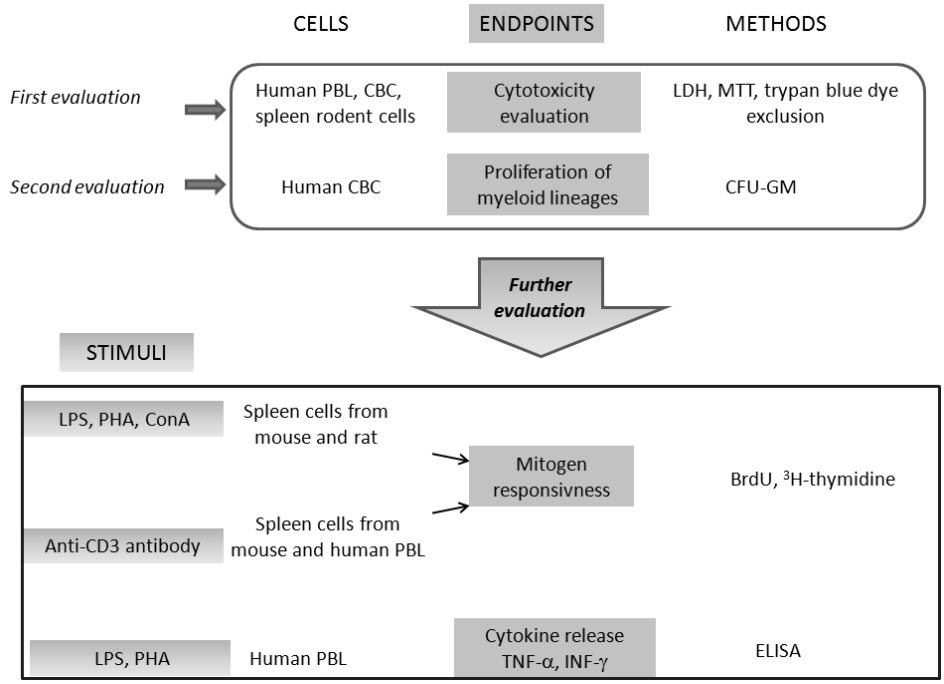

Figure 1. Approach to evaluate immunotoxicity *in vitro*. Methods used to study different endpoints are reported. Each endpoint has been analysed on different cells derived from rodents or human. The study was performed following these steps: first cytotoxicity evaluation, second myelotoxicity evaluation with CFU-GM assay on human CBC (cord blood cells), third T peripheral lymphocytes mitogen responsiveness, finally cytokine release from human PBL (peripheral blood lymphocytes). The mitogen responsiveness was induced by different stimuli (LPS, PHA, ConA, anti-CD3 antibody).

Despite the heterogeneity of cells and stimuli used, all tests provided similar classification of the compounds from the less toxic (Verapamil) to the most toxic (TBTC). The two negative compounds (Urethane and Furosemide)

were indeed not active in any of the assays. Importantly, the same classification was confirmed comparing data obtained with cells from different species (mouse, rat, human). In fact the IC50 values were very similar in the three species for Urethane, Furosamide, Verapamil and Benzo(a)pyrene. Human cells were however more resistant to Cyclosporin A and TBTC compared to rodents. Nevertheless, the IC50 calculation from the various assays may allow the comparison of the toxic concentration range between different species, offering the possibility to translate animal data to hazard assessment for human.

This approach, if validated, could allow at least the reduction of the number of animal used, as auspicated by the EU legislation (Council Directive 86/609/EEC).

Each test analyzes a particular aspect of immunotoxicity, for this reason it is important to develop an integrated system able to detect the most relevant parameters as suggested in Fig. 2.

