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Immunology in a Petri dish
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Discovery of the dendritic cell – from in vitro observation to clinical application
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Exploiting the diverse capabilities of in vitro monocyte-derived dendritic cells in the development of immunomodulatory drugs
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A molecular description of dendritic cell activation, maturation and migration
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Immunotoxicology and safety assessment of human pharmaceuticals
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Detection of pro-inflammatory ion channel activity in human microglia, the brain macrophages
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Immunforsvaret under udvikling og behandling af kræft – immunterapi
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Mesenchymal Stromal Cells and Immunological Potency Assays
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FORSIDE:
Mature T helper cell (Th cell) expressing the surface protein CD 4.
www.pixabay.com
Living organisms are made up of billions of cells, thousands of genes, proteins and small molecules, which makes the individual microenvironments complex and challenging to replicate in vitro. Focusing on a smaller number of components of such complex systems in in vitro systems simplifies the system under study. This has the advantage, that the basic functions of a defined and selective number of components can be investigated as reduced forms of what happens in vivo.

Extrapolations from animal studies directly to humans are problematic, as it is well known that cellular mechanisms are often species specific. Consequently, using human cells directly in *in vitro* methods overcome this translation problem. For compound screening and basic biological mechanistic studies, *in vitro* experiments are optimal experimental set-ups as they allow for high-throughput screening and essentially unlimited repetitive rounds of experiments, which is not feasible using animal models.

The life science industry in general and the pharmaceutical industry in particular, are increasing their focus on refined *in vitro* systems to improve early drug discovery and increase the understanding of basic disease mechanisms. Concomitantly, basic research in the field of immunology is moving rapidly and our understanding of the complexity in this field has increased significantly in the last decade. This new knowledge has paved the way for using in vitro immune cell-based models as tools in both traditional early drug discovery, vaccine development and novel stem cell therapies. The immune system is a complex network of specialized cells and organs that work together to protect us from getting ill by recognizing and destroying foreign invaders such as viruses, bacteria, parasites and fungi. Once immune cells receive the alarm of “danger”, they become activated and begin to produce the necessary signaling molecules that in turn allow the cells to regulate their own growth and behavior, enlist other immune cells, and direct the new recruits to the trouble spot.

As we progressively learn more about the intricate regulation of the immune system, we also become better at inventing sophisticated *in vitro* model systems to analyze the interaction, function and regulation of human immune cells and utilize the knowledge to create better and safer treatment for immune related disease. The need for reliable predictive models, especially in the field of drugs and vaccines, to improve efficiency of go/no-go decisions early in development is critical and will have a significant effect on the costs and efficiency of developing medical treatments for patients.

However, a good researcher must always be aware of the limitations of the used *in vitro* system designed and avoid to draw wrong correlations to the *in vivo* situation. Care must be taken not to end up like the famous joke of the man looking for a lost coin during the night beneath a streetlight (Figure 1).

In the issue of BioZoom no. 1, 2016, scientists from both industry and university have described their specific use of human immunological *in vitro* models within both drug discovery, vaccine development and stem cell therapy, covering some of the central elements of merging basic immunology into applied in vitro model systems.

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**Figure 1.** In 1942 a version of the joke appeared in the popular syndicated comic strip “Mutt and Jeff”. Three of the five panels in the strip are shown below. The gentleman in the top hat is Jeff, and in the final omitted panel the officer joins Jeff in the search beneath the streetlight. Published 1942 June 3, Florence Morning News, Mutt and Jeff Comic Strip, Page 7. Florence, South Carolina, USA.
The identification of immune cells began more than 150 years ago. The discovery of the dendritic cell in 1973 was a major breakthrough, and initiated a new era in cellular immunology. In recent years an increasing number of novel leukocyte subsets with specialized functions in the immune system have been described. These cells are categorized based on morphology and basic cellular functions like in vitro adherence, phagocytosis, cytotoxicity and suppressive functions. Today novel techniques like cell sorting, multicolor flow-cytometry, and RNA sequencing provides the immunologist with unique tools to dissect the cellular immune system.

Cellular immunology was born with the discovery of the microscope, the definition of the cell theory by Matthias Schleiden, Theodor Schwann and Rudolf Virchow (1821-1902), and the observation that pus cells are a mixture of dead tissue, bacteria, and white blood cells. Importantly, at that time the observation that an increased number of leukocytes in the blood is linked to pathology in the organism resulted in an increased interest in understanding the role of different leukocyte subsets.

Later, the improvement of the microscope by William Addison confirmed these observations by the demonstration of the diapedesis of white blood cells from the blood vessels into the tissue. Concepts like myeloid vs lymphoid cells soon followed based on the description of nuclear size and morphology. However, it was not until the emerging bacteriology around 1900 that a more detailed understanding of leukocyte subsets and their functional role in the immune system was achieved, in particular based on direct observations of phagocytosis performed by cells such as macrophages and granulocytes (1).

Although not standing alone, such relatively simple observations are still part of the discovery of novel cell subsets in immunology. In 1973, the simple in vitro discrimination of the ability of cells to adhere to glass and plastic was used for the groundbreaking definition of the dendritic cell (DC). During studies of mouse spleen cells and their adherence to glass and plastic, Steinman and Cohn identified the DC as a “large stellate cell with distinct properties from mononuclear phagocytes and granulocytes” (2). Based on the mixed leukocyte reaction (MLR) assay that involve co-culture of cells with allogeneic responder cells, Ralph M. Steinman demonstrated that DCs are the most potent cells for antigen presentation to T cells.

The importance of these findings was recognized when the Nobel Prize in Physiology or Medicine 2011 was awarded, one half to Ralph M. Steinman “for his discovery of the dendritic cell and its role in adaptive immunity”, and the other half jointly to Bruce A. Beutler and Jules A. Hoffmann “for their discoveries concerning the activation of innate immunity”.

