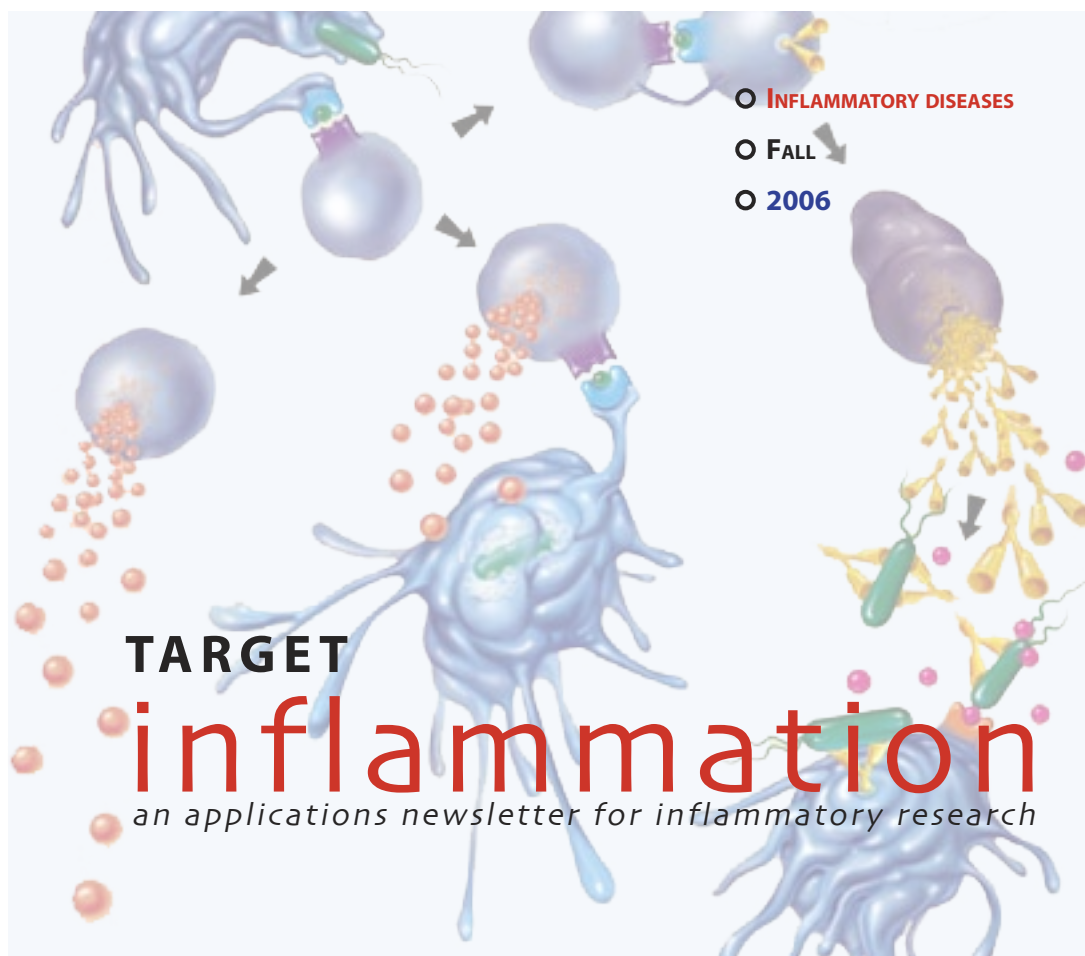


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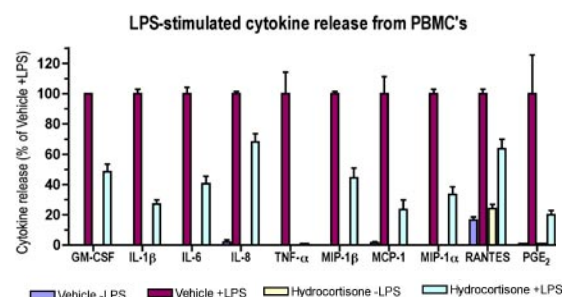
Increasing the therapeutic value of drugs. Applications in inflammatory diseases for proven anti-cancer agents

It is becoming increasingly clear that the link between inflammation and cancer is strong and that the interplay between the immune system and the development and remission of cancer is critical (Nature Reviews Cancer 2006 Jan;6(1):24-37). This development has encouraged increasing examination of the possible cross target application of anti-cancer and anti-inflammatory agents. The archetypical example of this is Methotrexate which was first developed as an anti-cancer agent but now forms the therapeutic mainstay of treatment for many auto-inflammation diseases. More recently anti-TNF therapies have been shown to have a beneficial effect on certain cancers as have cox-2 inhibitors of the NSAID family. The Thalidomide analogues have received much attention for their possible role in anti-inflammatory and anti-cancer

settings illustrating the possible cross application of agents that have been available for some time.