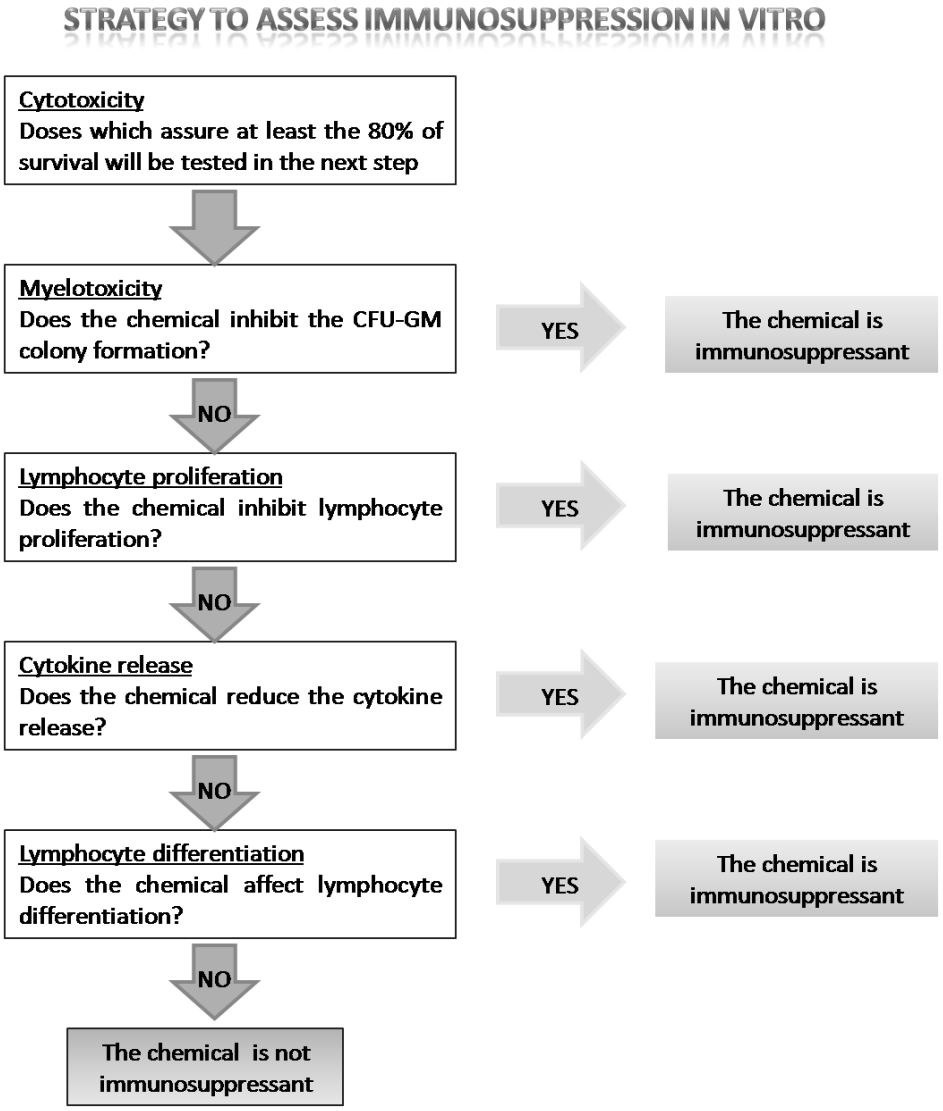

Figure 2. This figure is a strategy proposal to assess immunosuppression *in vitro*.

An important immunotoxicological target besides mature T cell activation is cell differentiation, being a fundamental step in the maintenance of an active and functional immune system.

Studies on lymphocyte differentiation have been performed using mouse cells (Dexter and Lajtha 1974; Dexter et al., 1977) because it was difficult to obtain lymphocyte differentiation and maintenance from human stem cells.

Different protocols have been used to optimize the long term culture (LTC) method, depending on the lineage of interest. Studies on the maturation of B lymphocytes were performed with mouse cells using LTC, that include a stromal cell line (S10 or S17) from myeloid long term bone marrow cultures (Collins and Dorshkind, 1987). Another option was to supplement the mouse bone marrow cell culture with 2-mercaptoethanol instead of high concentration HoS, with higher incubation temperature (37°C instead of 33°C) and low concentration FBS (Withlock et al., 1984). Whereas it was possible to obtain a LTC supporting B lymphopoiesis with mouse cells, LTC for B cell differentation with human bone marrow or cord blood hematopoietic progenitors required the addition of cytokines, such as GM-CSF (granulocyte-macrophage colony stimulating factor), SCF (stem cell factor), Flt-3 ligand, IL-7 (Canque et al., 2000) or the support of murine bone marrow stromal cell lines (Hao et al., 1998; Miller et al., 1999).

Chapter 3 describes the development of a LTC using only human progenitors, in a 2-stage culture design. Since it is known that B lymphocyte differentiation depends on bone marrow stroma (Nagasawa, 2006) we designed a protocol of LTC that allows the stroma formation in the first 14 days of culture. During this time, development of mesenchymal and myeloid lineages is stimulated by addition of hydrocortisone and horse serum (Fig. 3). Once the stroma is formed the medium is substituted with a fresh medium without hydrocortisone and horse serum, because these components are toxic to B cells (Phillips, 1980). Altering the medium allows the development of B cells, as evidenced by the increase in the percentage of $CD79^{+}\mu^{+}$ pre-B cells and $IgM^{+}CD19^{+}$ B cells, and the maintainance of $CD3^{+}$ T cells. We investigated also the effect of IL-7 on LTC, because this cytokine has been shown to be necessary in maintaining B cell differentiation from mouse common lymphoid progenitors (Dias et al., 2005). In addition, IL-7 contributes to human pro-B cell development, in a mouse stromal cell-dependent culture system (Taguchi et al., 2007). In our

study, IL-7 inhibited the percentage of $CD79^{+}\mu^{+}$ pre-B cells and $IgM^{+}CD19^{+}$ B cells compared to control, but appeared to stimulate $CD3^{+}$ T cells. Our data with human cells suggests that IL-7 activity on B cells is different from mouse, underlining species specificity also with regard to cytokines.

Our results encourage the use of the LTC model to study the potential xenobiotics toxicity on lymphocyte differentiation and proliferation.