The DCs are key players in antigen recognition in the innate immune system and perform efficient antigen-up-take, processing, presentation on MHC molecules and priming, differentiation and imprinting of T cells. A large part of the important insight has been achieved in part through in vitro observations of DC and T cell co-cultures in MLR’s. At the time of DC discovery a link between activation of the innate immune system and the subsequent priming of cells of the adaptive immune system was still missing. MLR and other functional assays like phagocytosis assays, cellular cytotoxicity assays, migration assays, together with ELISPOT and limiting dilution assays for enumeration of antigen specific B and T cells allowed immunologists to study a spectrum of function in cellular immunology. They defined the capacity of novel leukocyte subsets in relation to functions including phagocytosis, NK and complement function and cytolytic activity, lymphocyte activation, cytokine secretion, chemokine dependent migration, and cytotoxic T cell (CTL) mediated cytolytic activity. However, an important modern strategy for immune cell discovery is the selection based on surface markers.

A classic example is the discovery of regulatory T cells with pivotal functions in immune homeostasis. They were identified based on surface expression of CD4+CD25 followed by sorting of cells and use in in-vitro co-culture with effector T cells to elucidate their suppressive function.

Improved methods for cell sorting combined with mass cytometry (CyTOF), have overcome the limitations of spectral overlap for conventional fluorophore-based flow cytometry. With this advantage it is still likely that we will discover rare and specialized leukocyte subsets in the years to come, and obtain an improved understanding of their function by applying whole genome RNA sequencing. With such discoveries we may leverage and fine tune the
understanding of the immune system, exemplified by the current intense research in innate lymphoid cells subsets. However, the traditional protocols for cell extraction of solid tissues, including lymphoid organs, are still creating limitations for studying immune cell diversity, since many cells are not extracted from lymphoid tissues, or they are stressed and destroyed by the extraction procedure. However, novel methods that combine immunohistochemistry with multiplexing of surface markers at the single cell level, have the potential of detailed cellular phenotyping, which could revolutionize our understanding of immune cells and their interactions in the immune system.

As for other immune cells present in low numbers in the blood, studies of DC immunobiology have been hindered by difficulties of obtaining sufficient numbers of cells. The in vitro protocols for the differentiation human peripheral blood monocytes cultured with GM-CSF and IL-4 into DCs were a major breakthrough in DC research in 1994 (3).

These culture methods have allowed for improved understanding of DC immunobiology including the immature vs mature stage for tolerance induction vs immune activation, the link between innate antigen receptor ligation, DC cytokine secretion, and the priming of the increasing number of T cell subtypes such as TH1, TH2, TH9, TH17 etc., with different functions in the immune response. This field has recently been expanded with protocols that allow in vitro monocyte differentiation into various DC types as well as M1 and M2 macrophage subtypes.

Indeed, the in vitro differentiation of DCs combined with co-cultures of syngeneic or allogeneic T cells in MLR assays has advanced the ability to screen pharmacological compounds for inflammatory vs anti-inflammatory capacity, and for the ability to influence T cell priming in different directions, like TH1, TH2, TH17 etc. Using novel high throughput screening technology based on activity assays that apply differential scanning fluorimetry, fluorescence, absorbance, or microfluidic capillary electrophoresis, it is now possible to design suitable cell based assays (including DC assays) that can be screened against chemical library collections of more than 15,000 compounds at one time.

An important outcome of in vitro DC culture methods is the intelligent vaccine development strategy based on in vivo DC targeting. Thus, novel adjuvants can be tested and screened for effect on DC immunobiology, and the antigen can be tested for ability to target relevant receptors for uptake together with its route of antigen processing and presentation on MHC molecules to T cells. In example, we have characterized C-type lectin receptors on human DCs, and analyzed the binding of glycan-conjugated vaccine antigens, the intracellular location of such antigen and the capacity for DC cross-presentation of the vaccine epitope to specific CD4 and CD8 T cell hybridomas (4).

The protocols for human monocyte derived DCs also encouraged immunologists to develop DC based cancer vaccines based on the dogma that DCs are the best activators of tumor-specific CTLs, and that therapeutic DCs can bypass the tumor-mediated suppression of in vivo DCs in the tumor microenvironment (5). Researchers have invested massively in developing in vitro methods and validation for optimization of DC surface molecules, and cytokine profile of DCs to be used in cancer vaccines.

This insight has been translated to clinical trials where a large number of DC parameters have been tested, including vaccine administration route,
DC maturity, antigen loading such as HLA class I binding peptides, tumor lysates and DC fused tumor cells. Most recently, this effort has resulted in a DC-based cancer immunotherapy against prostate cancer, Sipuleucel-T (Provenge) that is now approved by the FDA.

Based on the observation that immature in contrast to mature DCs may play a role in peripheral tolerance as opposed to induction of immunity, early translational clinical studies demonstrated that immature DCs pulsed with antigen resulted in removal of antigen specific IFN-gamma producing CD8+ T cells from the blood and replacement with immunosuppressive IL-10 secreting T cells. The possibility to in vitro manipulate DCs with the immunomodulatory cytokines TGF-beta and IL-10 to generate DCs with ability to induce T cell hypo-responsiveness to autoantigens was recently tested in a vaccine for patients with early onset diabetes. Thus, DCs are together with other immune cells being increasingly tested as cytoktherapies including DC-based immunotherapy, adoptive T cell therapy, and NK cell therapy.

In conclusion, simple in vitro observations combined with modern sophisticated methods for cell sorting and phenotyping have the power to discover novel immune cell types. These advances may have implications for basic immunology as well as for improved cellular immunology assays for use in the pharmaceutical drug development, and even for cell-based immunotherapy on its own.

Reference List

The quality of an adaptive immune response is not based solely on specificity, but just as much the context in which the specificity is induced. Dendritic cells (DCs) play a fundamental role in the immune system by linking the innate and adaptive immune responses while continuously sampling the surrounding environment through among others pattern-recognition-receptors, which recognize specific chemical signatures mostly associated with pathogens. By doing so, DCs are key regulators of the specific responses of the immune system under both homeostasis and pathological conditions. For these reasons, DCs are attractive targets for immunomodulating drugs in various immune and inflammatory disorders. The present article reviews the use of a platform which exploits in vitro generated dendritic cells from human donors for prediction of the in vivo effects of e.g. immunomodulatory drugs, vaccine adjuvants or probiotics.