Proven therapies, such as anti-cancer agents, can be rapidly screened for cross application into inflammatory diseases using assay platforms such as ImmuneProfiler™. ImmuneProfiler™ is a series of assays that begins with a rapid anti-inflammatory screen allowing for a quick assessment of the potential anti-inflammatory properties of a therapy. Following the results of the initial screen, a series of assays will be determined and can lead to detailed in vivo dissection of the immunomodulatory mechanism and validation in cutting edge disease models. This results in an efficient and focused strategy for expanding into other diseases with your proven therapy.

ImmuneProfiler™: This system starts with an assay that is typically performed in human PBMC's (although other cells or cell lines are available). In this assay, PBMC's are stimulated with your choice of stimulants plus various concentrations of compounds or compound combinations (for those evaluating combination therapies). Cytokine levels are then evaluated and provide information on which cell based assays to perform next.



Asthma as a therapeutic target: choosing a pre-clinical model

The prevalence of asthma, along with asthma-associated morbidity and mortality, continues to increase worldwide. It is estimated that between 5-12% of the world's population now suffer from the disease. Asthma is therefore an important target for the biopharmaceutical industry. Development of new therapeutics depends upon suitable pre-clinical models. The goal of a pre-clinical asthma model is to reproduce the airway inflammation, mucus hypersecretion or airway hyper-responsiveness seen in human asthma.

Currently available pre-clinical models

The majority of pre-clinical models of asthma utilize rodents. Rodents are sensitized to a model antigen such as ovalbumin (OVA), house dust mite antigen or cockroach allergen in combination with an adjuvant such as alum. Most models utilize multiple sensitization steps, followed by one or more local challenges with the antigen into the lungs. The entire process typically takes approximately one month. Some of these models have the added advantage of being suitable for use in studying effects of test items in other pulmonary disorders such as rhinitis.

Typical endpoints measured

Lung function

It is possible to measure the functionality of the lungs, although the small size of rodent lungs has proven to be problematic. The most common measurement of lung function is to study airway

hyper-responsiveness (AHR).

Asthmatic immune response

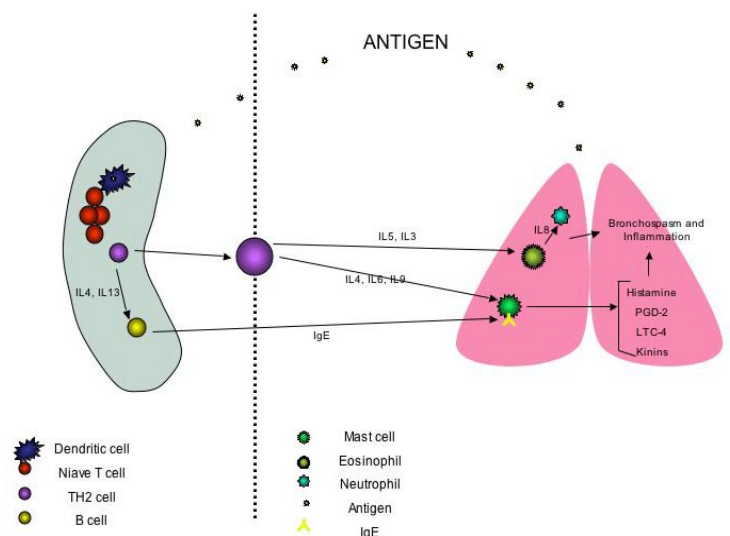
In contrast to lung function measurement, the immune response during asthma is well preserved between mice and humans. In human asthma, eosinophils and lymphocytes infiltrate the bronchial mucosa. Increased mucus secretion and production of Th2-associated cytokines such as IL-4, IL-5 and IL-13 are also found. IL-4 induces differentiation of CD4 T cells into Th2 cells, induces the proliferation of activated B cells and is the major cytokine involved in B cell class switching to IgE (the antibody isotype most associated with human asthma). IL-5 is involved in eosinophil activation and also facilitates B cell growth and antibody production. The activities of IL-13 and IL-4 show a high level of overlap, although it is thought that IL-4 acts primarily in the initial sensitization while IL-13 is more important during secondary exposure to the allergen. In addition to inducing IgE production, IL-13 can induce AHR, goblet cell metaplasia and airway glycoprotein hypersecretion, which all contribute to airway obstruction. All of these parameters can easily be studied in pre-clinical asthma models.