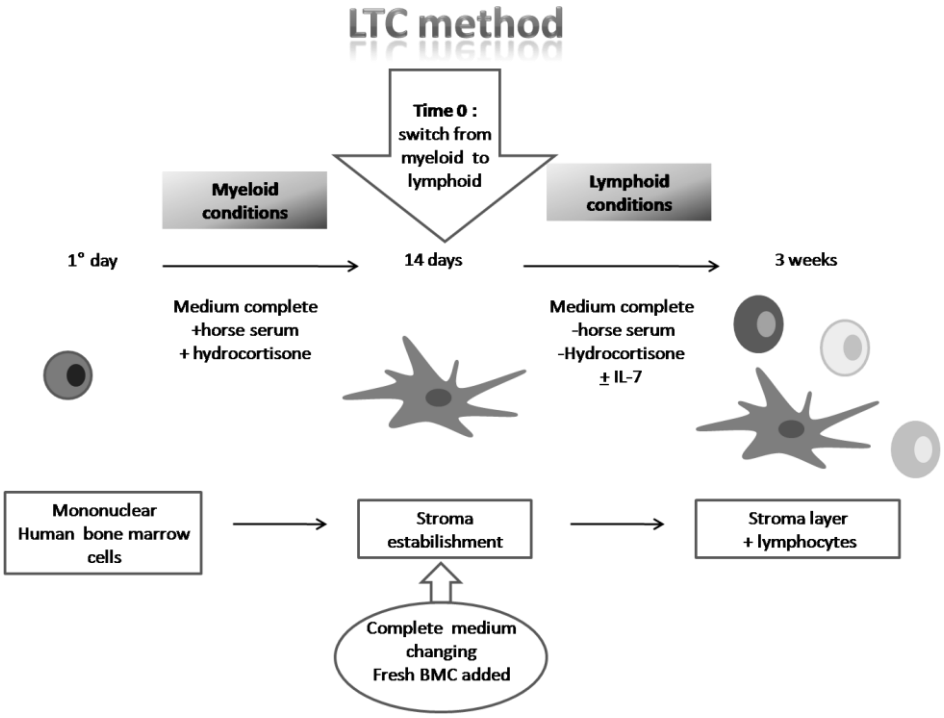

Figure 3. LTC method. Mononuclear human bone marrow cells are cultured in presence of horse serum and hydrocortisone for 14 days, allowing the stroma formation. After 14 days the medium is completely changed with a fresh one without horse serum and hydrocortisone. Fresh BMC are added to increase the number of stem cells which could be committed towards lymphoid lineage. Time 0 is the switching time point from myeloid to lymphoid conditions. Cells are cultured for 3 weeks with (IL-7) or without IL-7 (CTR) to study the effect of this cytokine on lymphocytes proliferation and differentiation. Lymphocytes were maintained for 3 weeks in both CTR and IL-7 treated cultures but with a different proliferation rate and immunophenotyping.

TBTC was chosen for its well-known toxicity toward rodent thymocytes and bone marrow (Snoeij et al., 1986). In addition, TBT has been shown to induce the conversion of fibroblasts (mouse 3T3-L1 cell line) into adipocytes (Inadera and Shimomura, 2005; Kanayama et al., 2005), through the activation of retinoid x receptor-α (RXRα) and peroxisome proliferator-activated receptor-γ (PPARγ). TBT compounds are high affinity ligands of these nuclear receptors and are able to activate the PPARγ pathway (Kanayama et al., 2005).

We investigated TBTC effects on the bone marrow stroma formation, in particular on adipocyte differentiation, because adipocytes may be important stromal cells for supporting growth and differentiation of bone marrow progenitors. Adipocytes are no longer considered as simple space fillers; instead, adipocytes participate in the overall lipid metabolism and in providing a local energy reservoir in the bone marrow (Marko et al., 1995). Adipocytes express adhesion molecules, produce cytokines, or hormones important in the hematopoietic process (Laharrague et al., 1998). Leptin, an important protein originating from adipocytes, has been demonstrated to be not only a regulating hormone for food intake and energy homeostasis, but also to be crucial in angiogenesis, reproduction, lymphoid organ homeostasis, and haematopoiesis (Zhang et al., 2005).

In chapter 4, we showed that TBTC dose dependently induces adipocyte differentiation in human bone marrow LTC, through up-regulation of the nuclear receptor PPARγ and aP2. On the contrary, the expression of leptin (mRNA and protein) as well as of a series of cytokines, involved in hematopoiesis (IL-7, IL-3, IL-6, GM-CSF, Flt-3, SCF) were down-modulated. These data suggest that TBTC exerts part of its toxicity by interfering with the normal cellular homeostasis of the bone marrow stroma and thus causing a reduction of the expression of hormones and cytokines, which support hematopoiesis.

Since TBTC modifies the stroma both at cellular and molecular level (chapter 4), we speculate that TBTC interferes with the physiological process of hematopoietic differentiation and maturation. We focused in particular on lymphopoiesis because lymphocytes are a well-known target of organotin toxicity (De Santiago and Aguilar-Santelises, 1999; Stridh et al., 2001). We wondered if it was possible to discriminate a chemical toxic effect on lymphocyte population in the same LTC system. Therefore, the same culture conditions used to study the TBTC effects on adipocyte formation were used to investigate TBTC activity on lymphocytes (chapter 5).

The total number of lymphocytes was reduced in a time-dependent manner, from 24h after cell thawing (basal condition) until 14 days LTC, confirming that these culture conditions do not favour lymphocyte maintenance, independently by the presence of TBTC. In addition, the analysis of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells revealed no differences between CTR and TBTC treated samples, whereas TBTC selectively damaged $CD19^+CD22^+$ B cells. In parallel, we established cell cultures added with a cocktail of hematopoietic cytokines, whose expression was reduced by TBTC. These cytokines stimulated $CD19^+$ B cell proliferation, maybe through the up-regulation of c-myc, as evidenced by the real-time PCR analysis.

Our results showed that when TBTC induced the highest cell death ratio (TBTC/CTR), cytokines prevented both $CD19^-$ and $CD19^+$ cell death. The different time-course of cell-death induction between $CD19^-$ and $CD19^+$ lymphocytes together with the highest cell death ratio detected within $CD19^+$ population, suggests a different sensitivity towards TBTC toxicity.

We hypothesized that TBTC could induce apoptosis in human B lymphocytes through the PPARγ pathway, as shown for other PPARγ agonists like ciglitazone (De Santiago et al., 1999; Schlezinger et al., 2004). But the real-time PCR analysis revealed that PPARγ was not present in $CD19^+$ B cells, while its expression was detected in mouse bone marrow B

cells (Schlezinger et al., 2004) and in human normal and malignant B cells (Ray et al., 2006). However, some authors suggested that apoptosis induced in human and mouse B cells by known PPARγ ligands, like ciglitazone or 15-Deoxy-Δ12,14-Prostaglandin J_2 (15d-PGJ_2) is independent by PPARγ (Piva et al., 2005; Ray et al., 2006). Using a dominant negative approach and an irreversible PPARγ antagonist, Ray (Ray et al., 2006) found that these inhibitors prevented PPARγ activation, but did not prevent B cell apoptosis induced by 15d-PGJ_2 or ciglitazone. Authors showed that these PPARγ agonists can activate other pathways: they found that 15d-PGJ_2 potently induced reactive oxygen species in B lymphocytes and caused an almost complete depletion of intracellular glutathione. These data suggest that also TBTC could induce apoptosis in B lymphocytes through a pathway independent from PPARγ.

