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Immunological substance testing on human lymphatic micro-organoids *in vitro*

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ABSTRACT

Pharmaceutical drugs and compounds used for consumer products may bear the risk of unexpected immuno-toxicological side effects, such as sensitization, allergy, anaphylaxis or immunogenicity. Modern biopharmaceuticals with high potency and target specificity, like antibodies and cytokines need to be tested for their therapeutical doses, their exposition regimens and their immune functionality prior to first-in-man applications.

For the latter, existing *in vitro* tests and animal models do not sufficiently reflect the complexity and specificity of the human immune system. Even novel humanised animal models have limitations in their systemic reactions. Monolayer or suspended cell culture possesses neither tissue functionality nor organ physiology, and also cannot be used for long term culture and experiments. In contrast, solid tissue biopsies, e.g. tonsil preparations of tonsillitis patients typically show inflammatory artefacts and degrade in long term culture due to preparation-induced damage.

The construction of tissue-like structures *in vitro*, so-called "micro-organoids", can overcome these limitations. Key structures of secondary lymphatic organs, e.g. lymph nodes or the spleen are the primary lymphatic follicles and germinal centres, in particular during the "activated state" of an inflammation or infection. To remodel lymphatic follicles, functional and structural cells, e.g. lymphoid cells derived from peripheral blood mononuclear cells (PBMCs) and stromal cells need to be combined with biogenic or artificial matrices and scaffolds to produce a suitable 3D tissue-mimicking environment.

Therefore, a unique human lymph node model (HuALN) was designed to operate over several weeks, and allow long term and repeated drug exposure to induce and monitor both cellular and humoral immune responses. Cellular immunity is monitored, for example, by cytokine release patterns; humoral immunity is analysed, for example, by B cell activation, plasma cell formation and antibody secretion profiles (IgM and IgG). Moreover, cellular composition and micro-organoid formation are analysed by flow cytometry, histology and *in situ* imaging.

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1. Introduction

All pharmaceutical drugs, cosmetic products and substances for use in consumer products must be tested for affects on the human immune system. All pharmaceutical drug candidates and modern biopharmaceuticals, in particular, must be tested in preclinical phases for adverse reactions, e.g. immunogenicity and immunotoxicity, according to ICH guidelines (ICH Harmonised Tripartite Guideline). If these drugs target the immune system specifically, e.g. certain antibodies or cytokines, their immune-therapeutical potency must be evaluated as well. Compounds for cosmetic applications and chemical substances for consumer products for the EU market must be tested for sensitisation effects, according to the Cosmetics Directive (Cosmetics, 7th Amendment of the Cosmetics Directive and EU Cosmetics Directive) or the REACH Regulation (Registration, Evaluation, Authorisation and Restriction of Chemical Substances).

These tests are based on animal models and *in vitro* methods which underestimate the complexity and diversity of mammalian immune systems and the remarkable target and species specificity of drugs.

Simple cell culture formats, for instance, using suspended PBMCs or in combination with monolayers of monocytes, macrophages or dendritic cells (DCs) in multiwell or transwell cultures, are designed for short term culture under static conditions and simple exposure regimes only (Holmes et al., 2009; Pörtner and Giese, 2007).

Existing animal models used for conventional immunotoxicity testing or sensitisation, e.g. the local lymph node assay (Basketter et al., 2002; Kimber et al., 2001), or delayed type hypersensitivity test (DTH test) on monkeys (Nyhus et al., 2006) have limitations in the predictiveness and comparability to the human situation (Westmoreland and Holmes, 2009). With the ban an animal test-

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ing at the end of March, 2013, for the safety testing of cosmetics (Westmoreland and Holmes, 2009) and the predicted increase of the safety testing of chemicals according to the REACH activities, more and efficient *in vitro* models to replace animal tests have become mandatory (Hartung and Rovida, 2009). Existing transgenic or xenogenic animal models, so-called *humanised animals*, which are modified with components of the human immune system, e.g. T cell functionality, do not reflect the systemic differences to humans (Suematsu and Watanabe, 2004).

Tissue biopsies, e.g. human tonsil preparations, are discussed as suitable for *ex vivo* testing applications, but their availability is limited and has underlying ethical restrictions. Moreover, damage and stress caused by preparation and logistics reduces the survivability and may lead to physiological artefacts.

Complex tissue-like culture systems with defined organ functionality, the *organotypic cultures*, may bridge this gap between animal tests and human applications. They are, with their controlled morphogenesis, in contrast to the *ex vivo* approach, expected to be better adapted to tissue-mimicking, yet artificial, *in vitro* conditions. The organotypic system evolves from the combination of relevant cell types, such as extra-cellular matrix (ECM) equivalents or scaffolds, controlled perfusion and suitable bioreactor hardware (Lutolf and Hubbell, 2005; Pörtner and Giese, 2007). Primary cells or immortalised cells, recombinant cell lines or tumour cells and/or co-cultures of these can be used as cellular substrates.

Organotypic cultures for *in vitro* testing have to focus on the relevant biomarkers of interest in order to reduce the complexity of the *in vivo* situation. Essential structures and smallest functional units, so-called micro-organoids, have to be identified and remodelled *in vitro* (Pörtner and Giese, 2007). For structural and accessory support these micro-organoids have to be embedded into a suitable stromal environment.

For the immune system, biochemical modifications by cellular processing are very important concerning the route of drug administration. Proteins applied subcutaneously are processed by antigen-presenting cells (APC), e.g. dendritic cells (DCs) or Langerhans cells (LC) in the peripheral non-lymphatic tissue. Small molecules (Haptens) may bind to a carrier protein when exposed to the skin and penetrate the epidermis. Hapten-carrier complexes or modified substances will then be carried to the target organ or tissue to induce the effect of interest.

