

## Contents

- 3 In vitro screening of potential compounds
- 4 MMPs
- 6 Induction of disease using PG-PS
- 7 Monoclonal Antibody Induced Arthritis
- 8 Animal COMP ELISA
- 8 Seminars



- DISEASE MODELS
- SPRING
- 2005

# TARGET inflammation

an applications newsletter for inflammatory research

## WHEN, WHERE AND HOW A POTENTIAL COMPOUND ALTERS THE IMMUNE SYSTEM

### THE T CELL B CELL TRANSFER MODEL:

Current methods used during *in vivo* immunological screening rely on *in vivo* parameters such as DTH, antibody production and *in vitro* parameters such as thymidine incorporation. These methods are all retrospective, thereby failing to give insight into the actual events as they occur. Furthermore, their insensitivity means they do not provide specific information about particular types of cells.

While these current methods are able to tell us if a potential immunomodulator alters the immune response they are unable to tell us when, where or how the immunomodulator accomplishes this. To answer this question,

we require the ability to dissect the immune system at each point on the immune response pathway. The T-B cell transfer model enables us to do this. This technology assists in predicting a compound's potential role in different inflammatory diseases.

The power of the T-B cell transfer model comes from the ability to detect antigen specific T and B cells. By inducing an immune response to a model antigen such as ovalbumin (OVA), we can then detect the progress and development of this immune response using a variety of techniques such as FACS analysis, CFSE analysis, Immunohistochemistry, Activation marker analysis, antibody/*in vitro* assays. In being able to track a developing immune response, we can simultaneously track the

effect of potential immunomodulator X on the immune response to a model antigen and thereby assess where, when and how immunomodulator X alters the immune system.

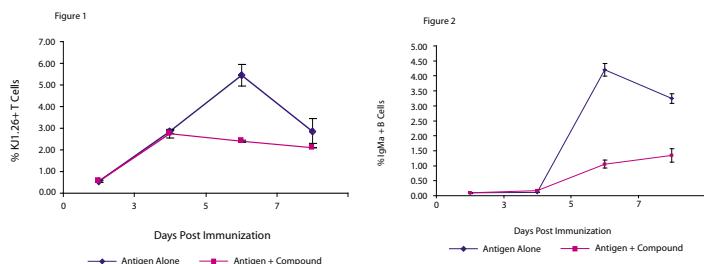
### Flow Cytometry

The technique of flow cytometry is used to detect and quantify the fluorescent staining of cell surface markers. We use this technique to detect and quantify antigen specific T and B cells as well as other cell surface molecules such as activation markers or the cell membrane dye CFSE. The immune response to the antigens, OVA and Hen Egg Lysozyme (HEL), was assessed in mice that had received antigen specific T and B cells.

### t cell b cell transfer model cont.

T and B cell accumulation in the lymph nodes was quantified using the flow cytometer. This was compared to the immune response to OVA and HEL in mice receiving compound X.

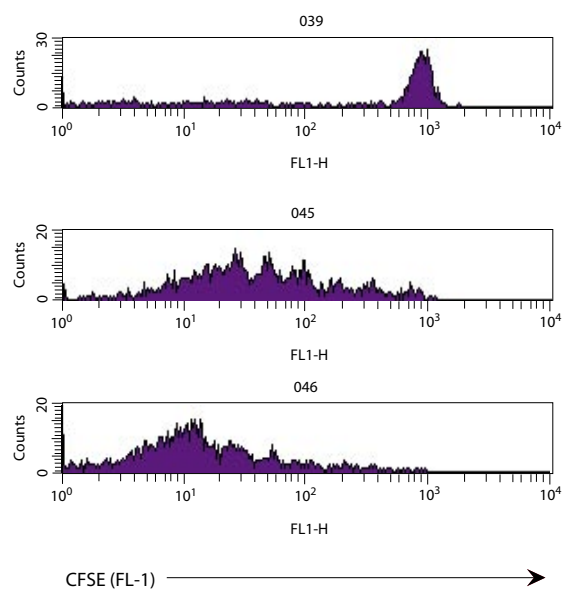
Figure 1 demonstrates that compound X inhibits T cell accumulation while Figure 2 demonstrates that compound X inhibits B cell accumulation significantly.



