# TREATING INFLAMMATION USING A BIOLOGICAL AGENT THAT CAUSES CELLS TO RELEASE CYTOKINE RECEPTORS

## RELATED APPLICATIONS

This application claims the priority benefit of a provisional application filed on September 23, 2003 in the U.S. Patent & Trademark Office, under U.S. Express Mail Label EK 304526603 US. The application was filed by Meyer Pharmaceuticals LLC (Docket Cyto-1), named Tetsuya Gatanaga as first inventor, and was entitled "Treating Inflammatory Conditions Using Cytokine Receptor Cleaving Enzymes".

For purposes of prosecution in the U.S., the priority application, and issued U.S. patents 6,569,664 and 6,593,456 are incorporated herein by reference in their entirety.

## BACKGROUND

Inflammatory events play a central role in the pathology of disease conditions that adversely affect a considerable proportion of the population in developed countries. This process is mediated by cytokines, a system of polypeptides that enable one cell to signal to initiate events in another cell that initiate inflammatory sequelae. Normally, the system acts as part of a defensive reaction against infectious agents, harmful environmental agents, or malignantly transformed cells. But when inflammation exceeds the requirements of its defensive role, it can initiate adverse clinical effects, such as arthritis, septic shock, inflammatory bowel disease, and a range of other human disease conditions.

Small-molecule antirheumatic drugs such as methotrexate and sulfasalazine are insufficient to control inflammation in about two-thirds of arthritis patients. New biological agents developed in the last decade have proved to be effective for a majority of patients unresponsive to traditional drugs. The target for such agents is often one of the cytokine pathways — either capturing the ligand conveying the signal from one cell to another, or blocking the receptor at the surface of the effector cell, preventing transduction of the cytokine signal, thereby forestalling the inflammatory events.

A leading biological agent for treating inflammatory conditions is *Enbrel* ® (Etanercept), marketed by Amgen Corp. It is a chimeric molecule comprising the extracellular portion of the human TNF receptor linked as a dimer to the IgG Fc region. The compound interferes with the binding of TNF to cell-surface TNF receptors — showing the importance of modulating the TNF pathway for clinical therapy of inflammatory conditions.

*Enbrel* ® is licensed in the U.S. for treatment of patients with moderate to severe rheumatoid arthritis, juvenile rheumatoid arthritis, and psoriatic arthritis. Approval is expected in 2003 for treating ankylosing spondylitis. Sales of *Enbrel* ® were $750 million in 2001. Scaling up production to meet

growing demand has been a challenge. The projected sales in the U.S. market for current indication is expected to reach at least $4 billion by 2005, just for current indications.

Other biological agents currently licensed in the U.S. for treating arthritis are *Remicade* ® (Infliximab), a chimeric antibody that binds the TNF-α ligand; *Humira*™, a humanized anti-TNF-α antibody, and *Kineret* ™ (Anakinra), a recombinant form of IL-1Ra, an antagonist of the interleukin-1 receptor.

As it happens, cytokine ligands are not the only component of the cytokine pathway released from cells involved in inflammation. Receptors for the cytokines on the target effector cell are also released in certain inflammatory conditions (Gatanaga et al., *Proc. Natl. Acad. Sci USA* 87:8781-8784, 1990; Brakebusch et al., J. Biol. Chem. 269:32488, 1994).

By 1997, Gatanaga and Granger had isolated a polypeptide that causes the human TNF receptor (both the p55 and p75 isoforms) to be cleaved from the cell surface (U.S. Patent 6,569,664). They demonstrated that the enzyme can be used as an anti-inflammatory agent for treatment of septic shock, and proposed that it be used to treat other inflammatory conditions, such as arthritis, cachexia, and inflammatory heart disease. Subsequently, Gatanaga and Granger isolated nine recombinant cDNA clones that encoded proteins implicated in TNF receptor release (U.S. Patent 6,593,456).

Some subjects having inflammatory conditions do not respond to the medicaments currently available, and the consumer cost of existing biological agents can be over $10,000 per year. There is a need for new biological agents that inhibit multiple cytokine pathways and which can be produced for more modest cost.

## SUMMARY

This disclosure provides inventions related to the use of biological agents that cause cytokine receptors to be released from the surface of cells.