In vitro monocyte-derived dendritic cells: a tool to mimic critical in vivo DC pathways

As an alternative to isolating dendritic cells, which are present in blood only in very low frequencies, in vitro generated monocyte-derived dendritic cells (MoDCs) are widely used. The reason for this is that MoDCs can be generated in high numbers with a great plasticity from blood monocytes. Despite the wide use of MoDC in in vitro mode-of-action studies and clinical cellular therapy as e.g. approaches in cancer treatment, coherent evidence of a direct translation to the in vivo situation is still missing, though commonly suspected. However, the presences of dendritic cells derived from monocytes have been confirmed in inflammatory conditions in vivo (1).

The standard protocol used to generate human MoDCs includes stimulation with GM-CSF and IL-4 in order to differentiate monocytes towards immature MoDC. During differentiation, different sets of cytokines and surface markers are induced as a reaction towards the presence of particular other environmental factors, which the dendritic cell senses through its cytokine- and pattern-recognition-receptors (PRRs) among others. As a consequence, the environmentally matured MoDCs critically determine the type of immune polarization of e.g. T helper (Th) cells, when brought in contact with these – in vivo this polarization takes place in the lymph nodes after having sampled the surroundings of the periphery.

At Bioneer, we have optimized combinations of cytokines, chemokines, PRR agonists and prostaglandins, which in the best combinations and concentrations induce specific phenotypes of MoDCs. These phenotypes – consequently – have the ability to induce specific Th cell phenotypes, which impact the immune responses (2). The optimized combinations have resulted in cocktails, which are applied in Bioneer’s human in vitro MoDC platform to induce specific polarization patterns – the character of the cocktails depending on the relevant pathway in question (Figure 1).

Dendritic and T helper cell phenotypes in autoimmune diseases, allergy and cancer

In certain immune disorders, the environmental priming of DCs in the body has skewed the DC phenotype towards an unfavorable response resulting in allergy, autoimmune disorders or cancer associated with a matching unfavorable Th cell response (3). Under normal conditions, the locally or systemically induced Th cell phenotypes are crucial for optimal fighting of infections, cancers or raising self-tolerances. However, in several diseases, the Th response is out of balance. In autoimmune diseases like rheumatoid arthritis or psoriasis, there is a skewed Th1- and Th17-response. In type I allergy, there is a Th2-skewed response, and in cancer diseases, the reponse is generally associated with an extensive upregulation of T regulatory (Treg) phenotypes, which induce tumor-tolerance.

These unfavorable immune responses – when developed – continuously reinforce the imbalance by further inducing the overproduction of the mentioned troublesome Th cell phenotypes. So far, various immunosuppressive and immunomodulating drugs have been developed – small molecules as well as biologics – in order to modulate the imbalanced immune response in al-
Figure 1. Bioneer’s human in vitro MoDC platform, which mimics disease conditions, can be used to predict how test substances such as potential drugs, probiotics or vaccine adjuvants can influence DC and T cell plasticity and counteract disease phenotypes in vivo. The DC phenotypes associated with e.g. psoriasis, rheumatoid arthritis, allergy and cancer are induced by the cocktail stimuli.

Allergy, autoimmune and transplantation diseases (e.g. anti-TNFα mAbs and Etanercept (TNFR2 ECD-Fc), Abatacept (CTLA4 ECD-Fc), Briakinumab (anti-IL-12 and 23 mAb), Omalizumab (anti-IgE mAb), corticosteroids, cyclosporines etc) (4). Recently, in cancer diseases, promising data are found when treating with a new class of biologics blocking checkpoint inhibitors (PD-L1, PD1, CTLA-4), thereby modulating and activating the immune system to circumvent Tregs and increasing the possibility of eradicating tumors.

Bioneer’s human in vitro MoDC and T cell platform

In Bioneer’s MoDC platform, the effect of potential drugs, probiotics or vaccine adjuvants can be measured on DC and T cell plasticity under mimicked disease conditions to predict how the test substances may counteract disease phenotypes in vivo (5). The non-cancer related pathways in the platform have been successfully validated by demonstrating that approved clinical drugs with known immunomodulating functions, such as dexamethasone, are able to suppress the cocktail-induced cytokine and surface molecule DC and T cell profiles associated with the disease pathway in question (2). The cancer related pathway is still under development and the blocking of checkpoint inhibitors (e.g. by Pembrolizumab, Nivolumab and Ipilimumab) are highly relevant in the present validation process to extend the platform.

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A MOLECULAR DESCRIPTION OF DENDRITIC CELL ACTIVATION, MATURATION AND MIGRATION

Revealing the power of innate responses to discriminate sensitizers from irritants.

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The innate immune response is often called for being ‘non-specific’. However, a lot of substance-specific information is lost when the ‘specific’ adaptive immune response pulls off. Innate responses provide an amazingly detailed picture of the characteristics of the challenging substance. A better understanding of how the early gene changes in keratinocytes, epithelial cells and dendritic cells contribute to the expression of maturation markers may help to understand and resolve the gaps that are currently existing in our understanding of the early events in sensitization. The presented results may have implications for the design of predictive in vitro tests for assessment of sensitization potency, and suggest that -omic approaches contain valuable mechanistic information associated with potency assessment of chemicals.

Introduction

Our understanding of the molecular mechanisms driving chemical skin and respiratory sensitization has reached a level that many consider sufficient for the development of advanced tools for testing and even risk assessment. However, recent genomics and proteomics-based studies targeting early innate events in epithelial cells (including keratinocytes) and dendritic cells have revealed mechanisms that might help filling out the gaps in our understanding of the relation between reactivity rate, mechanism of haptenation, protein target selection, pathway activation and T-cell skewing (1).