### CFSE Cell Membrane Staining

Staining T cells with the membrane dye CFSE allows the number of cell divisions to be recorded. This allows the kinetics of the T cell response to be dissected. For example in Figure 3 mice were fed either PBS, Ovalbumin or Ovalbumin + compound Y. Figure 3A demonstrates that there is no antigen specific T cell division when feeding PBS. Figure 3B demonstrates that some T cell division occurs when antigen is fed, however Figure 3C enables us to see that compound Y increases the division in response to feeding ovalbumin.

Figure 3 (a,b,c).



### Immunohistochemistry

The detection of specific cells in *ex vivo* tissue can reveal changes in cell distribution and cell migration. When using *ex vivo* lymph nodes we can detect the localisation of antigen specific T and B cells which may be altered by a potential immunomodulator. Figure 4 demonstrates the difference in localisation of tolerised and primed T cells within the lymph node. The tolerised T cells do not enter the B cell follicle (figure 4a) but the primed T cells enter the follicle (figure 4b).

Therefore the use of immunohistochemistry revealed that the difference between tolerised and primed T cells is their migratory ability.

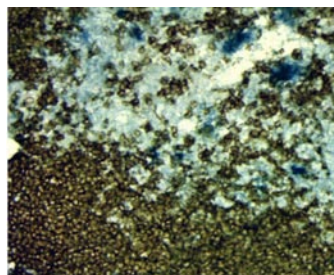


Figure 4a: Tolerised

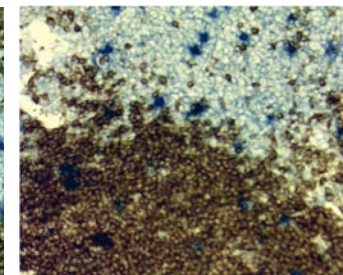


Figure 4b: Primed

The principles of Immunohistochemistry can be applied to provide more information by combining the technique with the Laser Scanning Cytometer (LSC). One of the key advantages of using the LSC is that it allows quantification and therefore statistical analysis of images. The use of the LSC also allows greater detection capability. This can be seen in Figure 5 where three colour detection has been carried out on tissue section. B cells are stained in red. Transgenic T cells are stained green. IFN- $\gamma$  is stained dark blue. Therefore the turquoise areas represent transgenic T cells that are expressing IFN- $\gamma$  while the red dots are Transgenic T cells that are not expressing IFN- $\gamma$ . The difference in localisation of these two cell types within the lymph node can thus be determined.

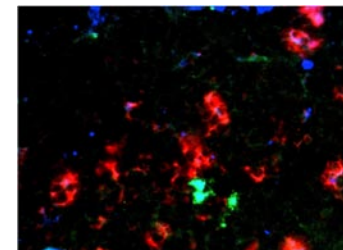


Figure 5.

B cell  
T cell  
IFN- $\gamma$   
T cell + IFN- $\gamma$

To read more about this T cell B cell Transfer model, log onto [www.mdbio-sciences.com](http://www.mdbio-sciences.com) and download our free white paper. ●

## IN VITRO SCREENING FOR INFLAMMATORY ACTIVITIES OF POTENTIAL DRUG COMPOUNDS

Cytokine and cell-based assays are quick and provide the ability to interpret and predict many biological properties of potential drug compounds. Cytokines and related molecules have been implicated in inflammatory diseases such as rheumatic disease, multiple sclerosis, asthma, inflammatory bowel disease, and autoimmune diseases as well as cancer and infectious diseases. These assays provide the ability to determine a compound's effect on cell proliferation, cytokine production and its effect on cellular responses. A sequential approach for determining inflammatory activities of potential drug compounds is described.

### Inflammatory Cytokine Assay

Used for investigating the inhibition of inflammatory cytokine production by test compounds.