One aspect of this invention is based on the unexpected finding that making exemplary biological agent MP8 longer, rather than shorter, improves the scale by which it can be produced by at least 10-fold.

This discovery enables the use of extended versions of MP8 (or nucleic acids encoding such proteins) for a number of new purposes: preparing a pharmaceutical composition, causing a cytokine receptor to be released from the surface of a cell, inhibiting signal transduction from a cytokine receptor into a cell, screening a substance for its ability to inhibit release of a cytokine receptor, producing a protein that causes a cytokine receptor to be released from the surface of a cell, or treating inflammation in a subject by administering the extended MP8 or a nucleic acid that encodes it.

A preferred embodiment of the extended MP8 in this context is a polypeptide comprising SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41. Fragments and variants can be defined as having a degree of sequence identity (as defined below) with SEQ. ID NO:53 not shared with SEQ. ID NO:41. Fragments and variants of the native sequence, and nucleic acids

encoding them, are also an aspect of the invention as compositions of matter, with the proviso that previously known sequences and products are not included.

Also contemplated are immunoassays, hybridization assays, and PCR assays using the extended form of MP8; and the use of extended MP8 and nucleic acids related thereto for medical use, or the preparation of medicaments for treating inflammatory disease.

Another aspect of this invention is based on the identification of biological agents that cause release of cytokine receptors not previously known as natural enzyme targets, such as the IL-1 Type I receptor, IL-1 Type II receptor and the IL-6 receptor.

One embodiment of this aspect of the invention involves contacting one of these receptors (or a peptide taken therefrom) with a composition comprising either a protein expressed from an encoding sequence selected from SEQ. ID NOs:1 to 29, a recombinantly produced protein containing an amino acid sequence that is at least 90% identical to any one of SEQ. ID NOs:30 to 59 and 80, or fragment thereof, optionally extended beyond SEQ. ID NOs:30 to 42.

A related embodiment a method for reducing inflammation, comprising contacting an inflammatory cell with an IL-6 receptor protease or an IL-1 receptor protease (or metalloprotease) generally, which can be referred to as a means for causing specific release of the IL-6 or IL-1 receptor. Another embodiment is the use of such proteases for preparation of medicaments for treating inflammatory conditions such as rheumatoid arthritis, ankylosing spondylitis, psoriasis, psoriatic arthritis, osteoarthritis, cardiac insufficiency, arteriosclerosis, asthma, myasthenia gravis, septic shock, ulcerative colitis, or Crohn's disease.

Another aspect of this invention is an assay method for determining cytokine receptor releasing enzyme activity in a solution. The solution is combined with a peptide consisting essentially of 8 to 20 consecutive amino acids of a human cytokine receptor, selected from p55 TNF receptor, p75 TNF receptor, IL-6 receptor, IL-1 type I receptor or IL-1 type II receptor, under conditions where the enzyme (if present in the solution) cleaves the peptide; cleavage of the peptide is measured, and enzyme activity is thereby determined. In a preferred embodiment, the peptide is labeled with a fluorescence emitter and a fluorescence quencher, and cleavage is measured by measuring change in fluorescence of the labeled peptide.

Other aspects of the invention will be apparent to the skilled reader from the description that follows, and the appended claims.

## DRAWINGS

**Figure 1(A)** shows isolation of cytokine receptor cleaving activity from human THP-1 cells. The activity was followed through purification on DEAE-Sepharose® and native gels by measuring the ability of the fractions to cause receptor release from the surface of transfected cells.

**Figure 1(B)** is a Western analysis of MP8, one of nine different cDNA clones that are associated with receptor releasing activity. The single MP8 band is shown beside molecular weight standards.

**Figure 2** is an alignment of full-length human MP8 (SEQ. ID NO:53) compared with species orthologs (SEQ. ID NOs:57 and 81-88). Dots indicate residues that are identical with the human sequence on the top line. The orthologs are remarkably similar, sharing a good deal of identity especially between vertebrates (SEQ. ID NO:89) and mammals (SEQ. ID NO:90). Motifs are identified (SEQ. ID NOs:91-102) that are shared throughout the family.