From dendritic cell ‘sensing’ to activation and maturation

It is generally accepted that activation of DCs results in prominent phenotypic and functional changes including enhanced levels of MHC class I and co-stimulatory molecules (e.g. cluster of differentiation (CD)54, CD80 and CD86, and receptors that are essential for migration) and antigen-presenting capacity. However, DCs have recently been shown to do a lot of sensing and tasting in order to match the right pathways for triggering these phenotypic and functional changes with the chemical challenge.

Preference of amino acid for haptenation. Extensive genomic analysis of monocyte-derived dendritic cells (MoDCs), human monocytic leukaemia cell line (THP-1) and MUTZ-3 cells exposed to skin sensitizers exerting cysteine and cysteine/lysine reactivity has identified genes describing the primary pathways of skin sensitization, i.e. signalling through transcription factors Nrf2 and aryl hydrocarbon receptor (AHR), and protein ubiquitination (2, 3). Lysine-reactive chemicals appeared to be less efficient (3). This difference in reactivity may explain the discrimination between small molecule skin and respiratory sensitizers.

Skin and respiratory sensitizers. Johansson et al. (2) published a list of 200 genes that with >95% accuracy (96% sensitivity; 95% specificity) describe skin, but not chemical respiratory, sensitizers (Fig. 1). The primary pathways involved in skin sensitization involved Nrf2, AHR, TLR and PKA signalling. In contrast, DC-based assays measuring phenotypic changes reveal 71-82% sensitivity, 70-75% specificity, and 71-84% accuracy.

Recently, Forreryd et al. (4) released a list of 389 differentially regulated genes for respiratory sensitizers. Several of these genes were involved in oxidative phosphorylation, ubiquinone metabolism and regulation of innate immune response signalling pathways leading to cell maturation, enhanced antigen presentation and interaction with other immune cells. Preliminary evidence is suggestion that a third gene signature is required to described allergenic proteins. Never the less, the overall key events of sensitization seems to be the same for the three groups of compounds (1).

The genomic data were backed by proteomic data. More than 200 proprietary skin and lung markers emerged from the EU funded FP6 project Sens-it-iv using the mass spectroscopy-based
proteomic biomarker discovery platform of Proteome Sciences (5). Specific assays were developed using its Tandem Mass Tagging technology combined with selected-reaction monitoring mass spec. SensiDerm™ applies a biomarker panel comprising ten proteins which were shown to be differentially expressed in MUTZ-3 cells in response to sensitisers compared to non-sensitisers.

**Chemical reactivity mechanism and rate.** By stratifying the sensitizing chemicals into chemical reactivity groups, a number of canonical pathways known to be involved in the biology of sensitization were confirmed, while novel pathways were identified (6). Sensitizers with different reactivity mechanisms were further shown to engage different pathways, indicating that the biological end-point of T-cell priming is achieved through different chemical-specific upstream mechanisms (Fig. 2A).

**Sensitizing potency.** Assessing sensitizing potency on the basis of the now generally accepted key events for sensitization, remains a challenge. However, 200 genes published by Johansson et al. (2) were found to correlate well with human potency of the tested substances (Fig. 2B). The general trend is that both metabolic and cell cycle associated pathways are engaged gradually, and in correlation with potency (6). In addition, to the genetic differentiation between substances of different potency, there is evidence suggesting that the observed effects are dose dependent. The dose required to observe cellular effects in

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**Figure 1:** 200 genes accurately (>95%) describe skin sensitizers. A. Principle Component Analysis (PCA) of the gene modifications triggered by 90 chemical substances. B. The stimulation index derived after application of the Genomic Allergen Rapide Detection (GARD) algorithm.

**Figure 2:** Chemical reactivity mechanisms and potency relate to quantitative and qualitative differences in gene responses. A. Genes affected by the different chemical reactivity mechanisms. B. The stimulation index derived after application of the Genomic Allergen Rapide Detection (GARD) algorithm using chemicals of different potency.
the GARD are inversely correlating with
the in vivo potency of the compound.

Dendritic cell migration: Where the
substance-specific information seems to
disappear.

Fibroblasts play a key role both as
advisors helping the KCs and Langer-
hans cells (LCs) to discriminate irritants
from sensitizers, which in many cases
are irritants themselves, and as guides
helping the LCs out of the epidermis
into the dermis and further towards
lymphatic vessels. Using a full-thickness
tissue-engineered skin model contain-
ing fully functional MUTZ-3-derived
LCs (MUTZ-LC), the MUTZ-LCs were
demonstrated to mature and to acquire
the ability to migrate towards C-X-C mo-
tif ligand (CXCL)12 and C-C motif ligand
(CCL)19/21 in a comparable manner
with primary LCs in skin explants. The
acquired knowledge has resulted in a
DC-migration assay which is based on
carboxyfluorescein succinimidyl ester
(CFSE)-labelled MUTZ-3 cells. The
discriminating feature of the assay is
that irritant induced migration is CCL5
dependent, while small compound skin
and respiratory sensitizers induced mi-
gration is CXCL12 dependent (7).

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IMMUNOTOXICOLOGY AND
SAFETY ASSESSMENT OF HUMAN
PHARMACEUTICALS

In vitro assays for pre-clinical safety assessment of mAbs

Pre-clinical toxicological safety testing of novel human pharmaceuticals is
a prerequisite before clinical testing. The objective is to identify target or-
gans and reversibility of toxicity in order to determine a safe starting dose
for clinical trials and identify parameters for safety monitoring in humans.
This article introduces in vitro immunotoxicological assays that can be
used when assessing pre-clinical safety of monoclonal antibodies (mAbs),
including methods for prediction of immunogenicity, cytokine release
and effector functions related to the biological properties of the antibody.

Immunotoxicology

Immunotoxicology is the specialized
area of toxicology that addresses poten-
tial adverse effects of xenobiotics on the
immune system. Inappropriate immune
activation can lead to hypersensitivity
reactions and autoimmunity, whereas
inappropriate inhibition may lead to in-
creased susceptibility to infections and
risk of cancer (Figure 1).
Immunotoxicology is the adverse or inappropriate change in structure or function of the immune system after exposure to a xenobiotic. The effect can manifest as inappropriate immune activation or inhibition and range from mild to severe effects (1).