The assays are performed using a mouse macrophage cell line (e.g. RAW 264.7). Cells are stimulated with LPS + various concentrations of test compound. Inflammatory cytokine production is measured by standard ELISAs such as IL-1, IL-6, and TNF- $\alpha$  or by using a multiplex panel (measuring IL-1 $\beta$ , IL-6, GM-CSF and TNF- $\alpha$ ). Lower cytokine levels in the presence of the test compound is indicative of an anti-inflammatory effect.

### B and T cell Function Assays

B and T cell function assays used for investigating the inhibition of B and T cell cytokine production and B and T cell proliferation by test compounds.

PBMC cells are isolated from whole blood from mice. Cells are stimulated with LPS to activate B cells or Concanavalin A to activate T cells + various concentrations of test compounds. Supernates are assayed for cytokine production (e.g. IL-2 and IL-10 or by multiplex assay for a wider selection of analytes).  $^3\text{H}$  thymidine is added to additional wells and incubated to study effects of test compounds on cell proliferation. Incorporation of thymidine into cells is measured. For cytokine production assay, lower cytokine levels in the presence of test compound would indicate an anti-inflammatory effect. For cell proliferation assay, lower thymidine incorporation in the presence of test compound would indicate an anti-proliferative effect.

### Adaptive Immune Response

*In vitro* screening of drug properties on cells involved in adaptive immunity using antigen specific transgenic T and B lymphocytes. This *in vitro* screening is performed prior to screening using *in vivo* models of adaptive immunity such as T and B cell transfer models.

Murine OVA-specific T cells are incubated with the antigen Ovalbumin, in the presence or absence of graded concentrations of test compound. Cultures can be carried for pre-defined periods between 24-72 hours. The cells and/or culture supernatants are subjected to the assays described below in a stepwise approach.

### Determination of clonal expansion of KJ+ antigen specific T cells using Flow Cytometry

Used to determine whether antigen specific T cell numbers increase or decrease in response to test compound. If the number increases, the test compound has potential pro-inflammatory effects, which could be tested by a cytokine/chemokine screen.

### Thymidine Incorporation Assay

Used to determine if the test compound affects T cell proliferation. This assay is a cost effective method of determining T cell proliferation, however it does not give information regarding the rate of cell division, which is offered by the Cell Division Assay.

### Cell Division Assay

Uses the cell marker CFSE to determine whether the test compound affects the rate of T cell division. If the cells are dividing less, the test compound may be anti-inflammatory or have regulatory effects. This could be further tested by looking at IL-10 levels using the Th1/Th2 cytokine screening assay.

### Pro-inflammatory Screening

Determination of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and GM-CSF levels present in cell culture supernates. Higher levels of these cytokines would indicate pro-inflammatory effects of the test compound.

### Chemokine Screening

Chemokines are involved broadly in homing of lymphocytes and innate immune cells to sites of inflammation. Therefore, testing levels of chemokines such as MIP-1 $\alpha$ , MCP-1, MIG, IP-10 and KL can provide specific information regarding the pro-inflammatory or anti-inflammatory effects of the test compound.

### Th1/Th2 Cytokine Screening

Determination of IL-2, IL-4, IL-5, IL-10 and IL-12 levels. Test compounds may have specific effects on Th2 responses therefore IL-4, IL-5, and IL-10 levels will be increased. Alternatively, the test compound may affect the Th1 response such as IL-12. ●

*For a listing of additional in vitro services, or to view additional in vivo disease models, visit [www.mdbiosciences.com](http://www.mdbiosciences.com)*

# MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) comprise a family of secreted and membrane-bound endopeptidases that hydrolyze extracellular matrix proteins (ECM) (1). Based on their preferred substrates and structural features, MMPs can be divided into collagenases, gelatinases, stromelysins, matrilysins and membrane-type matrix metalloproteinases. MMP mediated ECM degradation affects processes such as connective tissue remodeling, cell migration and cell micro-environment regulation. MMPs further affect cellular behavior by modulating the activities of cytokines, growth factors, cell surface receptors and other MMPs (2). In addition to their normal function in developmental and repair processes, inappropriate MMP activity also participates in disease processes including arthritis and cancer (3,4).