Mast cells are central to the development of asthma due to their ability to release an array of pre-formed and newly synthesized inflammatory mediators such as cytokines, leukotrienes and prostaglandins (Figure 1). Mast cells are also thought to be involved in the tissue remodelling that occurs later in asthma. It can therefore be of interest to study their location and degranulation in pre-clinical asthma models.

Recent developments in endpoints

The above readouts are well established and have been utilized for many years without significant alteration. In recent years models that make use of methods such as MRI or adoptive transfer have led to several advances in the end measurements.

Figure 1. The roll of T cells in asthma



Pre-clinical Asthma Models

Traditional 28 day OVA Allergic Asthma:

- Histology of Lung
- Flow cytometry and cytokine analysis of BAL
- Total & antigen specific IgG/E

Rapid 14 day OVA Allergic Asthma:

- Histology of Lung
- Flow cytometry and cytokine analysis of BAL
- Total & antigen specific IgG/E

OVA combined with adoptive transfer:

- Traditional assessments plus...
- Flow cytometry of BAL for proportion of Tg T cells
- Flow cytometry of draining & peripheral lymph nodes for proportion of Tg T cells
- IHC of lungs and lymph nodes for Tg T cells by light microscopy or LSC

In vitro asthma models:

- Cytokine stimulated human lung epithelial model
- Cytokine stimulated human bronchial smooth muscle cell

Download our white paper at www.mdbiosciences.com

Adoptive transfer

The use of adoptive transfer techniques in pre-clinical asthma models provides the ability to gather information on the mode of action upon the immune response of a test item. By utilizing transgenic T cells that are specific for OVA, it is possible to track these cells in the lungs and the peripheral and draining lymph nodes as asthma is induced. This model makes use of the fact that T cells are central to the immune response in asthma. As shown in Figure 1, T cells are involved from very early in the immune response, as soon as a dendritic cell presents antigen. The naïve T cells then differentiate into Th2 cells and release cytokines, which induce B cells to class switch to IgE. Binding of IgE to the high-affinity IgE Fc receptor on the surface of mast cells leads to cross-linking of IgE, which in turn activates mast cells causing them to degranulate and release a range of mediators such as histamine, prostaglandins and leukotrienes leading to bronchoconstriction. The activated T cells in the lungs also release cytokines such as IL-3 and IL-5, which act to recruit and activate eosinophils, mast cells, more lymphocytes and neutrophils both within the lymph nodes and lung. Thus T cells are involved at many stages in the development of asthma. By tracking T cells and any exerted effect upon them by a test item, we can determine at which stage and where a test item is affecting the antigen-specific immune response during asthma.

An additional benefit of adoptive transfer models is that they provide information on the immune response as it occurs, whereas traditional models only provide information on the final immune response (e.g. antibody levels). Using this technology, it is possible to determine whether a test item is able to affect a range of events during an immune response, such as the activation of T cells, the clonal expansion of T cells, the Th1/Th2 bias of an immune response (1). Of note are the facts that adoptive transfer technology can also be applied to other diseases such as rheumatoid arthritis and that this technology can be utilized as a powerful first step in identifying the mode of action of a test item in the absence of a disease setting. This technology can also provide information on what type of disease a test item is likely to be efficacious. For example if it is discovered that the test item modulates the cytokine balance towards a Th2 response, then it would not be sensible to test the item in an asthma model but rather in a disease with a Th1-mediated pathology such as the collagen-induced arthritis model of rheumatoid arthritis.

Conclusions

Pre-clinical asthma models remain an important tool for the pharmaceutical industry. MD Biosciences offers several models that offer well-established readouts such as pulmonary cell influx and antibody levels, which have good correlation with human disease. Additionally, we are able to offer researchers the ability to not only discover whether or not the test item is effective against asthma, but can also inform on timing, site and mode of action by utilizing the adoptive transfer asthma model.

References

1. Marshall FA, et al., (2005) J Immunol, 175:5817.

Inflammatory Bowel Disease

An early event thought to participate in the pathogenesis of inflammatory bowel disease (IBD) is the disruption of the gastrointestinal epithelial barrier. This disruption leads to the mixing of microbial pathogens from the lumen with antigen presenting cells in the lamina propria producing an inflammatory response. The resulting pro-inflammatory cytokines and chemokines recruit and activate leukocytes, regulate the integrity of the epithelial barrier and stimulate the production of chemokines from epithelial cells. Together, these events lead to chronic inflammation in the intestines.