The TBTC mode of action on human bone marrow B cells needs further investigations. Nevertheless, it was possible to conclude that the use of the long term culture method described in chapter 3 allowed studying different aspects of the TBTC induced immunosuppression already during the first 2 weeks of LTC. In fact, TBTC was tested both for its activity on a specific mesenchymal cell type (adipocytes) and on different lymphocytes population in the same culture conditions. This approach should be applied to a wider range of chemicals to evaluate if the long term human bone marrow culture *in vitro* can be considered a useful model for extrapolating data to *in vivo* bone marrow.

In conclusion, this work indicates that it is necessary to integrate different methods to cover the complexity of the immune system, in the evaluation of immunosuppression (Fig. 2). The results obtained by the comparison of different tests performed on three species are encouraging, because all the tests were able to classify the compounds chosen in a correct way. Moreover, the development of an in vitro method to maintain human lymphocyte is important to test the chemical compounds activity on specific

aspects of stromal formation and of lymphocyte differentiation or proliferation.

Further studies

The strategy proposed by this thesis to study immunosuppression *in vitro* should be evaluated through a pre-validation and validation process as indicated by ECVAM guidelines (Balls and Karcher, 1995). The LTC method should be further developed, and then tested with a wide range of chemicals to assess its efficacy in evaluating toxicity effects in bone marrow environment and hematopoiesis. This method could be also considered to study chemicals mechanism of action on specific bone marrow cell populations.

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SUMMARY

The immune system is an important target of xenobiotic toxicity. In fact, immunotoxicity evaluation is becoming a growing concern for international regulatory authorities. At present, immunotoxicity of chemicals is evaluated through standard toxicity studies (STS) as part of a repeated dose 28 days oral toxicity study in rodents (OECD, TG407). ICH (International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use) guideline S8 (ICH, S8) indicates that all new human pharmaceuticals should be evaluated for the potential to produce immunotoxicity, using STS. If a *cause-for-concern* is identified in STS, additional immunotoxicity studies should be performed to verify the immunotoxic potential of the compound.

Both the OECD and S8 guidelines are based on STS, which are performed on laboratory animals. European Union legislation aims at reducing the number of animals used for experiments, by requiring that an animal experiment should not be performed when an alternative method exists. Therefore, European Union is encouraging the development of alternative methods, which can replace, reduce or refine experiments with laboratory animals.

Except for the CFU-GM (granulocyte macrophage-colony forming unit) test, validated alternative methods to evaluate immunotoxicity are not available. Based on existing knowledge on immunotoxicity (e.g. immunosuppression) we here distinguished effects of chemicals on mature lymphocytes from effects of developing lymphocytes. Although we realize that these effects are not mutually exclusive, studies of the present thesis therefore aimed at:

- determination of the predictivity of a set of *in vitro* assays to evaluate suppressive potential on lymphocyte activation of compounds known for their effects on the immune system;

- developing a long-term culture method (LTC) using human mononuclear bone marrow cells to assess effects of chemicals on lymphopoiesis *in vitro*;
- performing a preliminary evaluation of the LTC developed, using the organotin compound tri-butyltin chloride (TBTC), which is known for its immunosuppressive activity in rats.

Given the complexity of the immune system, it was considered necessary to investigate different end-point parameters. In the second chapter, we evaluated the capability of several *in vitro* tests to classify a panel of compounds known for their immunosuppressive effects. Selected compounds included immunosuppressive and non-immunosuppressive pharmaceutical and chemical compounds. Cytotoxicity tests (LDH, MTT, trypan blue) were performed to select non-cytotoxic concentrations to be used in further tests on human (peripheral blood mononuclear cells) and rodent (rat and mouse, spleen) cells. After evaluating myelotoxicity with CFU-GM test, lymphocyte proliferation tests were performed using specific stimuli (e.g. LPS, PHA, ConA, anti-CD3 antibody). In addition, cytokine release (TNFα and INFγ) was measured. When possible, for each tested compound the IC50 was calculated and results were compared. Results show that *in vitro* tests performed well in classifying the selected compounds in all three species, although differences in sensitivity between species were apparent for some compounds. Data together indicate that it may be necessary to investigate different end-point parameters to study immunosuppression, possibly organised in a *tiered approach* starting from cytotoxicity and CFU-GM tests followed by more specific tests to evaluate cellular function of immune cells.

Most methods available use mature immune system cells. At present, there are no methods at all to study toxicity on human lymphoid progenitors, mainly because it is difficult to maintain human bone marrow *in vitro* for a

substantial amount of time. In the third chapter, we therefore set out to develop a long-term culture (LTC) from human bone marrow-derived mononuclear cells to maintain lymphopoiesis. During the first two weeks of culture, adherent cells differentiated to form a stromal feeder layer. Subsequently, the culture medium was replaced with a medium that specifically stimulated lymphopoiesis instead of myelopoiesis. Cultures were further supplemented with the hemopoietic growth factor IL-7 to investigate its effect on lymphocyte population. With this method B and T lymphocytes were maintained *in vitro* for five weeks. Although IL-7 induced the proliferation of the entire lymphocyte population, it was found to inhibit B cells and to stimulate T cells.