## Collagenases

MMP-1, MMP-8 and MMP-13 (also known as collagenase-1, collagenase-2 and collagenase-3, respectively) make up the collagenase subfamily of MMPs. Since the collagenases have the unique ability to cleave intact triple helical collagens, they are thought to play a key role in the irreversible destruction of cartilage in rheumatoid arthritis (RA) and osteoarthritis (OA). Of the three collagenases, MMP-13 has the highest activity towards type II collagen, the major collagen component in cartilage and is therefore thought to play a major role in the pathology of arthritis (5). The collagenases can also act on non-collagenous ECM components including the major cartilage proteoglycan, aggrecan (6-8).

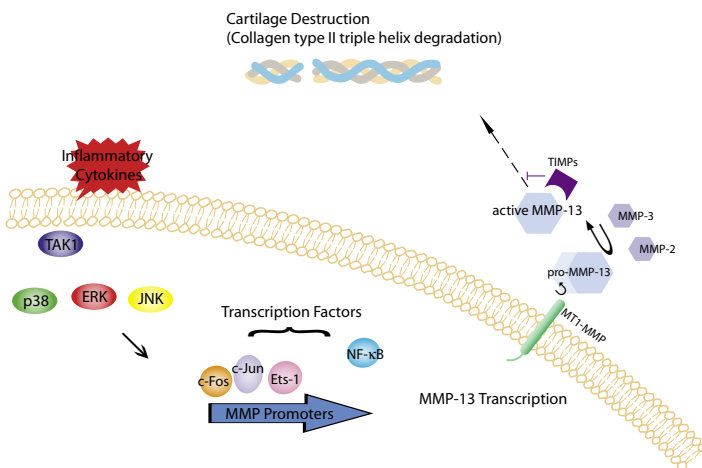


Figure 1. Regulation of MMP activity

MMP-1 is the most abundant collagenase and displays the broadest cell type expression pattern (9). In contrast, normal MMP-13 expression is limited to cartilage and bone during early development (10). Both MMP-1 and MMP-13 are expressed in RA and OA tissue (11-16). MMP-8 expression was initially thought to be restricted to neutrophils but has also been detected in chondrocytes of OA cartilage (12). However, the extent of MMP-8 participation in OA cartilage destruction is currently unclear (17).

## Regulation of collagenase activity

MMP activity is regulated at multiple levels (Figure 1). MMP-1 and MMP-13 expression is stimulated by IL-1 $\beta$  and TNF- $\alpha$ , two cytokines found in arthritic lesions (18). Cytokine induced MMP-1 and MMP-13 expression relies on a complex signal transduction cascade that is both MMP and cell type specific (19). Signal transducers include the MAP kinases p38, JNK and ERK and members of the NF- $\kappa$ B pathway. These proteins activate a number of transcription factors such as c-Fos, c-Jun, NF- $\kappa$ B and ETS proteins that ultimately affect MMP transcription (19-21). Cell specific MMP-13 induction appears to be regulated by the CBFA-1 (Runx-2) transcription factor (22). TGF- $\beta$  can also affect MMP-1 and MMP-13 expression through activation of the Smad proteins. This can have both negative and positive effects on MMP transcription (23, 24).

MMP activity is also post-translationally regulated. Most MMPs are secreted as inactive zymogens requiring proteolytic activation. MMP-13 activation is catalyzed by a number of compounds including MMP-2, MMP-3 and MT-MMP (5, 25). Once activated, MMPs can be inhibited by the binding of one of four tissue inhibitors of metalloproteinases (TIMPs) in a 1:1 stoichiometry (26). The balance between levels of MMPs and TIMPs is believed to play an important role in cartilage homeostasis.