**Figure 3** shows receptor cleavage sites determined by mass spectrometry and peptide sequencing of cleaved peptide substrates (SEQ. ID NOs:71, 73, 74, and 76).

**Figure 4(A), 4(B),** and **4(C)** shows receptor releasing activity measured in a peptide cleavage assay by fluorescence resonance energy transfer. Peptides having the sequence of the receptor cleavage site are labeled on opposite ends with a fluorescence emitter and fluorescence quencher, and metalloprotease activity is measured in the presence and absence of $Zn^{++}$ and $Ca^{++}$. Receptor cleaving enzyme clone MP8 shows specificity for several receptors involved in the inflammatory pathway (TNF-R p55 and p75 isoforms, IL-6 receptor and IL-1 receptors) in comparison with control substrates spanning known cleavage sites of other proteases. **Figure 4(B) 4(D)** illustrates the use of the FRET assay to assess potential inhibitors or activators of cytokine receptor cleavage activity. In this example, metal chelators have a strong inhibitory effect.

**Figure 5** shows cleavage of both the p55 and p75 isoforms of the TNF receptor by clone MP8 in vitro. As the receptor is cleaved from the cell surface, it accumulates in the culture supernatant where it can be measured by ELISA.

**Figure 6** shows cleavage of both isoforms of the TNF receptor by clone MP8 in vivo. Following subcutaneous injection into Balb/c mice, both isoforms accumulate in serum to a level that is over 100-fold above normal (saline control).

**Figure 7** is a compilation of data from two experiments, showing the kinetics of receptor release over a 48 hour period. In other experiments, released receptors persisted in the circulation for at least 6 days.

**Figure 8** shows results of a septic shock experiment. Sepsis was induced by injecting 10 μg LPS and 7 mg galactosamine intravenously into Balb/c mice. MP8 is fully protective against LPS-induced septic shock, whether given simultaneously with the LPS challenge, or 3 hours in advance.

**Figure 9** shows that the anti-inflammatory effect of MP8 is dose-dependent, showing partial protection at 30,000 FU, and complete protection at 300,000 FU.

**Figure 10** shows the receptor cleaving activity of purified MP8 (as measured in the peptide cleavage assay) when stored for an extended period at 4°C or -70°C.

**Figure 11** shows the therapeutic benefit of MP8 is also stable. After storage at 4°C or -70°C, the purified enzyme was still protective in the septic shock model.

**Figure 12(A)** shows that MP8 clears from the circulation in ~39 hours. **Figure 12(B)** shows the therapeutic benefit of MP8 is long-lived. It was protective when administered up to three days before the septic shock challenge — either because the remaining receptor cleaving activity is sufficient to cause ongoing cleavage, or because released receptor blocks signal transduction for several days.

**Figure 13** is taken from an experiment in which MP8 was tested in a model for collagen-induced arthritis (CIA). DBA/1LacJ mice were treated daily beginning 3 days before the disease-inducing agent. Eight out of 9 control mice were affected, but MP8 treated mice showed no joint swelling or other signs of arthritis. The treatment was at least as effective as a scaled dose of Enbrel® (etanercept).

**Figure 14** shows the average increase in joint swelling and arthritis index in each group. MP8 prevented the animals from showing any measurable signs of the disease.

**Figure 15** is taken from an experiment in which MP8 was tested for its ability to treat established disease. Animals with arthritis were randomized on day 22, and treated daily with MP8 or saline control. There was a highly significant reduction in swelling in the affected joints of the two MP8-treated groups compared with control ($p < 0.001$).

**Figure 16** shows the level of antibodies to type II collagen in the serum of the mice. The groups treated with MP8 had levels of pathogenic antibody that were almost half the control.

**Figure 17** is taken from an experiment where MP8 was found to inhibit paw edema in rats induced by injection of carrageenan. The effect was better than indomethacin, a small-molecule nonsteroidal anti-inflammatory agent.

**Figure 18** is taken from an experiment where MP8 was found to prevent development of Experimental Autoimmune Encephalomyelitis (EAE), an animal model for Multiple Sclerosis. The cloned enzyme delayed emergence of symptoms, and lowered disease severity by about 3-fold.