**Predicting cytokine storms**

Cytokine release syndrome is an adverse clinical event associated with release of pro-inflammatory cytokines from immune cells. After the clinical trial in London where the super-agonistic mAb TGN1412 induced a near-fatal release of cytokines in healthy volunteers despite inconspicuous preclinical testing, immense efforts have been made to improve the sensitivity of cytokine release assays. One successful improvement has been to pre-incubate blood immune cells at high density prior to drug testing in order to mimic the conditions leading to the lower activation threshold that tissue resident T cells have compared to blood T cells. Another successful improvement is the immobilization of the mAbs to wells using wet or dry-coating techniques to mimic mAb cross-binding or addition of accessory cells instead of testing in aqueous phase. Both changes greatly increases assay sensitivity and successfully predicts the cytokine storm seen with TGN1412 (3,4).

**Detecting Fc related effector functions and immunotoxicity of mAbs**

Certain antibodies can activate effector functions through the Fc part (the non-antigen binding part) of the antibody and induce antibody-dependent processes whereby the Ab-opsonized cells are targeted for lysis or phagocytosis by immune cells or the complement system. While these effector functions are exploited for mAbs targeting cancers, they are an undesired effect when targeting inflammatory disorders.

**In vitro** assays using primary human immune cells and serum can be used to test and select the desired mAb Fc profile for best safety of lead candidates. These include macrophage phagocytosis assays and cell-based cytotoxicity assays for detection of target cell lysis (5). Fc related mechanisms that lead to alterations in immune cell composition can be identified using flow cytometry by looking at absolute counts and percentages of different leukocyte subsets of whole blood after exposure to mAb.

**Designing the safety study**

Although guidelines for pre-clinical safety assessment of human pharmaceuticals are published, pre-clinical safety studies for mAbs must be designed on a case to case basis founded on the immunopharmacology. This requires an in-depth understanding of the target expression and mode of action, and a full functional characterization of the biological properties of the mAb in order to assess the immunotoxicological potential and ensure minimal risk for the first-in-human clinical studies.

**References**

DETECTION OF PRO-INFLAMMATORY ION CHANNEL ACTIVITY IN HUMAN MICROGlia, THE BRAIN MACROPHAGES

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A research group at Saniona A/S has established a method for studying functional ion channel expression and activity in human microglia, the macrophages of the brain, isolated from epilepsy patients. Various ion channels on microglia cells are thought to promote or dampen inflammation in the brain, and inhibiting or facilitating these ion channels by pharmacological means could serve as a novel handle to treat inflammatory diseases. Through an extensive collaboration between Saniona A/S, the Neurobiology Research Unit at the Copenhagen University Hospital (Rigshospitalet) and the EU consortium INMiND (HEALTH-F2-2011-278850), we have been able to study the functional expression of several ion channels in microglia derived from adult human CNS tissue. The new findings that these channels are in fact functionally expressed in human microglia is of crucial importance in order to enhance successful translation of previous findings in animal experiments to humans.

Microglia are the macrophages of the central nervous system

Microglia are the macrophages of the central nervous system (CNS), i.e. the brain and the spinal cord. They share a similar heritage to peripheral macrophages, however, during embryonic development the microglia migrate into the CNS where they remain after the blood-brain-barrier has sealed itself, and where the microglia subsequently survive through local self-proliferation. Microglia carry out many of the same functions as the peripheral macrophages do. They scaveng the CNS tissue to look for potential invading pathogens and/or damaged tissue. They can respond to pro-inflammatory stimuli and become activated, a process that is characterised by migration towards the stimuli, proliferation, secretion of pro-inflammatory molecules and phagocytosis of pathogens, cell debris and dead cells. They are also thought to play a role in facilitating the generation of neurons from progenitor cells in the brain. In other words, microglia cells are vital in maintaining homeostasis in the CNS.

Despite these many important functions, during pathology, microglia can easily become over-activated and harmful in their nature by the abnormal changes that occur in the brain. Plaque formation and excess levels of the peptide amyloid-beta in Alzheimer’s disease, a massive neuronal cell death in traumatic injuries (e.g. traumatic brain injury and stroke) and T cell infiltration in multiple sclerosis are all examples of pathological features in the CNS that trigger microglia to become over-activated (1,2).

Finding a treatment for inflammation in the brain

Activated microglia can greatly damage the healthy neuronal tissue. Since the CNS cannot easily regenerate itself, these harmful aspects of the microglia cells are usually detrimental and irreversible. Existing anti-inflammatory treatments (e.g. glucocorticoids) can reduce the activation profile of microglia but have both severe side effects as well as a limited treatment time span. However, finding an anti-inflammatory and neuroprotective treatment that does not undermine the natural healing ability of microglia and that does not prevent the microglia from their ability to detect and remove pathogens whilst at the same time dampening the harmful aspect of microglia over-activation remains a challenge.

An example of a putatively important ion channel target is the calcium-activated potassium channel, KCa3.1 (encoded by the KCNN4 gene), which is an ion channel present on rodent immune cells such as macrophages, T cells, B cells, neutrophils, mast cells and dendritic cells in the periphery and microglia in the CNS(1,3). It is thought to play an inflammatory role in these cells, by analogy to T cells, by facilitating an increase of intracellular Ca2+ levels, a typical sign and mediator of activation in these cells (Figure 1). In addition, KCa3.1 seems to participate in pro-inflammatory activities of rodent microglia such as the production of reactive oxygen species and other pro-inflammatory molecules, factors that are directly implicated in neurotoxicity. Many of these pro-inflammatory events can be directly prevented in both in vitro and in vivo models of neuroinflammation by blocking the KCa3.1 channel with e.g. the selective small molecule TRAM-34 (4). Most importantly, blocking KCa3.1 does not result in an impaired clearance of viral
particles suggesting that using $\text{K}_{\text{Ca}3.1}$ as an anti-inflammatory target could be safer than current treatment options (5).