Due to the multiple points of MMP regulation discussed above, there are a number of potential targets available for therapeutic development. Discovering ways to manipulate these pathways will reduce the level of MMP activity, which should decrease the amount of cartilage destruction associated with arthritis. Furthermore, understanding the MMP activation cascades in greater detail will inevitably uncover additional targets. ●

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## RESEARCH TOOLS: MMP PRODUCTS

### Active MMP-13 ELISA

Catalog # ACT-MMP13.96

- Serum, synovial fluid, and cell culture supernates
- Range: 32 - 2000 pg/mL
- Sensitivity: 7 pg/mL
- Sample Size: 100  $\mu$ L
- Assay Time: < 4.5 hrs



### Recombinant & Natural Matrix Metalloproteinases (MMPs)

Protein	Catalog #	Species	Source	Size(s)
ADAMTS1, truncated His-tagged	5028002	human	Sf	5/100 $\mu$ g
ADAMTS4 (aggrecanase 1), truncated His-tagged	5028001	human	Sf	5/100 $\mu$ g
Aggrecan interglobulin domain	5028003	human	E. coli	100/500 $\mu$ g
MMP-2 (gelatinase A)	5028013	human	Sf 9	10/200 $\mu$ g
MMP-9 (gelatinase B) monomer	5028012	human	natural human blood	10/200 $\mu$ g
MMP-13 (procollagenase-3)	5028014	human	Sf 9	10/200 $\mu$ g
MMP-13 (collagenase-3) catalytic domain	5028015	human	E. coli	10/200 $\mu$ g
MMP-14 (MT1-MMP) catalytic domain	5028004	human	E. coli	10/200 $\mu$ g
MMP-14 prodomain-catalytic domain	5028005	human	E. coli	10/200 $\mu$ g
MMP-14 prodomain-catalytic domain	5028006	human	E. coli	10/200 $\mu$ g
MMP-14 hemopexin domain	5028007	human	E. coli	20/200 $\mu$ g
MMP-15 (MT2-MMP) catalytic domain	5028008	human	E. coli	10/200 $\mu$ g
MMP-15 hemopexin domain	5028009	human	E. coli	20/200 $\mu$ g
MMP-16 (MT3-MMP) catalytic domain	5028010	human	E. coli	10/200 $\mu$ g
MMP-17 (MT4-MMP)	5028011	human	E. coli	10/200 $\mu$ g
MMP-24 (MT5-MMP) catalytic domain	5028016	human	E. coli	10/200 $\mu$ g

### Antibodies to MMPs

Antibody	Catalog #	Species	Type	Size(s)	Application
aggrecan N-terminal sequence of ARGSVIL	1028023	human	mouse IgG monoclonal	100 $\mu$ L	WB
Collagenase-3 (MMP-13)	2028019	human	rabbit IgG polyclonal	100 $\mu$ L	WB, ELISA
Collagenase-3 (latent & human)	1028020	human	mouse IgG monoclonal	100 $\mu$ L	WB, ELISA
pro-collagenase-3	1028021	human	mouse IgG monoclonal	100 $\mu$ L	WB, ELISA
pro-collagenase-3	1028022	human	mouse IgG monoclonal	100 $\mu$ L	Staining or detection
MMP-14	2028017	-	rabbit IgG polyclonal	100 $\mu$ L	WB, ELISA
MMP-15	2028018	-	rabbit IgG polyclonal	100 $\mu$ L	WB, ELISA

For research use only. Not for use in diagnostic procedures.



## Inflammatory Disease Models

### Rheumatoid Arthritis:

CIA model

- 42 days
- Induction w/collagen on day 0 and collagen boost on day 21

mAb model

- 11 days
- Induction w/mAb on day 0 with LPS boost on day 3

### Multiple Sclerosis:

EAE model

- 21 days
- Induction with PLP

### IBD:

DSS model

- 7 - 14 days
- Induction with DSS in drinking water.

### T cell B cell Transfer Model

- Know more about when, where and how a compound alters the immune system

## INDUCTION OF DISEASE USING A PEPTIDOGLYCAN FROM BACTERIAL CELL WALL

PG-PS (peptidoglycan-polysaccharide polymer) is purified fragments produced from the cell walls of *Streptococcus pyogenes* Group A, D58 Strain. Bacterial cell wall peptidoglycans possess proinflammatory properties and are toxic in that they are capable of stimulating or modulating an inflammatory response directly or indirectly (1).

Intact cell wall from bacteria such as streptococci consists of peptidoglycans that are linked to a polysaccharide polymer, which protects the peptidoglycan from in vivo degradation, and also enhances the biological effects (2 - 7).