TBTC was tested in LTC to verify if this culture system could be useful in the chemical toxicity characterization of TBTC (chapter 4). TBTC was chosen because its immunotoxicity in rodents is reasonably well-known; furthermore, it was the most toxic among compounds previously studied (chapter 2). TBTC was tested during the first two weeks of LTC to verify its effect on the formation of stroma, which is necessary for maintaining lymphopoiesis. This study showed that TBTC stimulated adipocyte differentiation causing an alteration of the stromal cellular components. TBTC-stimulated adipocyte differentiation was mediated by modulation of nuclear receptors PPARγ and RXRα inducing the transcription of aP2 gene, a marker of adipocyte differentiation. In addition, TBTC reduced the expression of some cytokines and of the leptin hormone, which is important for lymphopoiesis.

Whether TBTC had an effect on lymphocyte population in the LTC, effects on B and T subsets were analyzed (chapter 5). Remarkably, TBTC did not reduce the amount of total lymphocytes population or ($CD3^+CD4^+$ or $CD3^+CD8^+$) T lymphocytes. However, TBTC reduced the amount of $CD19^+CD22^+$ B lymphocytes, possibly by inducing cell death. This effect

was independent of PPARγ. Some of the cytokines that were considered important for lymphopoiesis and whose production was inhibited by TBTC (as described in chapter 4) were added to the medium to verify if they could prevent TBTC-induced effects on lymphocytes in LTC. In the presence of cytokines, the $CD19^{+}CD22^{+}$ B cell percentage increased, probably through the up-modulation of c-myc gene transcription. The cytokines also appeared to protect against TBTC-induced cell death of $CD19^{+}$ B lymphocytes.

Together, data indicates that TBTC promotes adipocyte differentiation in bone marrow stroma, reduces the production of different cytokines, and induces cell death in precursor B cells. To what extend various mechanisms are causally related and how these *in vitro* findings translate to the *in vivo* situation (TBTC is particularly known for its toxic effect on T cell differentiation) is not known and may be subject of future research.

In conclusion, this thesis provides new knowledge on various potential alternative tests for the evaluation of immunotoxicity of chemicals. The T cell proliferation assay is currently subject of pre-validation studies. The human LTC is yet far from applicable as alternative immunotoxicity test. However, LTC studies described in this thesis provides an important and promising first step towards designing an assay allowing detection of hematopoietic effects of chemicals. An important next phase in development of a suitable LTC would be a comparison study with a series of carefully selected compounds. In parallel, more in depth mechanistic studies are needed to understand the various processes occurring in the LTC. This knowledge is absolutely required to translate in vitro findings to the in vivo situation.

The current studies stress once more that identification of a chemical's immunotoxic potential demands a battery of assays, including the CFU-GM test, T cell proliferation assay and a LTC, possibly in a tiered approach.

SAMENVATTING

Het immuunsysteem is een belangrijk doelorgaan voor xenobiotische toxiciteit. Sterker nog, immunotoxiciteit is een toenemende zorg voor internationale regelgevende autoriteiten. Tegenwoordig wordt de immunotoxiciteit van chemicalien geëvalueerd door middel van standaard toxiciteitsstudies (STS) als onderdeel van 28 dagen orale toxiciteitsstudies in knaagdieren (OECD, TG407). De IHC (Internationale Conferentie voor Harmonisatie van technische eisen voor de registratie van pharmaceutische producten voor humaan gebruik) richtlijn S8 (ICH, S8) stelt, dat alle nieuwe humane geneesmiddelen geëvalueerd zouden moeten worden voor hun potentiële immunotoxiciteit met behulp van STS. Als er reden is tot zorg voor immunologische effecten, dan moeten additionele immunotoxiciteitsstudies worden uitgevoerd om de potentiële immunotoxiciteit vast te stellen.

Beide richtlijnen zijn gebaseerd op STS, welke uitgevoerd worden op proefdieren. Wetgeving van de Europese Unie richt zich echter op het verminderen van het aantal dieren dat gebruikt wordt bij laboratorium experimenten, door te stellen dat een dierexperiment niet uitgevoerd zou moeten worden indien er een alternatieve methode bestaat. Daarom moedigt de Europese Unie de ontwikkeling van alternatieve methoden aan, welke kan leiden tot het vervangen of verminderen van het aantal proefdieren, of het verfijnen van methoden waarbij proefdieren worden gebruikt.

Behalve de *CFU-GM (granulocyte macrophage-colony forming unit)* test zijn er momenteel geen gevalideerde alternatieve methoden voorhanden om de immunotoxiciteit van stoffen te evalueren. Uitgaande van wat bekend is ten aanzien van immunotoxiciteit, met name immunosuppressie, wordt hier voor het gemak onderscheid gemaakt tussen effecten op lymfocyten die functioneel uitgerijpt zijn en effecten op ontwikkelende lymfocyten. Dit proefschrift heeft als doel een bijdrage te leveren aan de ontwikkeling van *in*

vitro methoden voor het vaststellen van deze verschillende vormen van immunosuppressie. Studies zijn gedaan om:

- eerste aanzet te doen om de voorspelbaardheid van een aantal al bestaande *in vitro* methoden te bepalen met betrekking tot immunosuppressieve werking van stoffen;
- een langdurige kweekmethode (*long-term culture method*, LTC) te ontwikkelen uitgaande van humane beenmergcellen om effecten van stoffen op lymfopoiese *in vitro* te kunnen vaststellen; ,
- een eerste evaluatie op de ontwikkelde LTC uit te voeren met tributyltin chloride (TBTC), een stof waarvan de immunosuppressieve activiteit bij ratten redelijk bekend is.