**Figure 19** was obtained from an animal model for experimentally-induced Asthma. Mice were sensitized by immunizing with ovalbumin, and then challenged with the allergen in aerosol form. MP8 reduced the inflammatory sequelae, shown by fewer white blood cells migrating into the alveolar fluid, and a reduced proportion of eosinophils.

**Figure 20** compares full-length MP8 with the originally isolated clone and various control proteins. The bottom panel is a map of the vector used to express MP8-FL in *E. coli*.

**Figure 21** shows the remarkable degree of improvement in going to the full length protein for commercial production. *E. coli* express most of the shorter protein in inclusion bodies, allowing only a

small yield, even after extraction from the inclusion bodies and refolding. In contrast, the full length protein is produced in soluble form, and so can be produced 10 times more efficiently.

**Figure 22** is an SDS polyacrylamide gel stained with Coomassie Blue, comparing purified preparations of MP8 core protein, and full-length MP8.

**Figure 23** compares the activity of full-length MP8 with control protein in the peptide cleavage assay and cell surface cleavage assay. Full length MP8 retains the activity of the core protein.

**Figure 24** shows that full length MP8 is effective as an anti-inflammatory agent in the animal model for edema.

**Figure 25** shows that full length MP8 protects mice in a dose-dependent fashion against a lethal challenge with LPS in the septic shock model.

## DETAILED DESCRIPTION

The commercial potential for receptor cleaving enzymes and the proteins that regulate them has been described previously (U.S. Patent Nos. 6,569,664 and 6,593,456). This disclosure provides a number of milestone improvements that will serve to promote receptor cleavage as a clinically and commercially viable therapeutic strategy.

One of the discoveries of this invention ensued from development of the original exemplary clone MP8 (SEQ. ID NO:9). For research purposes, it was expressed in *E. coli,* and an amount suitable for in vitro and small animal testing was obtained. However, attempts to scale up the production for human clinical trials brought to light the fact that most of the expressed protein was produced in inclusion bodies, adversely affecting the yield. An extensive attempt to recover the protein from the inclusion bodies and refold it into active protein was disappointing — the protein was unstable, and only small amounts of biological activity were recovered. An alternative attempt to make MP8 in yeast was fruitless, as the protein was apparently degraded as fast as it was synthesized. At this point, the problem of how to scale up production was a considerable obstacle to commercialization of the product.

The solution came from quite an unexpected source. The original MP8 clone was the C-terminal portion of a longer reading frame (**Figure 20**). It turns out that when the full reading frame was expressed in *E. coli,* more than half of the protein was produced in the soluble fraction — allowing for straight-forward purification and scale-up. Accordingly, this disclosure provides full length MP8 and other variants extended beyond the core MP8 sequence at the N-terminal end, in order to enhance expression, production, and purification.

The finding that active full-length protein is considerably easier to produce than the truncated MP8 protein was quite unexpected. Smaller proteins are almost always easier to express and are more soluble, and so the usual prediction would be that full-length MP8 would be even harder to express and purify than the original MP8 clone. Furthermore, the otherwise unrecoverable MP8 core is still a component of the full-length molecule, so to the extent that self-catalysis contributed to the problem,

there is no reason to believe that the full-length protein would be any more stable. Using currently available expression systems, the switch from the original MP8 clone to the full-length sequence was a key turning point in the development of the product for clinical testing and use.

Another discovery of this invention ensued from further refinement of the specificity testing of the family of TNF receptor releasing proteins. In particular, it was found that MP8 cleaves not only the TNF receptor, but also the IL-6 receptor, and one or both isoforms of the IL-1 receptor.

This has several important implications. For example, it indicates that MP8 (SEQ. ID NO:41 or 53), and some of the other members of this family of proteins, can be developed for the treatment of conditions where the TNF pathway is not a critical part of the pathology. IL-6 has been implicated as playing a central role in multiple sclerosis and osteoarthritis. Thus, a biological agent such as MP8 that inhibit IL-6 signal transduction may be therapeutic agents for these conditions, and for other conditions that are refractory for therapy such as *Remicade*® and *Enbrel*® directed at the TNF therapy (or where the patient's condition has become refractory to such therapy). Indeed, MP8 has shown to be effective in Experimental Autoimmune Encephalomyelitis (EAE), an animal model for multiple sclerosis (Example 13, **Figure 18**).