Key structures for adaptive immune responses in mammalians are the secondary lymphatic organs, e.g. lymph nodes (LNs), and the spleen (Crivellato et al., 2004). Antigens and antigen-presenting cells drain from peripheral areas of the body towards the LNs via the lymphatic fluid. They enter the LN through afferent lymphatic vessels, the subcapsular and intermediate sinuses and swarm into the parenchymatic medulla and paracortex by lacunary channels. They migrate into the network of reticular cells and ECM occasional guided by a specialised cellular and fibrillary conduit system (Junt et al., 2008). T and B cells enter the LN via the blood flow, extravasate into the lymph node cortex under control of the high endothelial venules (HEV), migrate and seek for the right predisposed peptide presented by antigen-presenting cells (APC). Activated T cells proliferate and migrate to the areas of B cell follicles. The subpopulation of TH4-helper cells play an important role in B cell co-stimulation, whereas TH1-helper cells guide towards a cellular immune response, e.g. by activation of cytotoxic T cells. B cells are clustered in B cell follicles in the cortex of the LN and are activated by direct contact with antigen or immune complexes under co-stimulation of TH4-helper cells. B cell follicles evolve into germinal centres (GC) by induced B cell proliferation and plasma cell differentiation, with the typical formation of a dark zone (DZ), a light zone (LZ) and a mantle zone (MZ) occurs. In B cell proliferation, the formation of centroblasts is localised in the DZ and plasma cell selection is in the LZ, and the final antibody producing plasma cells are in the MZ (Lindhout et al., 1997; Allen et al., 2004, 2007; Camacho et al., 1998).

The type and intensity of the induced immune response is controlled by numerous accessory cells, the local microenvironment of cytokines, chemokines and other co-stimulators, and act like danger signals via toll-like receptor (TLR) activation. This leads either to a cellular or a humoral response. At the end of an inflammation episode, T and B memory cells are produced, which exit the LN and home into the bone marrow, waiting for recall.

LNs are very dynamic organs. During infection they grow in size like an aggressive tumour, but show a fast and controlled regression at the end of an acute inflammation. The lymphatic cells in the lymph node tissue are highly active during, even in inflammation (von Andrian and Mempel, 2003). Migration, homing and segregation in certain areas are influenced by the parenchymatic organisation and the open reticular network and ECM (Junt et al., 2008). Moderate lymphatic draining ensures the formation of local microgradients for migration and homing. The lymphoid cells interact with a network of stroma cells by direct contact, soluble factors and immune complexes. The stroma network not only provides structure and spatial organisation, homing and segregation of cells, but also has immune-modulating influences, important for stimulation, suppression or cell survival and antibody refinement (Junt et al., 2008). A number of different stroma cell types are described for LNs (Mueller and Germain, 2009).

- Follicular dendritic cells (FDCs) are important for antigen-presentation to B cells for refinement, class switch and affinity maturation, and prevent apoptosis.
- Vascular endothelial cells of the high endothelial venules (HEV) are the gatekeepers for lymphocytes to lymphatic tissue.
- Fibroblastic reticular cells (FRC) form a conduit network for antigen direction and APC trafficking into lymphoid tissue.
- Lymph node medullary fibroblasts (MLF) are attractors for macrophages and plasma cells.
- Marginal reticular cells (MRC) support the conduit system and chemokine secretion.

In addition to structural properties, stroma and endothelial cells directly affect cellular immune responses by releasing co-stimulatory factors. Biopharmaceutical drugs which target lymphoid cells, e.g. superagonistic antibodies for T cell activation or suppression, may also interfere with the stroma or endothel. The tragic occurrence during the TeGenero's phase I clinical trial of the CD28-targeting antibody TGN 1412 (Suntharalingam et al., 2006) has shown that co-stimulation of other accessory, and non-lymphatic cells, e.g. endothelial cells, may result in a strong opposite effect to the intended therapeutical mechanism (Stebbings et al., 2007). Here, TGN 1412 induced a cytokine storm by misguided T cell activation in humans, but not in *Cynomolgus* monkeys (Schraven and Kalinke, 2008).

Even the structure of the LN is complex, and a pre-existing complexity seems to be crucial for GC formation; GC-like structures can also be found in the joints of rheumatoid arthritis (RA) patients. Ectopic GC can develop in the synovial gap affected joints *de novo* without any pre-existing stroma environment, except synovial fluid and cartilage matrix (Salomonsson et al., 2003).

A unique human lymph node model was therefore designed to operate over several weeks, and allow long term and repeated drug exposure to induce and monitor both cellular and humoral immune responses.

It has become obvious that in addition to efficient oxygen and nutrient supply, the local gradient establishment of metabolites, cytokines, chemokines and other (undiscovered) parameters, as well as structured surfaces for chemotaxis and local settlement (including intercellular cross-talk via tight junctions), are crucial

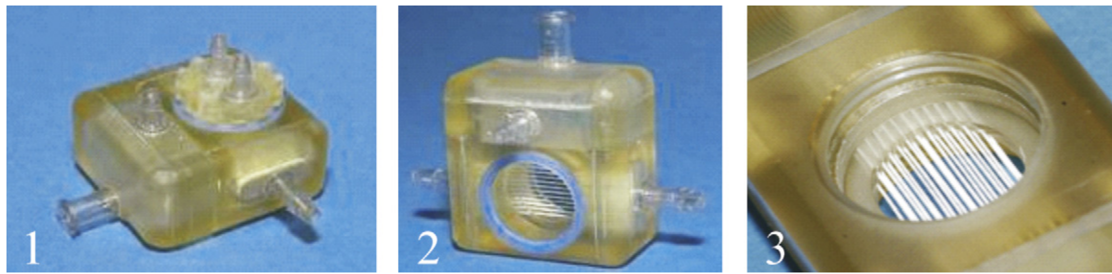

Fig. 1. Miniaturised bioreactor including the sidewise sample reservoirs (1 and 2). View of the culture compartment with a double layer of oxygenating membranes (3).

prerequisites for the proper emulation of *in vivo* environments (Griffith and Swartz, 2006) for suitable tissue culture techniques. This has provoked a shift from the development of homogeneous to heterogeneous culture systems and an emphasis on controlled, continuously adjustable, long term culture processes.