Human microglia in a Petri dish

Although we know quite a lot about the role of ion channels in rodent microglia, very little is known about their presence and role in human microglia. Studying human microglia has been greatly hampered by the fact that it is very difficult to get access to human CNS tissue. There are three potential sources of human microglia that have been previously used in the literature; microglia isolated from 1) foetal tissue, 2) postmortem tissue and 3) microglia isolated from epilepsy patients undergoing surgical treatment or from brain cancer patients. There is no doubt that all three methods have both pro et contra aspects to take into consideration but the choice is in the end usually driven by availability.

The CNS tissue used for our study came from the supposedly non-pathological cortex of epilepsy patients. In order for the surgeon to gain better access to the hippocampus (located closer to the middle of the brain) which is the pathological area in this group of epilepsy patients, a small part of the cortex (the outer layer of the neuronal tissue) has to be surgically removed. The cortex in these patients is considered healthy, consequently the microglia in this tissue should, in theory, be in a resting state. However, one has to keep in mind that it does come from patients that have severe epilepsy and most likely have received various different types of medication prior to surgery. All these things could ultimately affect both the neurons and the microglia in the tissue, which has to be taken into consideration when interpreting the results from the experiments using this method.

We isolate and generate pure human microglia cultures from the freshly dissected CNS tissue (>92% microglia cells). This is done through both enzymatic and mechanical homogenisation steps where bonds between tissue and cells are being dissolved. A cell suspension of dissociated microglia is subsequently plated in small petri dishes for electrophysiological recordings. The cell culture purity can be determined by immunofluorescent staining (Figure 2).

In culture, human microglia cells would typically display a large cell body with a few long processes of various lengths

Figure 1. Schematic illustration of the hypothesised mechanism of microglia activation. Ligand binding to pro-inflammatory receptors e.g. certain G-protein coupled receptors (GPCR) and toll-like-receptors (TLR) activates a cascade of intracellular signalling pathways ultimately leading to the release of $\text{Ca}^{2+}$ from the endoplasmic reticulum (ER) through the inositol triphosphate receptor (IP$_3$R). Reduced $\text{Ca}^{2+}$ levels in the ER initiates STIM1/2 to couple with ORAI1 allowing for a direct flow of $\text{Ca}^{2+}$ into the cell. Elevated intracellular levels of free $\text{Ca}^{2+}$ ([Ca$^{2+}$]) activates $\text{K}_{\text{Ca}3.1}$ to mediate K$^+$ flow out of the cell, thereby causing a hyperpolarization of the microglia membrane and an acceleration of the $\text{Ca}^{2+}$-influx which facilitate the activation process further. Increases in [Ca$^{2+}$], additionally triggers downstream signalling pathways including the enzyme iNOS and the transcription factor NFkappaB ultimately leading to the generation and release of pro-inflammatory molecules (cytokines) and nitric oxide (NO). Local net K$^+$ loss via $\text{K}_{\text{Ca}3.1}$, various chloride channels, and aquaporins, are also important for microglia migration (not illustrated). The photomicrograph below the illustration shows a real microglia cell in a petri dish. The photograph has been acquired from Figure 2.

Figure 2. Human microglia cells in vitro 6 days after they were isolated. The cells have been antibody-labelled with a red fluorescent dye and counterstained with a blue nuclear dye. The arrow indicates the microglia cell that can be seen at higher magnification in Figure 1.
stretching away from the cell body. Upon stimulation of these cells with the pro-inflammatory bacterial molecule lipopolysaccharide (LPS) we saw a strong pro-inflammatory cytokine response generated by these cells. There was no response in the cells that had not received LPS indicating that the cells were not already activated before the experiment started.

To test whether human microglia express specific ion channels we use an electrophysiological approach. Using this technique we can measure the electrical current across the plasma membrane that is generated by a flow of ions through a specific ion channel protein. By attaching a glass pipette to the cell membrane (Figure 3) and thereafter creating an approximately 1 µm hole in the membrane by applying gentle suction, the total amount of current across the microglia membrane can be measured (whole-cell current) in that particular cell. For isolation of the current components through specific ion channels we employ a variety of strategies, such as using special salt solutions on the extracellular and intracellular side of the membrane, specific activation protocols aimed at eliciting currents through specific channel types only (e.g. steps in membrane potentials for voltage-dependent ion channels, extracellular application of neurotransmitters for ligand activated ion channels, or intracellular application of Ca²⁺ for stimulation of Ca²⁺-activated ion channels), as well as pharmacological tool compounds developed for high selectivity for certain ion channels.

**Human vs. rodent microglia ion channel recordings are in for surprises**

An important conclusion of our studies is that some ion channel types identified are the “expected ones” as compared to rodent microglia, whereas others found are usually not considered to play a significant role in rodents. Another very important conclusion is that some channels (such as the previously mentioned KCa3.1) are expressed in much higher densities on the cell membrane of our adult human microglia preparation than on rodent microglia even after these have been stimulated with various pro- or anti-inflammatory agents, thus hinting to an accentuated physiological role for human microglia in situ. Even though further studies are needed in order to find out whether the observed differences are rodent/human-, young/old-, or health/disease-based variations, the results hitherto obtained clearly point to the need for much more human microglia-based information on expression and function, preferably from neuroinflammatory diseases, in order to validate specific ion channels for therapeutic use. We think that the established procedure for microglia isolation and ion channel recording represents a good foundation for such studies.

**References**

Immunsystegets samspil med kæftceller påvirker deres udvikling og udnyttes i dag til immunterapi.

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Immunterapeutisk behandling af metastatisk kæft har i de senere år haft sit helt store gennembrud i udlandet såvel som i Danmark. Robert Schreibers hypotese, om at immunforsvaret både overvåger og redigerer den ukontrollerede vækst af kæftceller, giver et indblik i den patologiske proces som immunterapeutisk behandling påvirker. På Center for Cancer Immunterapi ved Herlev Hospital arbejder vi med at forstå immunforsvarets rolle under behandling af kæft. Vi ved, at det har stor prognostisk betydning om en tumor er infiltreret af immunforsvarets T-celler og fra flere typer af kæft kan vi in vitro eksandere tumor-infiltrerende T-celler isoleret fra tumor-vævsprøver. I kliniske studier har vi foreløbig set ligeså gode resultater, som større studier har vist i USA, og vores håb er derfor at få behandlingen godkendt i Danmark.