Another implication is that the biological agents of this invention may effectively synergize with existing biological agents, by affecting cytokine pathways in addition to TNF. Other studies have demonstrated that *Enbrel*® (inhibiting the TNF pathway) and *Kineret*® (inhibiting the IL-1 pathway) are more effective together in certain biological and clinical situations than they are together. By combining MP8 with a TNF inhibitor, the clinician may be able to modulate a disease condition by affecting TNF, IL-1, IL-6, and perhaps other signaling pathways at the same time, for a more global approach to the underlying pathology.

Accordingly, this invention contemplates pharmaceutical compositions, kits, or methods of treatment in which a cytokine receptor releasing protein such as MP8 is used simultaneously or sequentially with TNF blocking agents, such as TNF-specific antibodies like *Remicade*®, TNF binding proteins having a soluble TNF component (such as *Enbrel*®), dominant negative mutants of TNF ligand, and other TNF inhibition means as may be known in the art or subsequently developed.

A further aspect of this invention that ensued from the commercial development of these biological agents is a peptide assay for measuring enzymatic activity causing cytokine receptor cleavage (Example 4). This allows the activity of certain receptor cleaving enzymes to be followed through purification, standardized by dose, and used for screening enzyme inhibitors.

This disclosure provides extensive animal model data demonstrating that biological agents mediating cytokine receptor cleavage have therapeutic and commercial potential for a number of different conditions. The data show that the biological agents of this invention have important advantages over the therapeutic products that are currently available to the general public for treatment of inflammatory diseases of various kinds. Some of the advantages are the following:

- Cleavage of cytokine receptors inhibits the TNF pathway in two ways: First, the receptor is removed from the membrane of the effector cell, so that it cannot participate in signal transduction. Second, the released ligand binding portion of the receptor neutralizes any incoming TNF ligand in a manner comparable with *Enbrel* ® and *Remicade* ®.
- Since receptor release is caused by enzyme cleavage, the biological agents of this invention have the potential to accomplish in catalytic amounts what receptor antagonists like *Enbrel* ® accomplish in stochiometric amounts. This means that a single molecule of enzyme should inactivate many TNF ligands and receptors, resulting in greater effect per molecule of administered drug.
- The biological agents of this invention can be formulated as naturally occurring human proteins that normally acts to regulate inflammation. This means they should not be immunogenic. Furthermore, the receptor that is released from the cell is an endogenous (non-recombinant) compound that neutralizes incoming cytokines in a physiologically natural way.
- The molecules of this invention share with other specific biological agents the potential for a low side effect profile. No safety issues have arisen in five different animal disease models. Specificity of biological agents facilitates rapid completion of clinical trials.
- Exemplary clone MP8-FL is a relatively small protein, causing greater effect per mass, while retaining the specificity and clinical benefit of other biological agents. The small size also provides a range of options for clinical formulation, including intradermal delivery, which would allow administration close to an inflamed joint.
- Data in this report indicate that the cytokine receptor released by the proteases of this invention persist for days after administration. This means that administration of the enzyme just once a week (or less) may be sufficient for a full therapeutic effect.
- Since the products of this invention work by different mechanisms than currently established drugs, it has potential not just as an alternative — it may also improve the effect of other therapeutic agents, increasing the number of indications and thereby expanding market size.
- All of the clones tested so far retain functional activity when produced by bacterial expression. The proteins apparently do not require glycosylation in mammalian cells like antibody products such as *Remicade* ®, or immunoglobulin derivatives such as *Enbrel* ®. The modest cost of production per dose will be an important competitive advantage.

Based on the summary of the invention and the appended claims, and guided by the illustrations in the example section, one skilled in the art will readily know what techniques to employ in the practice of the invention. The following detailed description is provided for the additional convenience of the reader.