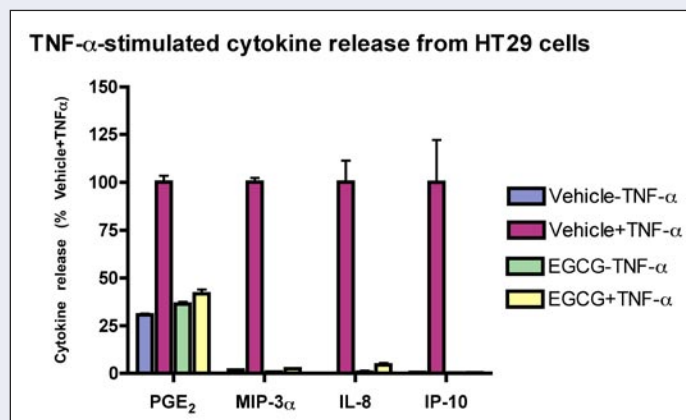
MD Biosciences offers two IBD in vitro assays designed to evaluate the effect of a compound on two of these events:

Macrophage-induced intestinal epithelial cell damage model.

A monolayer of human colon adenocarcinoma cells is cultured on a semipermeable support membrane above LPS-stimulated human macrophages in the presence and absence of test compound. Macrophage-induced epithelial monolayer damage is determined by measuring the transepithelial electrical resistance (TEER).

Cytokine-stimulated human colon adenocarcinoma cell model.

The human colon adenocarcinoma cell line HT29 is stimulated with TNF- α to mimic chronic inflammation in the presence and absence of test compound. Cell culture media is removed at 6 and 24 hours after stimulation and assayed for the following inflammatory mediators: PGE₂, IL-8, IP-10, and MIP-3 α .



In addition to the in vitro models, MD Biosciences performs the TNBS model for the study of IBD. TNBS is administered intrarectally to induce an intestinal pathology that is driven by IL-12. This model is a 7 day model using sulfasalazine as a control.

ArthritoMab™ Arthritis-Inducing Cocktail

Epitope specificity critical for disease pathology

Collagen-induced arthritis (CIA) in mice is widely used as an experimental model for rheumatoid arthritis (RA) in humans. CIA is mediated by autoantibodies, which bind to a particular region of type II collagen (CII). The ability to induce arthritis using this antibody cocktail provides an efficient protocol for the induction of antibody-mediated arthritis that can be used as a shorter, more synchronized alternative to the CIA model.

Epitope-specificity of the antibodies is critical for their pathogenicity in CIA. Evidence also suggests that an antibody response to certain epitopes is better associated with arthritis than other epitopes on the CII molecule. In the mouse CIA model, the antibody response that

is correlated with arthritis is mainly associated with binding to the epitopes of C1, J1 and U1. The ArthritoMab™ cocktail of four monoclonal antibodies developed by Prof. Rikard Holmdahl, binds to the well-defined epitopes C1^{1b}, J1, D3 and U1, which are spread over the entire CII region (CB8, CB10 and CB11 fragments), possibly encouraging better immune complex formation on the cartilage surface for the initiation of arthritis.

Benefits of ArthritoMab™ and the mAb-induced arthritis model:

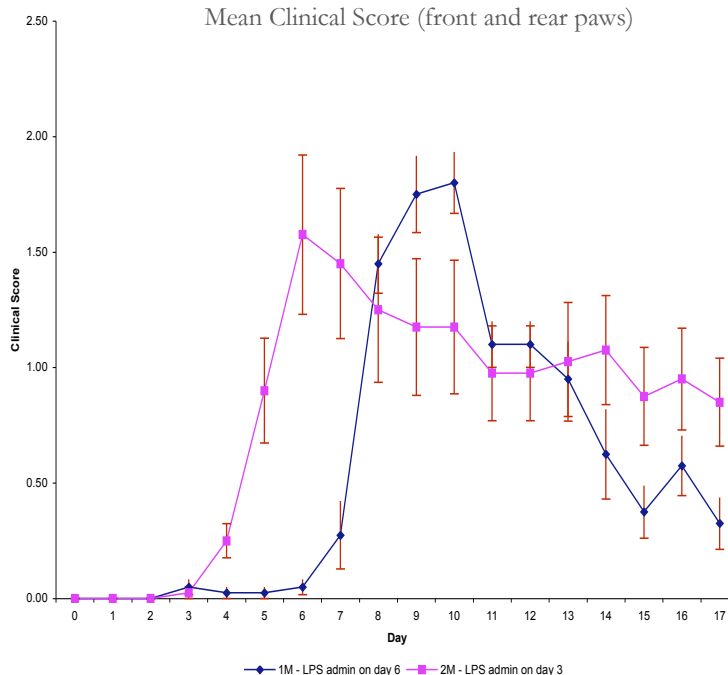
- **Length of study:** Arthritis develops in mice typically within days allowing the completion of a study within 2 weeks reducing the number of administrations, assessments and scoring periods.
- **Reduced group size:** Rate of incidence is nearly 100% depending on the strain allowing for smaller group sizes.
- **Synchronization:** onset of disease is synchronized between animals simplifying treatment schedules.
- **Inflammation:** swelling is visible in the front paws as well as the rear paws.
- **Flexibility:** Protocol can be altered for various study lengths, disease severity and disease pathology pathways.