Evidens for immunforsvarets påvirkning af kæftudvikling

I 1909 beskrev den tyske læge Paul Erhlich, hvordan immunforsvaret nødvendigvis måtte være en afgjørende faktor for at undgå udvikling af kæft i langtidslevende organer. Det var dog først ca. 50 år senere, at man kunne beskrive tumor-antigener i eksperimentelt udviklede kæftmodeller i mus. Omtrent samtidig formulerede Macfarlane Burnet og Lewis Thomas deres hypotese om, at det tilpassede (adaptive) immunforsvar genkender og eliminerer kæftceller (Cancer immunosurveillance). I 1990erne blev bedre musmodeller transmitteret udviklet og det blev påvist at såkaldte RAG2-/- mus uden IFN- receptor eller dens transkriptionsfaktor STAT1 var i større risiko for at udvikle spontan eller carcinogen-induceret kæft.

I 2001 formulerede Robert Schreiber en hypotese om at immunforsvaret ikke kun overvåger men også redigerer den ukontrollerede vækst af kæftceller (The cancer immunoediting concept, Figur 1). I et afgørende forsøg blev tumorer udviklet i immunkompetente versus -deficiente mus (vildtype vs RAG2-/- mus). Herefter blev tumorerne transplanteret på syngene mus (genetisk identisk) med et normalt tilpasset immunforsvar (vildtype). Der sås en spontan afvisning af næsten halvdelen af alle RAG2-/- tumorer mens ingen vildtype-tumorer blev afvist (2). I tidligere studier med mere klassiske modeller for immunkompromit gik vi sig i lignende opdagelser (3). Nøgne og SCID (Severe combined immunodeficiency) mus mangler henholdsvis thymus eller dele af det adaptive immunsystem, og det giver anled-
ning til kun delvis immunitet medieret via T-celler. I overensstemmelse med Dr. Schreibers teori, kunne vi i disse modeller observere, at mere end dobbelt så mange tumorer fra immunkompromitterede mus blev afvist i forhold til tumorer fra immunkompetente mus efter transplantation. Resultaterne ansåker det tilpassede immunforsvarevne til at redegøre (edit) tumoren i en selektionsproces som tumorer fra RAG2−/−, SCID eller nøgne mus ikke eller i mindre grad udsættes for.

Konceptuelt kan samspillet mellem tumor og immunforsvar inddeles i tre faser. I eliminationsfasen (1. fase) er både det medfødte og det tilpassede immunforsvar i stand til at overvåge og eliminere transformerede celler, inden de giver anledning til klinisk manifeste kraftekündere. Varianter af transformerede celler vil dog overgå til en ligevægtstilstand (2. fase) med immunforsvar der overvåger og forhindrer ucontrollerede vækst, men samtidig selekterer de mindre immunogene varianter af kræftcelle. Som påvirkning af denne udsættelse for et immunologisk selektionspres og redigeres herved til at blive mindre immunogene, og undslipper immunologisk kontrol i flugtfasen, karakteriseret ved et immunosupressivt miljø og klinisk manifesterede kraftek (2).


Immunterapi med checkpoint inhibitorer

I relation til klinisk behandling af metastaseret kraftek er den mest interessante mekanisme til immun-flugt opregulering af inhibitoriske receptorer på T-celler, de såkaldte checkpoint-inhibitorer henunder CTLA4 og PD-1. Antistof-baserede behandlinger rettet mod disse receptorer har vist sig at være meget effektive og i det nyeste kombinations-studie med anti-CTLA4 og anti-PD1 i malignt melanom ses en responsrate på op til 60%.
% (4). Langtidsoverlevelsen for patienter med malignt melanom efter behandling med anti-CTLA4 er estimeret til ca. 21% estimeret ud fra en puljet analyse af næsten 5000 patienter hvilket er bemærkelserigt for denne patientgruppe.

Immunterapi med patientens egne celler efter *in vitro* ekspanzion

På Center for Cancer ImmunTerapi (CCIT) ved Herlev Hospital arbejdes der med at forstå immunforsvarets rolle under behandling af kræft. Vi ved, at det har stor prognostisk betydning om en tumor er infiltreret af T-celler (1), og igennem flere år har vi arbejdet med ekspanzion af tumor-infiltrerende T-celler isoleret fra tumor-vævsprøver. Processen består af flere trin i laboratoriets forløb (Figur 2). Når en tumor modtages fra operationssuiten skærres den ud i små fragmenter, som dyrkes i høj dosis af støtte-celler med krydsbundet antistof mod T-celler (anti-CD3) samt IL-2. Patienten er forinden infusion af TIL blevet behandlet med lymfo-ablativ kemoterapi og gives efterfølgende høj dosis IL-2 (Oversigt over TIL behandling er udarbejdet af læge og Ph.D. studerende Magnus Pedersen, CCIT).


Referencer

Cellular therapy has received increasingly more attention the past decade. This is due to the possibility of providing treatment for diseases rather than addressing the symptoms of a disease, which is what most medicine is currently doing. Mesenchymal stromal cells (MSCs) are "supportive cells" located next to the blood vessels throughout the tissues in the body. They are unspecialised cells characterised by a number of surface proteins, their plastic adherence, and their ability to divide asymmetrically. Asymmetrical cell division is a cellular division resulting in one cell similar to the parent cell, and one cell which is more differentiated towards a certain tissue lineage.

It is proposed that the main purpose of the MSCs is to function as supportive cells for other tissues in case of injury. Following injury, the MSCs enter the bloodstream and home towards the affected area. If you think of the MSCs as micro-engineers, you are not far from the current opinion in the field. Practicing their function, the MSCs appear in the affected area, sense what is wrong, resolves what is possible, and disappears from the area again when their job is done.