Definitions and basic techniques

Agents of this invention that act to reduce inflammation are referred to variously in this disclosure as cytokine receptor cleaving or releasing enzymes or proteins. The terms are interchangeable, and not meant to require any particular biochemical or biological activity, except where explicitly required. For example, cloned proteins of this invention (such as MP8-FL2 and its derivatives) may themselves have proteolytic activity, or they may cause release of one or more unspecified cytokine receptors from an inflammatory cell in a less direct fashion (such as by causing expression or activation of another protein). Demonstration of apparent proteolytic activity may be attributable directly to an enzymatic function of the protein, or may be due to a copurifying product that acts as a proxy to determine the relative concentration of the active biologic agent. The terms used to refer to the principal product are not meant to limit the therapeutic use of the compounds of this invention where not explicitly indicated, since the therapeutic benefit may be determined empirically without understanding the mechanism by which a compound is effective.

Designations used in general description of the invention are meant to include all functionally equivalent fragments, variants, and homologs, unless otherwise explicitly stated or implied. For example, reference to "core MP8" in specific working illustrations means a polypeptide having the sequence of SEQ. ID NO:41 (usually with a HIS tag); Otherwise it generally refers to fragments and homologs having a degree of identity with SEQ. ID NO:41 and an appropriate biological function. Reference to "full length MP8" or "MP8-FL" in specific working illustrations means a polypeptide having the sequence of SEQ. ID NO:53 (optionally with a HIS tag). Otherwise it generally refers to any fragment of SEQ. ID NO:53 that has sequence extending beyond that of SEQ. ID NO:41 by any length, up to the complete SEQ. ID NO:53, and homologs having a degree of identity therewith and an appropriate biological function. Reference to "MP8" outside the working illustrations refers interchangeably to full length MP8 and core MP8 (including fragments and variants thereof), unless otherwise explicitly stated or implied.

The term "polynucleotide" refers to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Non-limiting examples include: a gene or gene fragment, mRNA, cDNA, other forms of recombinant or synthetic polynucleotides, plasmids, vectors, nucleic acid probes, and primers. The term refers interchangeably to double-and single-stranded molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form, and each of two complementary single-stranded forms known or predicted to make up the double-stranded form

"Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. Hybridization reactions can be performed under conditions of different "stringency". Relevant conditions include temperature, ionic strength, and the presence of additional solutes in the reaction mixture such as formamide. Conditions of increasing stringency are 30°C in 10x SSC (0.15 M NaC1, 15 mM citrate

buffer); 40°C in 6x SSC; 50°C in 6x SSC, 60°C in 6x SSC, or at about 40°C in 0.5x SSC, or at about 30°C in 6x SSC containing 50% formamide.

The percentage of sequence identity for polynucleotides or polypeptides is calculated by aligning the sequences being compared, and then counting the number of shared residues at each aligned position. No penalty is imposed for the presence of insertions or deletions, but are permitted only where required to accommodate an obviously increased number of amino acid residues in one of the sequences being aligned. When one of the sequences being compared is indicated as being "consecutive", then no gaps are permitted in that sequence during the comparison. The percentage identity is given in terms of residues in the test sequence that are identical to residues in the comparison or reference sequence.

As used herein, "expression" of a polynucleotide refers to the production of an RNA transcript. Subsequent translation into protein or other effector compounds may also occur, but is not required unless specified. "Genetic alteration" refers to a process wherein a genetic element is artificially introduced into a cell, which may result in expression or replication of the genetic element, or inheritance of the element by progeny of the cell.

The terms "polypeptide", "peptide" and "protein" are used interchangeably to refer to polymers of amino acids of any length, and their homologs and derivatives. They may be isolated from natural sources, or produced by recombinant expression or chemical synthesis.

It is understood that the folding and the biological function of proteins can accommodate insertions, deletions, and substitutions in the amino acid sequence. Some amino acid substitutions are more easily tolerated. For example, substitution of an amino acid with hydrophobic side chains, aromatic side chains, polar side chains, side chains with a positive or negative charge, or side chains comprising two or fewer carbon atoms, by another amino acid with a side chain of like properties can occur without disturbing the essential identity of the two sequences. Methods for determining homologous regions and scoring the degree of homology are described in Altschul et al. *Bull. Math. Bio.* 48:603-616, 1986; and Henikoff et al. *Proc. Natl. Acad. Sci. USA* 89:10915-10919, 1992. Substitutions that preserve the functionality of the polypeptide, or confer a new and beneficial property (such as enhanced activity, stability, or decreased immunogenicity) are especially preferred.