The basic aim of this cell culture device and process developments is the creation of an architecture and homeostasis mimicking the relevant human microenvironment for self-organisation of a specific tissue.

Cellular immunity is monitored, for example, by cytokine release patterns; humoral immunity is analysed, for example, by B cell activation, plasma cell formation and antibody secretion profiles (IgM and IgG). Moreover, cellular composition and micro-organoid formation is analysed by flow cytometry, histology and *in situ* imaging.

2. Materials and methods

Four programmes, including the qualification of human leukocyte sources and preparation procedures, the evaluation of material candidates for matrix-assisted culture design, prototyping of disposable perfusion bioreactors and qualification of various in-process and end point analyses were simultaneously carried forward. This general approach can be applied to the design of any complex tissue culture system developments, with obvious applications in the fields of pharmaceutical drug testing, organ research and tissue regeneration. Feasible technical equivalents of human habitat and homeostasis of primary follicles in human lymph nodes were developed. They comprise macro-porous matrix sheets, incorporated in a disposable miniaturised bioreactor, to enable wide ranges of cell feeding rates, gas supply and cell recycling.

2.1. Cell preparation

PBMCs of healthy, adult donor leucapheresis material, provided by a commercial blood bank, were separated by density gradient centrifugation as described previously (Giese et al., 2006). The selected human donor material was seronegative for HepA- or CMV-infection. Monocytes and, if required, T cells and B cells were separated by surface marker-specific magnetic bead separation (T cells: CD4; B cells: CD19; monocytes: CD14; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cryopreservation in liquid nitrogen was used to store selected cell populations for later use. RPMI1640 + 10% FCS medium (foetal calf serum, Fisher, Biochrom) was used as culture medium for all cell cultures.

2.2. Generation of antigen-specific dendritic cells

Human monocytes are differentiated into mature dendritic cells (mDCs) using a 7-day protocol. Immature DCs (iDCs) are generated by a cocktail of cytokines and growth factors as described (Giese et al., 2006). Mature DCs (mDCs) are induced by antigen-priming, using cytomegalovirus (CMV) lysate (EastCoastBio, North Berwick, USA) or vaccines (Havrix™; Glaxo-Smith-Kline, Munich, Germany) by incubation for 24 h at concentrations of 10 μg/mL and 50 relative units (RU), respectively. Control bioreactor runs were operated with TNF-α matured DCs.

Maturation and phagocytotic capabilities of generated DCs were tested by a panel of CD markers (CD1a, CD14, CD40, CD83, CD86 and HLA-DR; BD Biosciences, Heidelberg, Germany) (Banchereau and Steinman, 1998) and by phagocytosis assay, respectively (Sallusto et al., 1995). Briefly, a fraction of DCs was incubated for 24 h with CMV lysate-coated microbeads (latex microspheres, diameter 1 μm; Polysciences Europe GmbH, Eppelheim, Germany). Following the fluorescence signal of the beads allowed easy microscopic evaluation of phagocytosis.

For selected experiments the micro-organoid cultures were continuously exposed to dexamethasone (Sigma–Aldrich) by culture medium supplementation (1 μM dexamethasone; Sigma–Aldrich).

2.3. Cell-staining

Prior to the inoculation of the bioreactor system B cells, mature dendritic cells (mDCs) and CD14-and CD19-depleted PBMCs were labelled with CMFDA, CMTMR and CMPTX, respectively (all Invitrogen, Karlsruhe, Germany). Briefly, 1 E6 viable cells were stained with 1 μM (B cells and depleted PBMCs) and 5 μM (mDCs) dye solutions for 30 min at 37 °C. Cells were washed three times with PBS after staining.

2.4. Bioreactor perfusion systems

In order to mimic the physiological process of immunological responses *in vitro*, two disposable perfusion bioreactor systems were designed: the HIRIS™ III (Fig. 1) and the IG-Device™ (see Fig. 3). The bioreactors ensure continuously perfused long term cultures of lymphocytes and accessory cells and multiple or constant drug exposition have been designed according to the EU medical device directive ISO 13485.

The HIRIS™ III bioreactor enables matrix-assisted cultures of more than 1 E8 cells/bioreactor and allows the circulation of B cells emulating the situation *in vivo*. This bioreactor combines a large T cell and enables repeated restimulation with antigen and previously antigen-primed mDCs.

The HIRS III bioreactor is made of polysulfone (PS), and houses two culture compartments which are separated by a double layer of oxygenating membranes (hollow fibre module, HFM; Accurel PP 50/200, Membrana, Germany), which are continuously perfused with a gas mix (5% CO_2/20% O_2).