The ability of the MSCs to adapt to their surroundings has been extensively studied in vitro. As an example, when the cells are exposed to lowered oxygen tension they respond by secreting proteins responsible for the sprouting of new blood vessels. A similar specific response is seen when the MSCs are faced with apoptotic cells or an inflammatory environment. The fact that the MSCs can adjust and repair in this manner makes the MSCs a very interesting candidate for the treatment of a great variety of diseases.

Cellular therapies using MSCs have proven beneficial for patients who were otherwise beyond medical treatment, such as patients with severe heart disease or graft versus host disease following transplants. At Cardiology Stem Cell Centre, patients with severe heart disease are treated with cell therapy. In a placebo controlled double blinded randomized trial, an increase in pump function and decreased scar area was found using MSC therapy (1,2). This cannot be achieved by medical treatment, emphasising the promise of cellular therapy. For the first clinical studies, the patients’ own cells were extracted, cultured, and administered into the heart muscle. This requires a lot of resources, and is far from feasible. Therefore, in order to increase the number of patients to be treated, treatment with a standardised cell product from a cell bank is the next step. To make this possible, MSCs will have to be isolated from a donor for treatment of one or more other recipients; in other words, a shift from autologous to allogeneic transplantation is needed.

Allogeneic cell therapy is only possible because MSCs are generally perceived to be immunoevasive. This means that the MSCs do not express the proteins of "the usual suspects", whereas an array of active immunosuppressive paracrine mechanisms can be activated. Hence, they are able to evade recognition by the immune cells of the recipients. Despite the growing attention and clinical evidence much remains unknown about exact mechanisms of action behind allogeneic use and immunosuppressive capabilities of MSCs. Some can be attributed to expression of membrane receptors and direct cell-to-cell contact. Contact serves as a mean for transmission of signals, and brings MSCs in to close contact with immune cells, which potentiates the effect of secreted soluble factors. Several adhesion molecules have been described as being important for the immunosuppressive potential of MSCs, while a low expression of major histocompatibility complex and co-stimulatory molecules render the initiation of an immune response unlikely.
Aside from the contact-associated attributes, several secreted factors have been identified as central to modulation of different parts of the immune system. For instance, some factors target innate immune cells, such as macrophages and NK cells, while others preferentially affects the adaptive branch, e.g. T cells or B cells; however, most factors has some influence on both (3).

A key cell type for conveying information from the innate to the adaptive immune system is the dendritic cell, the most potent antigen-presenting cell. This function makes the dendritic cell pivotal for cell-based therapies and as such, efforts have been made to generate dendritic cell assays in vitro.

Circulating monocytes can be isolated from the blood and differentiated into a mature phenotype. Upon activation, these cells express a number of maturation markers and cytokines required to stimulate leukocytes. Analysis of markers and cytokines provides quantifiable data, and the addition of MSC in a co-culture setup can be used to document the immunosuppressive capabilities of these (4).

In supplement to specific mechanisms of action from the co-cultures, the dendritic cells can be transferred to purified T cells where the resulting proliferation offers functional insights to MSC immunomodulation.

A less refined, yet more multifaceted setup utilizes peripheral blood mononuclear cells (PBMCs) to model a mixed leukocyte reaction. Based on the high alloreactivity of this heterogeneous population, PBMCs from one donor can be challenged by those of another. Measuring the proliferation of leukocytes in general and subtypes in particular provides valuable information of how MSCs can affect even such complex interplays and skewer the “distribution” of cell types towards more regulatory phenotypes (4).

From the more elaborate models, it is becoming more evident that inflammatory signals affect MSC, and that the MSC respond in return. This opens for intriguing possibilities to pre-condition MSCs to heighten their potential as active immunosuppressors rather than a one-fits-all or ‘passive’ treatment.

At Cardiology Stem Cell Centre, the primary aim of MSC treatment is to regenerate damaged myocardium. Given inflammation is part of normal wound healing and due to the allogeneic approach, elucidation and documentation of regenerative and immunological modes of action of the cell product are essential.

Expansion of cells for clinical use is classified as production of a medicinal product, and as such is bound to comply with rules for pharmaceutical manufacturing as issued by the European Medicines Agency. Critical elements of pharmaceutical production are to characterize the product, identify its biological function and assure quality, reproducibility, and stability during production and storage. At this point above mentioned in vitro assays will come in...
handy, not just for the sake of scientific curiosity and basic research, but as necessary tools during production.

Regulatory authorities use the term “potency”. It essentially means that you should identify which biological function of your cell product is relevant for its clinical purpose and be able to test this function in a quantitative and reproducible manner. This is an extraordinary challenging task working with cell products because cells use multiple and complex mechanisms of action and respond to their environment in equally complex manners. As such, a single assay will never suffice (5).

Identifying immunosuppressive activity of a cell product, whether it is for the sake of allogeneic use or active immunosuppression per se, means that you need to have a thorough understanding of not just your own product, but also disease mechanisms and the inflammatory environment in which the product is meant to make a difference. The use of PBMCs is a useful tool for screening, but establishing assays with purified effector cells important for the particular disease in mention are necessary supplements. First encounter of allogeneic MSCs in a chronic ischemic myocardial environment will activate innate immunity. As such, mentioned dendritic cell assays are meaningful supplements to PBMC screening for the sake of ischemic heart disease.

The goal of a potency assay is to predict clinical efficiency and it should be used as a tool during production to assure that all batches released are equally effective. Predicting immunosuppressive activity of every batch of a cell product means that potency assays should be performed at multiple steps during production, including screening of donor material, in-process controls, and final release. To assure consistency of measures, traditional in vitro assays need translation into standard analytical methods. This means that assays must be feasible, robust, and fully validated – not to mention low-cost. A full validation includes identification of accuracy, precision, specificity, linearity, range and suitability. As such, once you have documented mechanisms of action during pre-clinical work, you most likely still have a lot of quality work ahead of you. As clinical stem cell research disseminates, the regulatory requirements for production of cell products will increase, and there is no doubt that the need for analytical assays for quality control and quality assurance will grow with it.

References

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