front paw inflamed

normal rear paw

Arthritis Research	
ELISA's	
Animal COMP	A-COMP.96
Aggrecanase Activity	ACT-AGG.96
Sensitive Aggrecanase Activity	SEN-AGG.96
Arthritis Induction	
Soluble Bovine Collagen II	804001-Sol
Lyophilized Bovine Collagen II	804001-Lyo
Lyophilized Chicken Collagen II	804002-Lyo
ArthritoMab™ Cocktail	CIA-MAB-50
Adjuvant	
Complete Freund's 4 mg/mL	501009
Incomplete Freund's	501011
Proteoglycans	
Septum Cartilage Extract	5028044
Joint Cartilage Extract	5028045
In Vivo Disease Models	
Collagen-induced arthritis	
Monoclonal Antibody Induced Arthritis	
Adjuvant Induced Arthritis	
OVA-Induced Arthritis	
In Vitro Disease Models	
Osteoclast Resorption/Differentiation Assay	
Matrix Degradation Assay	
Synoviocyte Culture Assay	



Relapsing experimental autoimmune encephalomyelitis (R-EAE) model for the study of MS

Due to the clinical and pathological similarities of Experimental Autoimmune Encephalomyelitis (EAE) and Multiple Sclerosis (MS), EAE has been used as a model for the study of human demyelinating disease. Both EAE and MS are characterized by a relapsing-remitting disease course with subsequent progressive disability. EAE is characterized by chronic inflammatory demyelination of the central nervous system (CNS) and involves autoimmune CD4⁺ Th1 cells. These cells develop in the peripheral lymphoid organs and travel to the CNS causing an autoimmune response. The development of T cells is controlled largely by the expression of various cytokines as well as cellular adhesion molecules.

Products and Pre-clinical EAE Models for MS Research

- **MOG-Induced EAE model.**
The MOG-induced model is a chronic progressive model that is completed in 35 days. Control compound is copaxone.
- **MBP-Induced EAE model**
The MBP EAE model in rats is a self-limiting model that is completed in 21 days. This model is often used for preliminary screening.
- **Relapsing PLP-Induced EAE model**
This model allows researchers to evaluate the effect of a compound on the initial peak and if compound is efficacious, the model can be continued to evaluate the effect of the compound on ameliorating the second disease peak.

Research Products for MS	Catalog #	Size
Myelin Proteolipid Protein (PLP 139-151)	301008	15 mg
Myelin Oligodendrocyte Glycoprotein (MOG 25-55)	3038001	25 mg
Complete Freund's Adjuvant, 4 mg/mL	501009	5 mL
Complete Freund's Adjuvant, 3 mg/mL	501010	5 mL
Incomplete Freund's Adjuvant	501011	5 mL