PG-PS produces a biphasic response that is highly representative of the which is caused naturally. Acute inflammation of joints is observed 3 - 5 days after the injection and then recedes. Around 3 weeks, chronic remittent inflammation is observed and is associated with destruction of cartilage, which eventually progresses to ankylosis of the joints.

### Experimental diseases induced by PG-PS in rats:

- Acute and chronic, remittent arthritis
- Granulomatous enterocolitis (resembling Crohn's disease),
- Granulomatous hepatitis
- Intestinal hemorrhage
- Vasculitis

### Benefits of PG-PS:

- Rapid: Produces acute inflammatory response in 3 - 5 days.
- Ready to use: No special preparation, just a brief sonication prior to injection.
- Biphasic model: study acute and chronic inflammatory disease states.

PG-PS Particle	Catalog #	Size	MW*	Concentration
PG-PS 10S	3036001	10 mg	5x10 <sup>6</sup> - 5x10 <sup>8</sup> Da	3 - 6 mg/mL
PG-PS 100P	3036002	10 mg	5x10 <sup>7</sup> Da	5 - 8 mg/mL

\*Measured in mg of rhamnose per mL of material.

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## MONOCLONAL ANTIBODY INDUCED ARTHRITIS

A SHORTER MORE SYNCHRONIZED ALTERNATIVE TO THE TRADITIONAL COLLAGEN INDUCED ARTHRITIS (CIA) MODEL.

### In vivo service: An overview of mAb induced arthritis model.

Initial symptoms of inflammation in the collagen induced arthritis (CIA) model occur at approximately Day 21-24. The induction of the disease is typically at 80% of the animals and varies from laboratory to laboratory and with the use of different collagen sources. The disease symptoms also appear at slightly different times in different animals, making therapeutic administration protocols more demanding technically. CIA studies in mice are typically completed in 42 days, requiring significant amounts of compounds to be prepared for use over the study duration. Furthermore, the lengthy study period also requires increased number of measurements and scoring periods increasing the cost of the study further.

The above points illustrate some of the key issues in running a typical CIA model. The monoclonal anticollagen antibody induced model presents a real alternative to the CIA, significantly reducing the study duration and cost while increasing disease induction and synchronicity among individual animals. This model enables the study of compounds in typically a 10-day protocol with either a therapeutic or prophylactic regimen. The required amount of compounds as well as number of measurements is significantly reduced when compared to the CIA model. The short study duration also means that smaller studies can be performed with results that would be available to plan and perform a next-step study in short intervals.

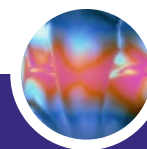
### Antibody cocktail

Initial work by Terato et al. (J. Immunology, 148:2102-2108,1992) showed that antibody-mediated CIA can be induced by iv injection of a combination of 4 different monoclonal antibodies generated by mouse B hybrid-

oma cell lines. Three of the mAbs recognize autoantigenic epitopes clustered within an 84 amino acid residue fragment, LyC2 (the smallest arthritogenic fragment of type II collagen) of CB11 and the fourth mAb reacts with LyC1. Importantly, all 4 mAbs recognize the conserved epitopes shared by various species of type II collagen and cross-react with homologous and heterologous type II collagen. These arthritogenic mAbs alone or in combination with bacterial LPS, can induce severe arthritis within days instead of weeks (classic CIA model).

The monoclonal antibody induced arthritis model is ideal for rapidly screening and evaluating anti-inflammatory therapeutic agents. It is also relevant for studying inflammatory mediators such as cytokines, chemokines, matrix metalloproteinases and other factors such as the role of bacterial flora and their by-products in triggering and exacerbating arthritis.