An "antibody" (interchangeably used in plural form) is an immunoglobulin molecule capable of specific binding to a target, such as a polypeptide, through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term encompasses not only intact antibodies, but also antibody fragments, chimeras, and equivalents that include at least one antigen combining site of the desired specificity.

An "isolated" polynucleotide, polypeptide, protein, antibody, or other substance refers to a preparation of the substance devoid of at least some of the other components that may also be present where the substance or a similar substance naturally occurs or is initially obtained from. Thus, for example, an isolated substance may be prepared by using a purification technique to enrich it from a

source mixture. Enrichment can be measured on an absolute basis, such as weight per volume of solution, or it can be measured in relation to a second, potentially interfering substance present in the source mixture. A substance can also be provided in an isolated state by a process of artificial assembly, such as by chemical synthesis or recombinant expression.

The term "clinical sample" encompasses a variety of sample types obtained from a subject and useful in an in vitro procedure, such as a diagnostic test. The definition encompasses solid tissue samples obtained as a surgical removal, a pathology specimen, or a biopsy specimen, cells obtained from a clinical subject or their progeny obtained from culture, liquid samples such as blood, serum, plasma, spinal fluid, synovial fluid and urine, and any fractions or extracts of such samples that contain a potential indication of the disease.

Unless otherwise indicated, the practice of the invention will employ conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology.

Polynucleotides

Polynucleotides of this invention can be prepared by any suitable technique, including but not limited to chemical synthesis or recombinant expression.

Using the data provided in this disclosure, sequences of less than ~50 base pairs are conveniently prepared by chemical synthesis, either through a commercial service or by a known synthetic method, such as the triester method or the phosphite method. A suitable method is solid phase synthesis using mononucleoside phosphoramidite coupling units (U.S. Patent No. 4,415,732). For use in antisense therapy, polynucleotides can be prepared by chemistry that produce more stable in pharmaceutical preparations. Non-limiting examples include thiol-derivatized nucleosides (U.S. Patent 5,578,718), and oligonucleotides with modified backbones (U.S. Patent Nos. 5,541,307 and 5,378,825).

Polynucleotides of this invention can also be obtained by PCR amplification of a template with the desired sequence. Oligonucleotide primers spanning the desired sequence are annealed to the template, elongated by a DNA polymerase, and then melted at higher temperature so that the template and elongated oligonucleotides dissociate. The cycle is repeated until the desired amount of amplified polynucleotide is obtained (U.S. Patent Nos. 4,683,195 and 4,683,202). Suitable templates include the Jurkat T cell library and other human or animal expression libraries that contain genes that cause release of cytokine receptors. The Jurkat T cell library is available from the American Type Culture Collection, 10801 University Blvd., Manassas VA 20110, U.S.A. (ATCC #TIB-152). Production scale amounts of large polynucleotides are most conveniently obtained by inserting the desired sequence into a suitable cloning vector and reproducing the clone. Exemplary cloning and expression methods are illustrated in Example 2.

Preferred polynucleotide sequences are 50%, 70%, 80% , 90%, or 100% identical to one of the sequences exemplified in this disclosure; in order if increasing preference. The length of consecutive residues in the identical or homologous sequence compared with the exemplary sequence can be about

15, 30, 50, 75, 100, 200, 500 or 1000 residues in order of increasing preference, up to the length of the entire clone. Nucleotide changes that cause a conservative substitution or retain the function of the encoded polypeptide (in terms of hybridization properties or what is encoded) are especially preferred substitutions.

The polynucleotides of this invention can be used to measure altered receptor releasing activity in a cell or tissue sample. This involves contacting the sample with the polynucleotide under conditions that permit the polynucleotide to hybridize specifically with nucleic acid that affects receptor release, if present in the sample, and determining polynucleotide that has hybridized as a result of step a). Specificity of the test can be provided in one of several ways. One method involves the use of a specific probe — a polynucleotide of this invention with a sequence long enough and of sufficient identity to the sequence being detected, so that it binds the target and not other nucleic acid that might be present in the sample. The probe is typically labeled (either directly or through a secondary reagent) so that it can be subsequently detected. Suitable labels include $^{32}P$ and $^{33}P$, chemiluminescent and fluorescent reagents. After the hybridization reaction, unreacted probe is washed away so that the amount of hybridized probe can be determined. Signal can be amplified using branched probes (U.S. Patent No. 5,124,246). In another method, the polynucleotide is a primer for a PCR reaction. Specificity is provided by the ability of the paired probes to amplify the sequence of interest. After a suitable number of PCR cycles, the amount of amplification product present correlates with the amount of target sequence originally present in the sample.