Each side of the culture compartment is closed by a cap, allowing the application of two fluidic systems to the bioreactor (Fig. 2), one for cell culture medium-perfusion and one for circulating suspended cells. Additionally, the caps can be equipped with sensor spots for non-invasive online control of pH and pO_2 (Presens GmbH, Germany): The culture medium is pumped into the culture com-

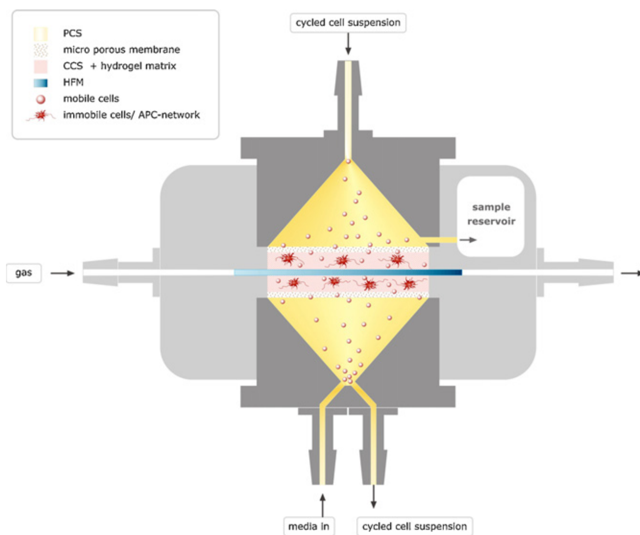

Fig. 2. Schematic drawing of the bioreactor in operation. Cell culture media and cell suspension flow vertically, and the gas supply perfuses the system horizontally. The oxygenating membranes are encompassed by the immobilized matrix-assisted cell suspension.

partment during the bioreactor operation by a peristaltic pump, using the cap at the bottom of the reactor. Cell culture media subsequently perfuse the matrix-assisted co-cultures (central culture space, CCS) with a flow rate of 45 μL/h, and exit the peripheral culture space (PCS) through a small hole into an integrated sample reservoir. Samples can be taken semi-continuously from this reservoir at pre-defined time points via a needle-free sampling port. The fluidic system is equipped with an injection port at the bioreactor's medium inlet port, where defined concentrations of antigen, for example, can be administered. A separated fluidic system with a second peristaltic pump allows the continuous cycling of a B cell suspension in order to mimic lymph flow and allow B cell contact with preliminary antigen challenged DCs and primed T cells. Design, prototyping and manufacturing of the bioreactors are performed under ISO standards for the development of medical devices (ISO International Standards Organization, 1999). To set up the system, two agarose matrix discs (Giese et al., 2006) with 1 E8 *in vitro* differentiated DC and lymphocytes are cast in a special mould and transferred into the culture compartment after gelling. The matrix is covered by an 80 μm polyamide sieve (Stockhausen, Germany) for mechanical stability and subsequently fixed and sealed by inserting the cap. The fluidic system is connected to the ports, and cell culture medium is pumped into the system (Giese et al., 2006). The cells are challenged with a defined amount of antigen after 2 h; optionally B cells are cycled, using a separate closed loop fluidic system. Medium perfuses the bioreactor system at a rate of 1 mL/day and fills the incorporated sample reservoir. During the culture time of 14–30 days, further antigen together with mDCs can be applied to the agarose discs via the ports.

Micro-organoids of selected runs were continuously exposed to dexamethasone (Sigma–Aldrich) by culture medium supplementation to demonstrate immunosuppressive effects.

By contrast the IG-Device^{TM} houses 12 individually perfused miniaturised culture compartments and is designed for multiparallel exposure of different drugs and drug concentrations to matrix-assisted co-cultures. The miniaturised design of the bioreactor enables microscopic analysis of micro-organoid formation *in situ*. The culture compartments are embedded in a 3 mm polycarbonate base plate in a separate cassette. Each culture compartment of 3 mm × 7 mm × 7 mm and a culture volume of approximately 150 μL is encompassed by two hollow fibre membranes (2 mm Micro PES Capillary Membrane, Membrana, Wuppertal, Germany) for the supply and conduction of cell culture media and test compounds. The first hollow fibre membrane is connected to the channels leading to a media inlet port and a venting port. The second hollow fibre membrane is connected to channels leading to a media outlet port and a venting port. The outlet port is connected via a conduct with a sample collection vial. The culture compartment is connected to a matrix port allowing liquid, gel-forming matrix to be added. The culture compartment and the channels for venting, media supply and probe sampling are covered with a 1 mm polycarbonate plate, with Luer ports for connection to tubing on the top, and a gas-permeable foil for the oxygenation of the cell-matrix culture (Lumox, Greiner Bio-One, Frickenhausen, Germany) at the bottom. The 3 mm base plate and the 1 mm cover plate are joined together with silicon adhesive. The 3 mm base plate and the gas-permeable foil are joined together with two-component liq-