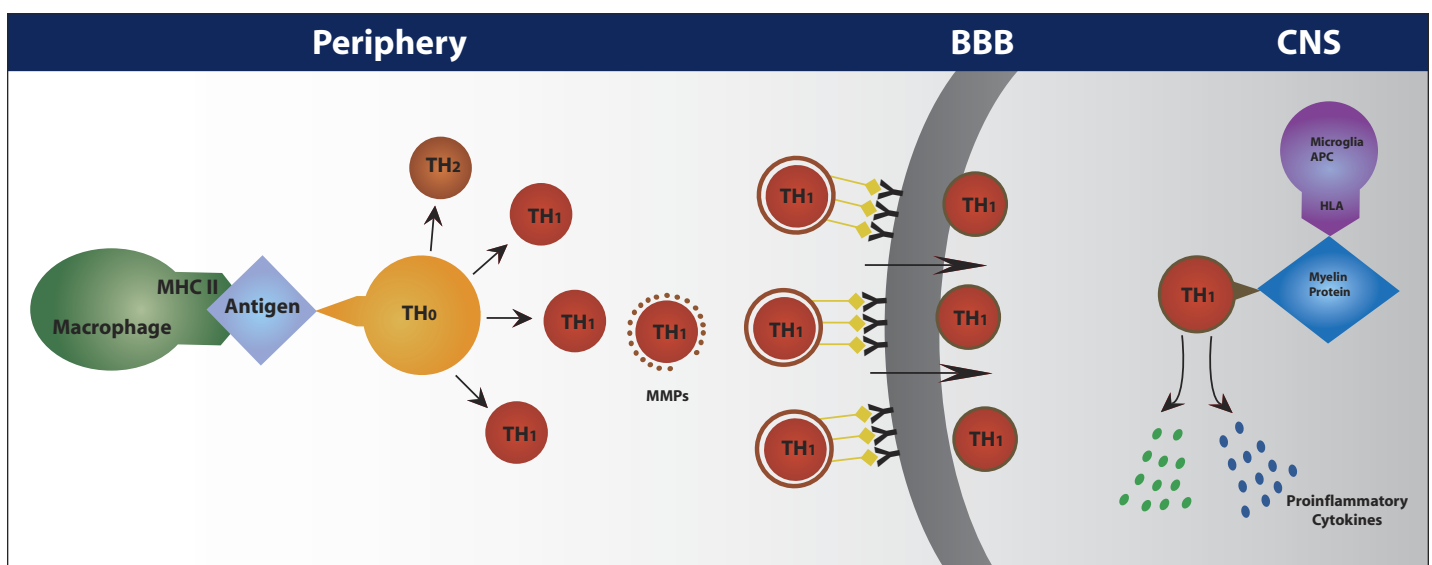
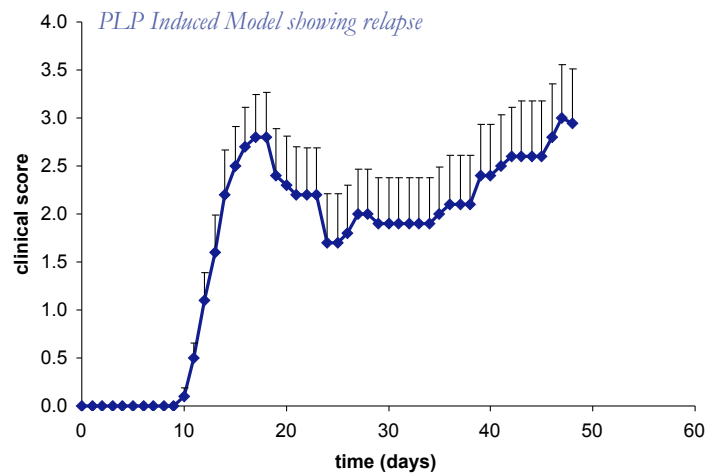


Figure 1. Pathology of MS. In the periphery, antigen is bound by APC via MHC II. TH₀ cells bind to the antigen and causes it to undergo activation and differentiation. Adhesion molecules and MMPs help the TH₁ cells stick to and penetrate the blood brain barrier (BBB). Once TH₁ cells cross the BBB into the central nervous system (CNS), they engage antigen-MHC complexes and produce pro-inflammatory cytokines leading to damage in the CNS. The autoimmune system recognizes myelin proteins as foreign and begin to attack.

Nociception, Neuropathic and Inflammatory Pain

Pain, being one of the most uncomfortable sensations we experience, is a critical component of our body's defense system. It is a mechanism that allows us to remove ourselves from dangerous situations as we move away from noxious stimuli, prevents further damage as we escape stimuli that causes pain after an initial insult, and promotes the healing process as we take great care to protect an injured body part. Pain is divided into two main categories: acute and chronic pain.

Acute or nociceptive pain is part of a rapid warning relay instructing the motor neurons of the central nervous system to minimize detected physical harm. It is mediated by nociceptors, on A- δ and C fibers. These nociceptors are free nerve endings that terminate just below the skin, in tendons, joints, and in body organs. They serve to detect cutaneous pain, somatic pain and visceral pain. Nociception can be associated with nerve damage caused by trauma, diseases such as diabetes, shingles, irritable bowel syndrome, late-stage cancer or the toxic effects of chemotherapy. It typically responds well to treatment with opioids and NSAIDs.

Chronic pain, however, serves no biologic function as it is not a symptom of a disease process but is a disease process itself. There are two types of chronic pain: inflammatory nociceptive pain and neuropathic pain. Inflammatory nociceptive pain is associated with tissue damage and the resulting inflammatory process. It is adaptive in that it elicits physiologic responses that promote healing.