*To see data from the mAb-induced arthritis model, log onto [www.mdbiosciences.com](http://www.mdbiosciences.com) and check out our white paper. ●*



## Typical Protocol

The typical protocol\* for a mAb cocktail induced arthritis model is as follows:

### Groups: (n=8/group)

- Negative control group: Vehicle
- Positive control group: Dexamethasone or etanercept
- Test compound group(s)

### Induction phase:

- Day 0: Injection of mAb cocktail per i.v.
- Day 3: Injection i.p. of LPS

### Inflammation measurement and scoring:

- Day 5, 6, 7, 9 (peak: Day 6)
- Paw measurements using a microcaliper
- Arthritis scoring on a 0 to 4 scale
- Body weight
- Clinical observations

The study is terminated on Day 10-14.

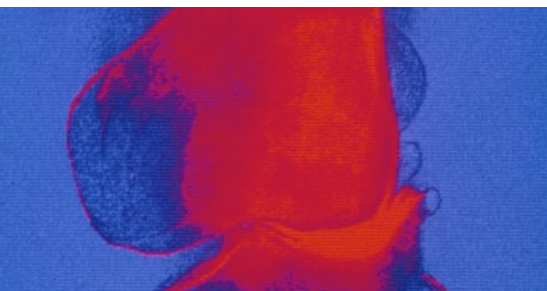
*\*Custom protocols may be designed based on individual need. E-mail us at [info-us@mdbiosciences.com](mailto:info-us@mdbiosciences.com) for more information.*

## Finding the right service provider that fits your needs...

When deciding on a pre-clinical CRO to assist you with your discovery/development project, consider the length of time from contract to the start of the study. Now more than ever, you are under increasing pressure to keep the drug pipelines full. Time lost waiting on a service provider to process the contract and schedule the study is time that could be spent getting your potential compound through development and eventually to market. Ask the service provider about the current case load and the average contract-to-study delay to get an idea of how your project will be handled.

## ANIMAL COMP ELISA

COMP: A POTENTIAL BIOMARKER FOR MONITORING CARTILAGE DEGRADATION IN INFLAMMATORY JOINT DISEASES.



Cartilage oligomeric matrix protein (COMP) is a pentameric glycoprotein originally isolated in cartilage. COMP is produced by cells in the synovial membrane and the concentration present in other tissues such as tendon, ligament and meniscus varies, the highest being found in cartilage.

When cartilage matrix is degraded by disease, protein fragments are produced that are released into the joint fluid. These protein fragments can then be found circulating in the blood and an increase has been correlated to the development of cartilage damage.

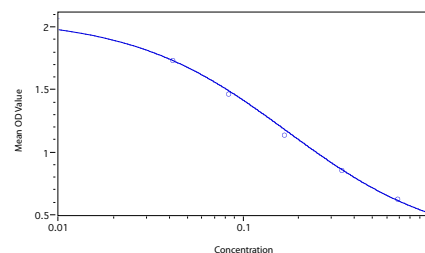
COMP therefore shows promise as a potential biomarker for monitoring cartilage degradation in inflammatory joint diseases such as rheumatoid arthritis and osteoarthritis.

An ELISA specific to COMP provides an accurate, sensitive, and precise method to quantifiably measure COMP levels in serum.

Animal COMP ELISA  
Catalog # A-COMP.96

- Rat, mouse, sheep, and bovine serum
- Easy to use
- Precise, accurate & reproducible results in 3.5 hours
- Measure levels up to 0.9 U/L
- Sensitivity of <0.2 U/L
- 50  $\mu$ L of sample size

Typical Standard Curve



Log onto [www.mdbiosciences.com](http://www.mdbiosciences.com) to view data and ELISA protocol. ●



## Seminars

MD Biosciences will be at the following conference:

**ACR/AHRP Annual Scientific Meeting  
November 12 - 17, San Deigo**

### Have you missed our recent seminars?

This spring, MD Biosciences gave the following seminar at the Inflammation & Immune Disease Summit in Philadelphia and the Anti-Arthritic Agents Conference in London.

*"Where, When, and How - the Importance of Advanced Immunological Screening In Vivo in Drug Discovery"* by Prof. Paul Garside, MD Biosciences Inflammatory Drug Discovery Services.

If you've missed these events, but would like to attend a web seminar, e-mail us at [info-us@mdbiosciences.com](mailto:info-us@mdbiosciences.com) and we'll be sure to send you the event details.

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