Gezien de complexiteit van het immuunsysteem werd het noodzakelijk geacht om verschillende parameters als eindpunt te onderzoeken. In het tweede hoofdstuk wordt het vermogen van enkele bekende *in vitro* testen geëvalueerd met aan aantal stoffen, waarvan het effect op het immuunsysteem bekend was op basis van *in vivo* studies of eerdere *in vitro* studies. Cytotoxiciteitstesten (LDH, MTT, Trypan Blue) werden uitgevoerd om de niet-cytotoxische concentraties te bepalen voor verdere experimenten op humane (uit bloed) en knaagdier (rat en muis, milt) lymfocyten. Na de myelotoxiciteit te hebben bepaald met behulp van CFU-GM testen werden lymfocytproliferatie experimenten uitgevoerd gebruikmakend van bekende stimulaties (LPS, PHA, ConA, anti-CD3 antilichaam). Vervolgens werd ook de afgifte van cytokines (TNFα en INFγ) door lymfocyten gemeten.

Van elke geteste stof werd de IC50 berekend waarna de resultaten vergeleken werden. Resultaten wezen uit dat de *in vitro* methoden goed in staat waren om de geselecteerde stoffen te classificeren. Dit gold voor alle drie de soorten (mens, rat en muis) al was er wel sprake van verschillende gevoeligheid tussen de soorten. Resultaten geven aan dat het wellicht noodzakelijk is om verschillende functionele eindpunten van lymfocyten te

bestuderen om remming van lymfocytenactivatie goed te kunnen vaststellen. Wellicht is een stapsgewijze benadering hiervoor geschikt, beginnend bij testen voor het vaststellen van de cytotoxiciteit, gevolgd door testen om proliferatie en cytokine release te bepalen.

De potentieel alternatieve testen voor immunotoxiciteit die hierboven genoemd worden gaan uit van volwassen immuuncellen. Op dit moment zijn er geen methoden voorhanden om de toxiciteit op de ontwikkeling van humane lymfocyten, lymfopoiese, te bepalen. In het derde hoofdstuk wordt de ontwikkeling van een LTC beschreven uitgaande humane mononucleaire beenmergcellen. Gedurende de eerste twee weken van de kweek bleken hechtende (stroma) cellen te differentiëren tot een ondersteunende cellaag. Vervolgens werd het medium vervangen door een ander medium, waardoor de hechtende cellen niet verder doorgroeien, maar juist de lymfocyten kunnen ontstaan. Met deze methode konden B en T lymfocyten gedurende 5 weken *in vitro* worden doorgekweekt. Aan celkweken werd ook de groeifactor IL-7 toegevoegd om het effect op de lymfocyten te bestuderen. IL-7 bleek de vorming van B cellen te remmen en die van T cellen juist te stimuleren.

TBTC werd getest in de LTC methode om na te gaan of dit systeem bruikbaar is voor het vaststellen van effecten of lymfocytenontwikkeling (hoofdstuk 4). TBTC werd gekozen vanwege de bekende immunotoxiciteit in knaagdieren. Ook was het de meest toxische van de eerder geteste stoffen (hoofdstuk 2). TBTC werd eerst getest gedurende de eerste twee weken van de LTC om de toxiciteit vast te stellen op de hechtende cellen, welke noodzakelijk zijn ter ondersteuning voor de lymfocytenontwikkeling. Deze studie toont aan dat TBTC de differentiatie van vetcellen (adipocyten) stimuleert en zo een verandering in de cellulaire componenten van de voedingslaag veroorzaakt. De ontwikkeling van vetcellen is mogelijk het gevolg van een effect van TBTC op de nucleaire receptoren PPARγ en

RXRα, waardoor ook het aP2 gen actief werd. aP2 is van belang bij de ontwikkeling van vetcellen. TBTC verminderde ook de expressie van sommige cytokines en van het hormoon leptine, welke belangrijk zijn voor lymfocytenontwikkeling.

Om na te gaan of TBTC ook *in vitro* een effect had op de lymfocytenontwikkeling, werden B en T lymfocyten nader geanalyseerd (hoofdstuk 5), gebruikmakend van dezelfde kweekcondities zoals beschreven in het voorgaande hoofdstuk. TBTC bleek geen invloed te hebben op de hoeveelheid van de totale lympfocytenpopulatie en ook niet op de hoeveelheid $CD3^+CD4^+$ en $CD3^+CD8^+$ T lymfocyten. Maar TBTC veroorzaakte wel een opvallende vermindering van de hoeveelheid $CD19^+CD22^+$ B lymfocyten, mogelijk door een specifieke toxisch effect van TBTC in deze cellen. Dit effect bleek onafhankelijk van PPARγ. Sommige cytokines, die belangrijk geacht werden voor lymfocytenontwikkeling en waarvan de productie geremd werd door TBTC (hoofdstuk 4), werden aan het medium toegevoegd om na te gaan of deze de specifieke toxische effect van TBTC op B cellen konden beïnvloeden. In de aanwezigheid van deze cytokines, nam het $CD19^+CD22^+$ B cel percentage toe, waarschijnlijk door verhoogde transcriptie van het c-myc gen, en bleek het effect van TBTC inderdaad minder. Deze resultaten suggereren dat cytokines deels de celdood door toxiciteit van TBTC kunnen voorkomen.

De bevindingen in de LTC duidden erop dat TBTC de vorming van vetcellen stimuleert, productie van sommige cytokines remt en celdood veroorzaakt in B cellen. Het is onduidelijk uit de gepresenteerde studies in hoeverre deze verschillende effecten causaal met elkaar in verband staan. Ook de relevantie van de huidige *in vitro* bevindingen voor de *in vivo* situatie is niet duidelijk, vooral ook omdat TBTC vooral bekend staat vanwege specifieke effecten op de ontwikkeling van T lymfocyten. Beide aspecten, ophelderen van een

eventueel causaal verband en van de relevantie voor de *in vivo* situatie, vragen om verder onderzoek.