Neuropathic pain is produced by damage to the neurons in the peripheral and central nervous systems and involves sensitization of these systems. In peripheral sensitization, there is an increase in the stimulation of peripheral nociceptors that amplifies pain signals to the central nervous system. In central sensitization, neurons that originate in the dorsal horn of the spinal cord become hyperstimulated, increasing pain signals to the brain and thereby increasing pain sensation. It is most commonly associated with chronic allodynia and hyperalgesia.

One of the challenges for researchers and clinicians alike is that chronic pain may involve a mix of both inflammatory and neuropathic components. In inflammatory nociceptive pain, inflammation may cause damage to the neurons and produce neuropathic pain. Likewise, neuronal injury may cause an inflammatory reaction (neurogenic inflammation) that contributes to inflammatory pain.

Animal Models for Nociception:

Tail Flick: this model is typically use to measure the response to noxious stimuli. It utilizes thermal and mechanical stimuli and measures the latency time until the animal responds.

Visceral Pain: this model is used for screening the effectiveness of analgesic agents. It utilizes noxious chemical irritation of the peritoneum and measures the pain response.

Moderate pain: Post-operative pain models are utilized for screening the pathophysiology of hyperalgesia and can be performed in the rat or pig via incision or surgical procedure respectively. Pain response in rats is evaluated by the response to Von Frey filament stimuli. Pain response in pigs is measured by evaluating the physiological changes in the animals pre- and post-procedure. Three major observations are part of the pain scoring system:

- Animal solitary performance (walking and vocalization)
- Animal social behavior
- Time an animal was able to stay on a sling

Inflammatory Pain:

Carrageenan-induced inflammatory pain: Inflammatory pain is induced by SC intraplantar injection of 1 μ l of 2% carrageenan in saline solution into the hind paw of dryice sedated rats (Sprague Dawley male 200 g). Only one paw is injected with carrageenan while the other paw remains intact as control. Pain threshold is evaluated in both hind paws before carrageenan injection and 180 minutes after the injection using thermal and mechanical stimuli.

Animal Models for Neuropathic Pain:

Chronic constriction injury neuropathic pain in rats: This model involves inflammation around the nerve giving it both a neuropathic and inflammatory component. It can be used as a secondary phase if compounds show effectiveness in both a nociceptive and inflammatory model.

The model is based on chronic constriction of the sciatic nerve, known as the Bennet and Xie model. Sprague Dawley male rats (250 g) are anesthetized with a combination of sodium pentobarbitone sodium and xylazine HCl. Under anesthesia the right sciatic nerve is explored at a location above the femoral joint. Four loose knots are applied to the sciatic nerve. The wound is then closed and animals are treated once with antibiotics. Two weeks later, the rats are tested for their pain threshold using thermal and mechanical tests and the Von Frey filament test. Animals that demonstrate reduced threshold in the operated leg are included in the study. Twenty four hours later animals are divided into different treatment and dose groups. Their pain threshold is re-measured prior to and following treatment.

Taxol-induced neuropathic pain: This model involves the nerve endings and does not contain an inflammatory component. Rats are injected with taxol on days 1, 3, 5, 7, and 9 and changes in pain threshold are measured.

Models of Parkinson's Disease that mimic the depletion of dopaminergic cells

Parkinson's disease (PD) is a neurodegenerative disorder characterized by reduction in striatal dopamine (DA) content caused by the loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc) and their projections to the striatum. Several neurotoxins induce Parkinson's-like neuropathology in animals, including the neurotoxins 6-hydroxydopamine (6OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These models mimic the depletion in dopaminergic (DAergic) cells.

MPTP-induced PD-like disease:

MPTP is an effective dopaminergic neurotoxin that selectively destroys dopamine neurons in the substantia nigra, resulting in a Parkinson's-like syndrome. This model is an acute histology-based model mimicking a depletion of 50-60% in Tyrosine-hydroxylase (TH) immunoreactive cells at the level of the Substantia Nigra pars compacta (SNpc) without affecting the general motor activity of the mice. The effect is less pronounced in the nucleus accumbance, olfactory tubercle and ventral tegmental. Since in MPTP lesioned mice the biochemical and morphological effects are bilateral, the detection of motor deficit is based on spontaneous locomotion observation such as open field test or on accelerating rotarode test.

Acute MPTP model:

Disease induction: Male mice (C57/bl) are administered via four IP injections of MPTP or saline control at 2 hour intervals on day 0.