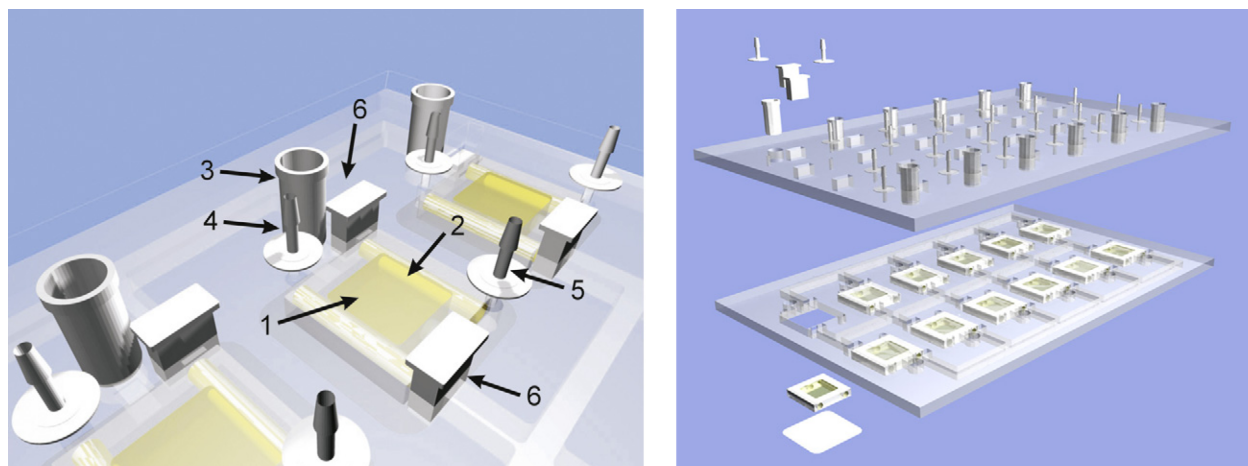

Fig. 3. Schematic drawing of the IG-Device^{TM} (detail, left; exploded drawing, right). 12 parallel culture units are realised on a base plate with microtitre plate format. Each individual culture compartment (1) is filled via the matrix port (4) with cell-matrix suspension. After this port is closed, the controlled gelling of the matrix can be induced by a temperature shift. The peripheral fluidic conducts are connected to the inlet (4) and outlet port (5). Cell culture media and test compounds are delivered to the culture chamber through hollow fibre membranes (2). The supplying and the conducting hollow fibre membranes are each filled by pumping cell culture media into the system while the ports for air outlet (6) are open. Using this protocol, perturbing air bubbles in the fluidic system are avoided. The air outlet ports are closed, after filling the system with cell culture media, and the incoming cell culture media can now perfuse the culture compartment homogeneously. Thereafter, the media pass through a second hollow fibre membrane which retains the cells and is drained via the port for probe sampling. The samples are collected in an individual probe sampling container.

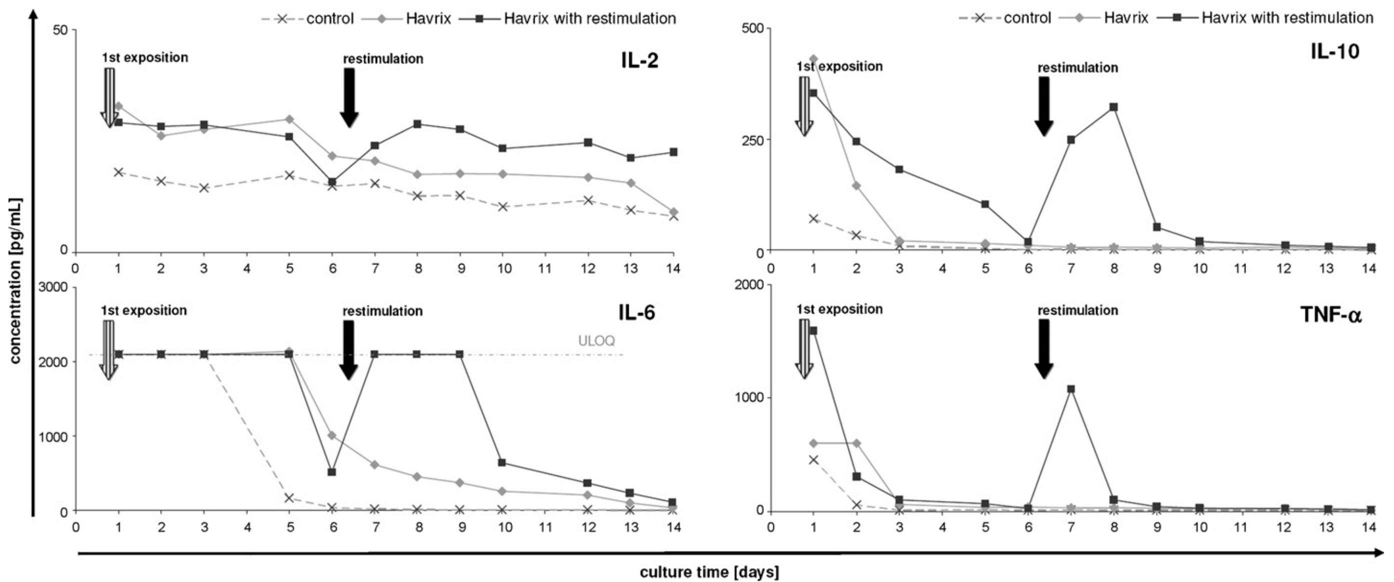

Fig. 4. Secretion profiles of three bioreactor runs operated with different donor materials and different stimulations. Daily samples of culture supernatant were analysed for a set of four cytokines by multiplex technology. The control run was carried out with TNF-α matured DC (without antigen exposure), whereas the other two runs were carried out with Havrix™ matured DC either without (◇) or with restimulation (□; as indicated with arrows). Prior to inoculation the cells are prepared in exactly the same way except from the maturation substance and are supplied with fresh medium so that the cytokine secretion results from the stimulation (selection of data of the first 14 days of culture).

uid polyurethane. 12 parallel culture units are realised on one base plate with outer dimensions corresponding to a multiwell plate (Fig. 3).

2.5. In situ imaging

The B cells and rest PBMCs were pooled after staining and mixed with the mDC preparation for inoculation. The bioreactor was inoculated with 1.5 E8 viable cells/mL (2.2 E7 viable cells/culture compartment) in RPMI1640 + 10% FCS in gelated agarose matrix (Giese et al., 2006). The culture compartments were perfused with RPMI1640 + 10% FCS at 13.1 μL/h using syringe pumps (Giese et al., 2006). After 7 days in culture, micro-organoid formation was explored using a LaVision TriM Scope™ 2-photon microscope (Goettingen, Germany). Images were generated using the Imaris™ software (version 6.2.1, Bitplane, Zurich, Switzerland).