Samenvattend, het werk gepresenteerd in dit proefschrift levert nieuwe technische en mechanistische kennis ten aanzien van mogelijke alternatieve testen voor het vaststellen van immunotoxische potentie van stoffen. De genoemde proliferatie test met T lymfocyten is momenteel onderwerp van een "pre-validatie" studie. Voor de LTC, welke interessante aanwijzingen voor mechanismen op heeft geleverd, is het nog een verre weg naar validatie. Eerst zal de test verder geoptimaliseerd moeten worden en zullen de effecten van meer bekende immunotoxische stoffen getest moeten worden. Verdere optimalisatie-studies zullen zeker ook bijdragen aan uitbreiding van de noodzakelijke mechanistische kennis met betrekking tot de LTC. Die kennis is van groot belang om de *in vitro* bevindingen te kunnen vertalen naar de *in vivo* situatie.

De huidige studies tonen duidelijk aan dat voor het *in vitro* vaststellen van immunosuppressieve effecten van stoffen een enkele test niet volstaat, maar dat hiervoor een reeks van slim gekozen testen, mogelijk in een stapsgewijze aanpak, absoluut noodzakelijk is.

RIASSUNTO IN ITALIANO

Il sistema immunitario è un importante bersaglio della tossicità indotta da xenobiotici. Infatti, la valutazione dell'immunotosicità sta diventando, di crescente interesse tra gli enti regolatori internazionali.
Attualmente, l'immunotossicità indotta da sostanze chimiche viene valutata attraverso studi standard di tossicità (STS) che fanno parte dello studio di tossicità a somministrazione orale ripetuta per 28 giorni nei roditori (OECD, TG 407).

La linea guida S8 (ICH, S8) dell'ICH (conferenza internazionale sull'armonizzazione dei requisiti tecnici per la registrazione dei prodotti farmaceutici per uso umano) indica che tutti i nuovi prodotti farmaceutici per uso umano, dovrebbero essere testati per la loro potenziale immunotossicità utilizzando gli STS. Se da questi test, si evidenzia un effetto tossico della sostanza testata, ulteriori studi dovranno essere condotti per verificare il potenziale immunotossico del composto.

Entrambe le linee guida prevedono lo svolgimento di studi di tossicità su animali da laboratorio. La legislazione dell'Unione Europea prevede la riduzione dell'uso degli animali da laboratorio, richiedendo che l'esperimento sull'animale non debba essere svolto, nel caso in cui siano disponibili test alternativi validati, capaci di indicare la tossicità della sostanza in esame con la medesima attendibilità scientifica. Per tale regione, l'Unione Europea, sta incoraggiando lo sviluppo di test alternativi che possano sostituire, ridurre o migliorare l'utilizzo degli animali da laboratorio.

Eccetto che per il test CFU-GM (unità formante colonia- granulocita macrofago), metodi alternativi validati per la valutazione dell'immunotossicità, non sono disponibili. Basandosi sulle attuali conoscenze sull'immunotossicità (ad esempio l'immunosoppressione), nel presente studio abbiamo distinto gli effetti delle sostanze chimiche sui

linfociti maturi dagli effetti sui linfociti in fase di differenziamento. Sebbene sia stato evidenziato che tali effetti non sono mutualmente esclusivi, il presente studio è stato sviluppato secondo i seguenti obiettivi:

- determinazione della predittività di alcuni test *in vitro* nella valutazione del potenziale soppressivo sull'attivazione linfocitaria di composti conosciuti per i loro effetti sul sistema immunitario;
- sviluppo di un metodo di coltura a lungo termine (LTC), usando cellule mononucleate di midollo osseo umano, per determinare gli effetti delle sostanze chimiche sulla linfopoiesi *in vitro*;
- valutazione preliminare del metodo di coltura sviluppato, usando l'organostannico TBTC, sostanza chimica nota per la sua attività immunosoppressiva nei ratti.

Data la complessità del sistema immunitario, è necessario investigare diversi parametri. Nel secondo capitolo abbiamo valutato la capacità di una batteria di test nel classificare una serie di sostanze note per i loro effetti immunosoppressivi. Tra queste sostanze positive o negative rispetto all'immunosoppressione, erano inclusi sia prodotti di uso farmacologico che chimico. Alcuni test di citotossicità (LDH, MTT, trypan blue) hanno permesso di scegliere concentrazioni non citotossiche da utilizzare nei test successivi, su cellule di origine umana (cellule mononucleari da sangue periferico) o di roditore (cellule di milza di ratto e topo). Dopo aver valutato la mielotossicità con il test CFU-GM, sono stati eseguiti test di proliferazione su lifociti a seguito di appropriato stimolo mitogeno (es. LPS, PHA, ConA, anticorpo anti-CD3). Inoltre, è stato misurato il rilascio di citochine (TNFα e INFγ).

Quando possibile, per ciascun composto testato, è stata calcolata l'IC50 e i dati ottenuti confrontati. I risultati mostrano che ogni test ha consentito la classificazione corretta delle sostanze studiate all'interno delle tre specie. Allo stesso tempo, è stata evidenziata una diversa sensibilità tra le specie nei

confronti di alcuni composti. Questi dati indicano che potrebbe essere necessario investigare diversi parametri per studiare l'immunosoppressione, possibilmente organizzati in un approccio sequenziale, partendo dalla citotossicità e dal test CFU-GM, seguito da test più specifici per valutare la funzionalità delle cellule costituenti il sistema immunitario.

I metodi oggi in uso sono stati sviluppati prevalentemente su cellule mature del sistema immunitario. In questo momento, non vi sono metodi per studiare la tossicità sui progenitori linfoidi, perché è molto difficile mantenerli *in vitro*, partendo da midollo osseo umano. Nel terzo capitolo abbiamo sviluppato una coltura a lungo termine (LTC) da cellule mononucleate di midollo osseo umano per mantenere la linfopoiesi. Il metodo permette il differenziamento di cellule adese che formano lo stroma durante le prime due settimane di coltura. In seguito, il terreno è sostituito con un tipo di terreno che stimola in modo particolare la linfopoiesi anzichè la mielopoiesi. Questo metodo permette il mantenimento dei linfociti B e T in coltura per cinque settimane. L'interleuchina 7 (IL-7) è stata aggiunta alle colture, per investigare il suo effetto sulla popolazione linfoide. L'IL-7 inibisce la popolazione B mentre stimola i linfociti T, inoltre induce la proliferazione della popolazione linfocitaria totale.