Readout: One week later the animals are sacrificed and their brains are removed for the presence of TH immunoreactive cells at the level of the SNpc.

Chronic MPTP model

In addition to the acute model, we have developed a chronic MPTP induced model that combines the depletion in the TH-immunoreactive cells with a decrease in motor activity as observed in animals treated with MPTP vs. saline treated animals. The benefit of the chronic model is that we are able to monitor motor function in addition to the histological assessment of DAergic neuron depletion.

Disease induction: Male mice (C57/bl) are administered via IP four injections of MPTP once every 2 weeks.

Read out: The animals are pre-trained in the rotarod test for a period of 4 days before the first MPTP injection. Following injection, the animals are evaluated every 2 weeks for motor function using 2 tests:



- the open field test
- the accelerated rotarod test

At the end of the study the animals are culled and their brains are removed for TH immunoreactive analysis at the level of the SNpc.

6OHDA-induced PD-like model

The 6OHDA-induced PD model in rats is a unilateral lesion model of which the Nigro-Striatal pathway is damaged. In this model, unilateral dopamine (DA) denervation induces a number of behavioral deficits similar to those observed in patients with PD, and a variety of tests have been developed to evaluate spontaneous and drug-induced behavioral changes.

Disease induction: SD rats are anesthetized and are stereotactically injected with 6OHDA hydrobromide into the right Nigro-Striatal pathway (SN, mbf or Stratum). Lesion coordinates are set according to bregma and dura in mm. The injection rate is 1 μ L/min and the cannula is left for another 5 minutes to avoid backflow.

Readout: At least 14 days after the induction, the animals are tested for drug-induced rotation and complement behavioral tests such as the staircase test, open field test and rotarod test. Animals are culled at termination and brains are removed for histological analysis.

Pig models for acute cardiac infarct

Pigs share important characteristics with the human's anatomy and physiology of the cardiovascular system, making them useful models for the study of human diseases. Additionally, pigs respond in a similar manner to humans with acute myocardial infarction. The following section describes the pig myocardial infarct models available from MD Biosciences in collaboration with the Heart Institute, Sheba Medical Center of the Tel Aviv University, Israel.

Pig models for acute cardiac infarct:

1. Ligation induced acute cardiac infarction: Under anesthesia and sterile conditions, the chest is opened and an incision is made in the pericardium. The infarct is induced by ligation of the left anterior descending coronary artery (LAD). After the procedure, the chest is closed and the animals are allowed to recover. Since this procedure is accompanied by opening of the chest, the recovery period is longer.

2. Coil-induced, acute cardiac infarction: A coronary guiding catheter is introduced into distal LAD through the right carotid artery. A superselective infusion catheter is inserted into the LAD. A myocardial infarction is created by occlusion of the LAD using an intraluminal coil forming a thrombus surrounding it and causing acute occlusion of the artery. The main advantage of this model is avoidance of cardiothoracic surgery.

3. Balloon-induced cardiac ischemia reperfusion: Myocardial ischemia reperfusion is created by inflating an angioplasty balloon for 60-90 minutes in the proximal LAD immediately distal to the first septal perforator. This model is mimicking catheterization of a relatively new cardiac infarct in humans. Another variation to the

balloon-induced ischemia-reperfusion is the injection of alcohol to the same area of the balloon. This procedure causes damage to the endothelial cells as well.

Both versions of this model are very common for the evaluation of drug treatment to myocardial infarct since the artery is functional and open after the procedure is terminated.

4. Induction of multi-focal myocardial infarct: The animals are subjected to acute myocardial infarction using distal microembolization. This is carried out by injection of bead suspension through the distal lumen of the a catheter to the distal left LAD territory.

During surgery, all animals are monitored for ECG, invasive blood pressure, body temperature, SpO₂, ET_{CO}₂ and PPV. Readouts include ECG, Echo, Angiography, cardiac MRI and blood tests for relevant parameters such as CPK. All tests are analyzed by expert technicians and cardiologists. According to protocols, animals may be treated before, during and after the procedure with various medications such as anticoagulants, anti-arrhythmic drugs, β -blockers and antibiotics. The animals can be hospital-treated and followed for long periods of time.

Induction of vascular disease:

Atherosclerotic stenosis and plaques are created with balloon denudation followed by a high-cholesterol diet which leads to damage to the intima. Readouts include Intimal thickening measured by light microscopy with a semiquantitative scale.

Contact MD Biosciences for further information on the pig models available.

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Discover the mode of action while simultaneously determining target disease choice using Senega Mode of Action Platform.

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