2.6. In-process controls and end point analyses

Daily samples of culture supernatant harvested from the sample reservoir were subject to cytokine analysis (GM-CSF, IFN-γ, TNF-α, IL-4, IL-1β, IL-2, IL-5, IL-6, IL-8 and IL-10); analysed by a services provider using the flow cytometric bead array technology (Luminex, Invitrogen), as well as immunoglobulin (IgM and IgG) analysis, using standard ELISA methods.

After the completion of each bioreactor run, matrix sheets were removed from the bioreactor housing and screened to evaluate the nature and arrangement of *in vitro* formed tissues. For agarose sheets, paraformaldehyde fixation, ethanol dehydration, followed by paraffin embedding and sectioning (8 μm), as well as subsequent immunostaining, using ABC-method (avidin–biotin-complex and the chromogenic substrate Sigma Fast Red) and haematoxylin (Merck, Darmstadt, Germany) for counterstaining of the nuclei were applied for end point analysis.

3. Results

Bioreactors were consistently and repeatedly operated over period of 14–30 days each. Early T cell activation could be detected consistently by IL-2 responses. Long term viability and reactivity of lymphatic tissue was proven by IL-2, IL-4, IL-5, IL-6, IL-10, IL-1β, IFN-γ and TNF-α responses (Fig. 4, not all data shown). Restimulation on day 7 led to an increase of cytokine secretion.

The donor-to-donor variation could be observed on the basis of cytokine secretion. Bioreactor runs with Havrix™ priming and restimulations were subsequently operated with material from five different donors. The release of selective cytokines on the culture day before (day 6), and directly after first restimulation (day 7), was compared and showed the distinctive donor responses (Fig. 5).

In another set of bioreactor experiments, the immunosuppressive effect of dexamethasone was determined. A suppressive effect on cytokine secretion (pro-inflammatory and TH1 cytokines) compared to the Havrix™ bioreactor run could be determined (Fig. 6).

Beside the analysis of cytokine patterns, the immunohistochemical staining of the paraffin slides of the matrix sheets evaluates the generation of micro-organoid structures, cellular organisation and plasma cell formation. On the basis of various markers (especially the CD marker), a verification of cluster formation and proliferation was successful. B cell maturation up to plasma cell development, and even antigen-specific antibody expression could be demonstrated (Fig. 7).

The bioreactor IG-Device™ is designed for multi-parallel drug evaluation and *in situ* microscopy during the cultivation of preliminarily stained and antigen-primed lymphocyte subpopulations. Microscopic control directly after inoculation revealed homogeneously distributed cell in matrix-assisted culture. After 7 days in culture, organoids are formed, revealing close contact of lymphocyte subpopulations as well as B and T cell proliferation in cell clusters. Organoid formation was visualized using 2-photon microscopy (Fig. 8).

Focussing on profiling the antibody secretion (Fig. 9), Havrix™ treatment results in the early secretion of immunoglobuline (hIgM) into the supernatant. The restimulations maintain the IgM secretion over a period of 2–5 days, which leads to a delayed decrease. In contrast to the suppressive effect of dexamethasone on cytokine releases (see Fig. 5) the IgM secretion is enhanced (broken line).

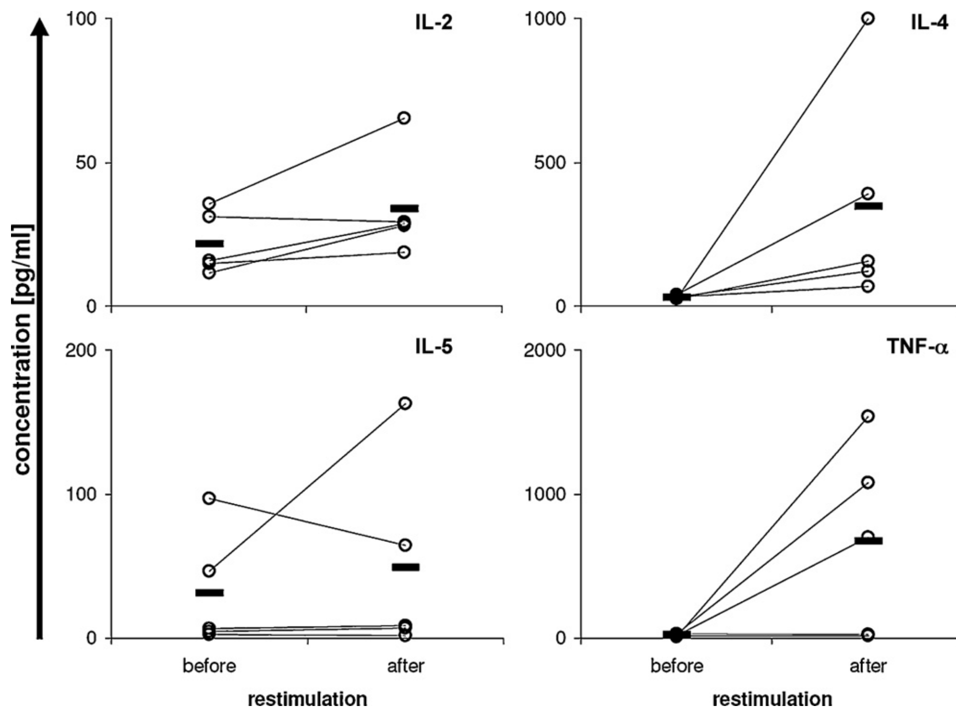

Fig. 5. Cytokine secretion (IL-2, IL-4, IL-5 and TNF-α) before and after restimulation within Havrix™ bioreactor runs of five different donors (○ single donor values; – arithmetic mean) demonstrates the donor-to-donor variation (selection of data of the first 14 days of culture).