Per verificare se questo metodo può essere utilizzato per caratterizzare la tossicità di una sostanza chimica, è stato testato il TBTC (quarto capitolo), perché è noto per la sua immunotossicità nei roditori; inoltre si è dimostrato essere la sostanza più tossica nel precedente studio, come descritto nel secondo capitolo. Il TBTC è stato testato nelle prime due settimane di LTC per verificare la sua tossicità sulla formazione dello stroma, indispensabile per la linfopoiesi. Lo studio ha rilevato che il TBTC induce il differenziamento degli adipociti causando uno squilibrio nella componente cellulare dello stroma. Il differenziamento adipocitico indotto dal TBTC è mediato dalla modulazione dei recettori nucleari PPARγ e RXRα che

inducono la trascrizione del marcatore di differenziamento adipocitico aP2. Il TBTC riduce la produzione di diverse citochine e in particolare dell'ormone leptina, importante per la linfopoiesi.

Al fine di verificare se il TBTC abbia realmente un effetto soppressivo sulla popolazione linfoide nella LTC, nel quinto capitolo, sono state esaminate alcune sottopopolazioni cellulari di tipo B e T. Il TBTC non inibisce la popolazione linfocitaria totale, nè i linfociti T ($CD3^+CD4^+$ o $CD3^+CD8^+$), ma provoca una riduzione dei linfociti B $CD19^+CD22^+$, probabilmente inducendone la morte. Questo effetto è indipendente dall'interazione con PPARγ.

Alcune citochine importanti per la linfopoiesi, la cui produzione è risultata inibita dal TBTC come descritto nel capitolo 4, sono state addizionate al terreno di coltura, per verificare se possano prevenire la tossicità del TBTC sui linfociti. In presenza delle citochine la percentuale di cellule B $CD19^+CD22^+$, aumenta, probabilmente attraverso l'aumento della trascrizione di c-myc. Inoltre, le citochine sembrano proteggere i linfociti B $CD19^+$ dalla morte cellulare indotta dal TBTC.

Questi dati suggeriscono che il TBTC promuove il differenziamento adipocitico nella parte stromale del midollo osseo, riduce la produzione di diverse citochine e induce la morte cellulare dei precursori B.

Come i vari meccanismi siano causalmente correlati e come, i risultati ottenuti *in vitro* possano essere tradotti in osservazioni che si otterebbero *in vivo* (il TBTC è conosciuto specialmente per il suo effetto tossico sul differenziamento dei linfociti T) non è noto e potrebbe essere oggetto di ricerche future.

In conclusione, questa tesi offre nuove conoscenze su diversi potenziali test alternativi per la valutazione dell'immunotossicità delle sostanze chimiche. Il saggio di proliferazione delle cellule T è attualmente in una fase di pre-

validazione. Il metodo LTC su cellule umane è ancora lontano dal poter essere applicato come test alternativo d'immunotossicità. Comunque, lo studio sul metodo LTC, descritto in questa tesi, offre un importante e promettente primo passo verso il disegno di un saggio che permetta l'identificazione degli effetti dei composti chimici sull'ematopoiesi. Il prossimo importante stadio nello sviluppodi un idoneo metodo LTC potrebbe essere uno studio di confronto con una serie di composti opportunamente selezionati. In parallelo, studi meccanicistici più approfonditi sono necessari per predirre le osservazioni in vivo partendo dai dati in vitro.

Il presente studio conferma che l'identificazione del potenziale immunotossico richiede una batteria di saggi, incluso il test CFU-GM, il saggio di proliferazione delle cellule T e un LTC, possibilmente in un approccio sequenziale.

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CURRICULUM VITAE

Maria Carfi' was born on August 20, 1978 in Somma Lombardo (Varese, Italy). In 1997, she graduated at the Liceo Scientifico Statale of Somma Lombardo and in 2002 she took a degree in Biotechnology (medical field) at the University of Milan, Italy.

Between 2000 and 2002 she worked for the preparation of her degree thesis, in the laboratory of Prof. G. Tredici at the Department of Neuroscience and Biomedical Technology, University of Milan Bicocca, Italy. After the degree in Biotechnology, she did a six months training at DG-JRC, IHCP-ECVAM (European Centre for the Validation of Alternative Methods) - Ispra, Italy, under the supervision of Dr. L. Gribaldo.

At the end of this training period, she found a job as sales representative for a pharmaceutical Company. After six months, she had the opportunity to attend a one year Master in Bioinformatics. During the master she worked at the Department of Biotechnology and Biosciences, University of Milan Bicocca, Italy. She worked in the molecular modeling laboratory of Prof. P. Fantucci.

As the contract in Milan expired, she had the opportunity to come back working at ECVAM, under the supervision of Dr. L. Gribaldo. At the same time she was enrolled as PhD student at IRAS, Utrecht University, under the supervision of Dr. R.Pieters. The work conducted in ECVAM was finalised for the preparation of the PhD thesis.

In 2009 she spent six months at the OECD in Paris, where she worked on the Test Guidelines Programme, under the supervision of Dr. L. Musset.

From December 2009 to October 2010 she will work in the laboratory of Prof. E. Corsini, at the Department of Pharmacological Sciences, University of Milan.

In November 2010 she will defend her thesis in Utrecht.

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