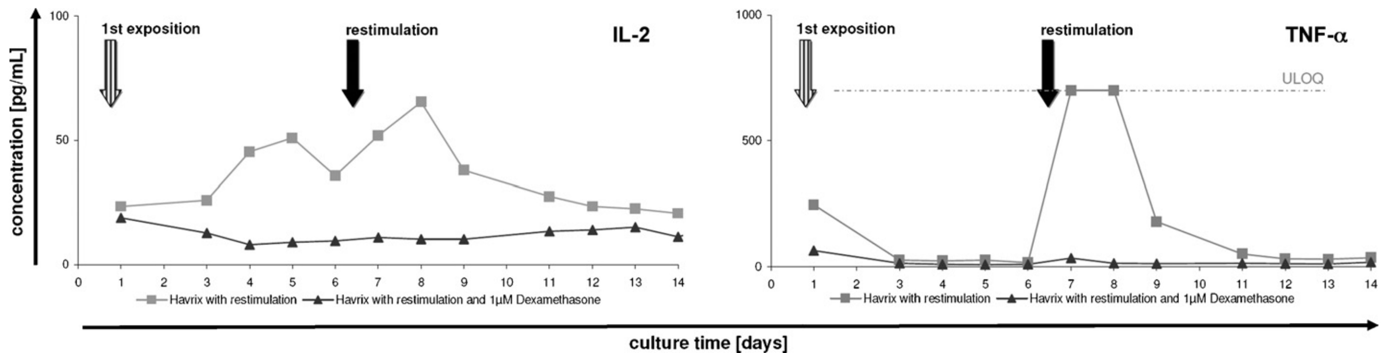

Fig. 6. Secretion profiles of two bioreactor runs, operated with identical donor material and Havrix™ stimulation; one run was supplemented with 1 µM dexamethasone, which suppresses the secretion IL-2 and TNF-α (selection of data of the first 14 days of culture).

4. Discussion

The model of the human artificial lymph node (HuALN) was used for testing vaccination *in vitro*. A commercial viral vaccine Hepatitis A (Havrix™) and a CMV virus preparation were used as antigens. The human donor material prepared for the co-culture was seronegative for HepA- or CMV-infection. Human immune responses to viral antigens in long term culture using antigen restimulation were investigated using the HIRIS™ III and IG-Device™ bioreactor platforms. Cytokine secretion profiles were monitored for the analysis of cellular and early humoral responses. Havrix™ induces a very early pro-inflammatory response (TNF-α) that decreases rapidly during the first days of culture.

Restimulation with antigen and loaded DC on day 6 induces a second release of TNF-α, but also of the TH2 promoting cytokines

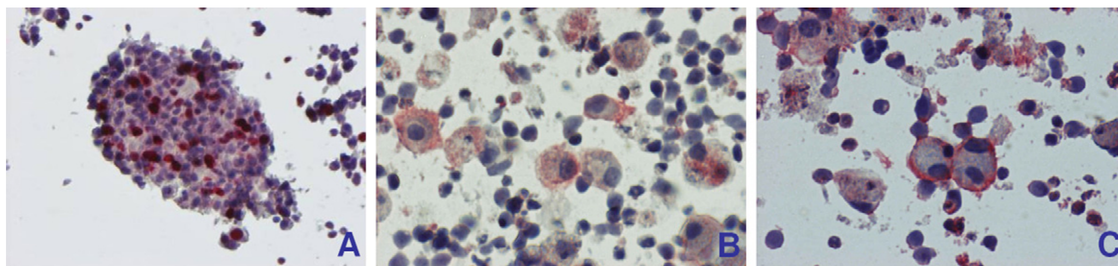

Fig. 7. Histological preparation and analysis of 3D matrix culture: organoid formation and proliferation during cultivation (A: Ki67, red), plasma cell differentiation (B: CD138, red), and antigen-specific binding on plasma cells (C: biotinylated antigen (CMV lysate), red); counterstained by haematoxylin (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

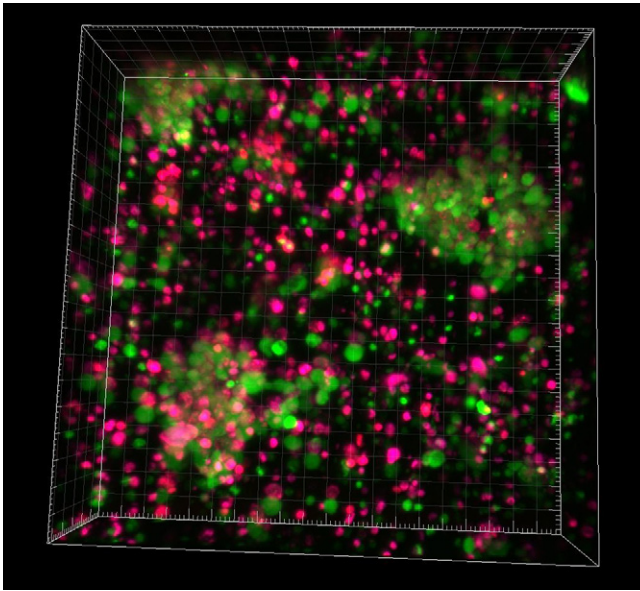

Fig. 8. Formation of micro-organoids. *In situ* 2-photon microscopic image of mDCs (CMPTX, red), B cells (CMFDA, green) and PBMCs (CMTMR, pink) in a matrix-assisted co-culture after 7 days in the constantly perfused bioreactor IG-Device^{TM}. The 3D reconstructed volume has a dimension of 200 μm × 200 μm × 54 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

IL-6 and IL-10. The release of IL-2, a typical mediator of cellular immune responses is very moderate.

There is a considerable variation in the level of cytokine release from different donors, but a reproducible dynamic behaviour and peak response which can be used for screening donor-to-donor variability.

The *de novo* formation of plasma cells for the CMV model is shown. Specificity of the plasma was demonstrated by antigen binding, using the most prominent antigen of CMV, the pp65 antigen. The formation of micro-organoid structures in both viral models is shown.

The immune suppressive drug dexamethasone reduces cytokine secretion within this *in vitro* model completely, especially pro-inflammatory and TH1 cytokines (Torres et al., 2005), but enhances the production of IgM antibodies as described for *in vivo* (Fleshner et al., 2001) as well as for *in vitro* (Sakuma et al., 2001). Therefore this HuALN model physically reflects the immunological effects of vaccines and virus preparations and immune-modulating substances.

2-Photon microscopy and histological endpoint analysis of the co-cultures revealed a highly self-organising model system, allowing for cell migration and antigen induced proliferation of B and T cells. We have monitored cytokine secretion profiles for the analysis of cellular and early humoral responses. Havrix^{TM} induces a very early pro-inflammatory response (TNF-α that drops down rapidly during the first days of culture). Restimulation with antigen and loaded DC on day 6 induces a second release of TNF-α again but also of TH2 promoting cytokines IL-6 and IL-10. The release of IL-2, a typical mediator of cellular immune responses is very moderate. There is a considerable variation in the level of cytokine release of different donors, but a reproducible dynamic behaviour and peak response that can be used for screening donor-to-donor variability.

For the CMV model the *de novo* formation of plasma cells is shown. Specificity of the plasma cells was demonstrated by antigen binding using the most prominent antigen of CMV, the pp65 antigen. In both viral models the formation of micro-organoid structures is shown.

The described HuALN model is the first 3D-organoid system of a human lymph node for *in vitro* testing of immune functions. Improvements for fully organ equivalency are mandatory. Based on the autologous concept of the co-culture model and the use of PBMC-based cell material only, the major and pivotal reactions of cellular and humoral immune responses could by induced and monitored. To improve the antigen-specific antibody responses of the model, e.g. increased. The antibody secretion performance seems to be adjustable by the optimisation of drug exposition and restimulation regime. For the induction of antibody class switch (e.g. IgM to IgG) and affinity maturation on the level of plasma cells the implementation of stromal cells is crucial. The stromal environment induces homing and segregation of lymphocytes, structural organisation and tissue formation of lymph node organoids and modulates the balance steady-state and inflammation. Human stromal cell can be prepared from human lymph node or bone marrow biopsy material. Alternatively, human fibroblasts or animal derived stromal cell lines are available. The use of allogeneic or xenogenic cell material may induce inflammation, cell killing or background noise. Intrinsic inflammation or background noise

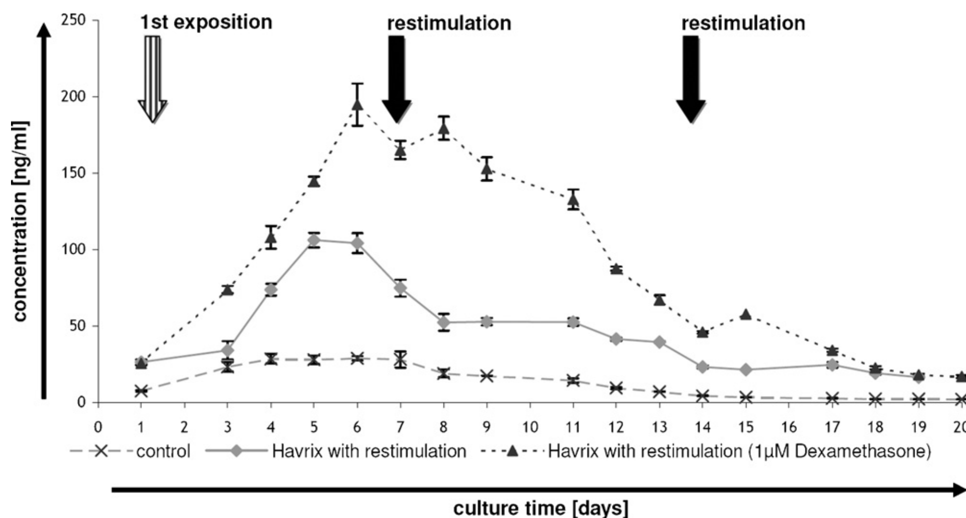

Fig. 9. Secretion of human IgM over a HuALN culture period of 20 days, including two restimulations. Exposition to results in increased IgM secretion compared to the control, whereas dexamethasone further enhances secretion (broken line) The control run was inoculated with TNF-α matured DC, without antigen exposure.

would be a problem for immunogenicity testing and may interfere with low and moderate immunogenic to-be-tested substances. Cell killing of non-self stromal cells could be managed by depletion of NK cells and cytotoxic T cells prior to co-culture, but depletion of cells may affect the PBMC pool.

The HuALN model will be improved in cell composition and tissue complexicity. The model and the experimental design need to be optimised for defined applications and standardised testing programmes.

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