Such tests are useful both in research, and in the diagnosis or assessment of a disease condition. For example, cytokine signaling plays a role in eliminating tumor cells, and a cancer may evade the elimination process by activating cytokine receptor release in the diseased tissue. Hence, under some conditions, high expression of molecules that affect receptor release may correlate with progression of cancer. Diagnostic tests are also of use in monitoring therapy, such as when gene therapy is performed to increase receptor releasing activity.

Polynucleotides of this invention can also be expressed in a eukaryotic or prokaryotic expression system for production of polypeptides; or used for the preparation of medicaments, as explained below.

Polypeptides

Short polypeptides of this invention can be prepared by solid-phase chemical synthesis. The principles of solid phase chemical synthesis can be found in Dugas & Penney, Bioorganic Chemistry, Springer-Verlag NY pp 54-92 (1981), and U.S. Patent No. 4,493,795. Automated solid-phase peptide synthesis can be performed using devices such as a PE-Applied Biosystems 430A peptide synthesizer (commercially available from Applied Biosystems, Foster City CA).

Longer polypeptides are conveniently obtained by expression cloning. A polynucleotide encoding the desired polypeptide is operatively linked to control elements for transcription and translation, and then transfected into a suitable host cell. Expression may be effected in prokaryotes

such as E. coli (ATCC Accession No. 31446 or 27325), eukaryotic microorganisms such as *Pichia pastoris* yeast:, or higher eukaryotes, such as insect or mammalian cells. A number of expression systems are described in U.S. Patent No. 5 ,552,524. Expression cloning is available from such commercial services as Lark Technologies, Houston TX. The protein is purified from the producing host cell by standard methods in protein chemistry, such as affinity chromatography and HPLC. Expression products are optionally produced with a sequence tag to facilitate affinity purification, which can subsequently be removed.

Preferred sequences are 40%, 60%, 80 , 90%, or 100% identical to one of the sequences exemplified in this disclosure; in order if increasing preference. The length of the identical or homologous sequence compared with the native human polynucleotide can be about 7, 10, 15, 20, 30, 50, 100, or 200 residues in order of increasing preference, up to the length of the entire encoding region.

Polypeptides can be tested for an ability to modulate release of cytokine receptors in a peptide cleavage assay. The polypeptide is contacted with the receptor (preferably expressed on the surface of a cell, such as a C75 or THP1 cell), and the ability of the polypeptide to increase or decrease receptor cleavage and release is determined.

Polypeptides of this invention can be used as immunogens for raising antibody. Large proteins will raise a cocktail of antibodies, while short peptide fragments will raise antibodies against small region of the intact protein. Antibody clones can be mapped for protein binding site by producing short overlapping peptides of about 10 amino acids in length. Overlapping peptides can be prepared on a nylon membrane support by standard F-Moc chemistry, using a SPOTS™ kit from Genosys according to manufacturer's directions.

Polypeptides of this invention can also be used to affect cytokine signal transduction in a therapeutic context, as explained below.

Variants

The user may decide to make fragments, variants, or other homologs of the prototype sequences provided in this disclosure in order to improve the activity to mass ratio, to alter glycosylation sites, improve production efficiency, or for any other worthwhile purpose.

Fragments of proteins disclosed in this invention (e.g., SEQ. ID NOs:41 and 53) that cause TNF receptor release can readily be identified by employing standard methodology for mapping function. Recombinant protein which is trimmed at the N- or C-terminus, and then test it for function using a suitable assay: such as the peptide cleavage assay or receptor release assay illustrated in Examples 4 and 5, respectively, which can be run in a high throughput manner. Trimming would continue until activity is lost, at which point the minimum functional unit of the protein would be identified. Fragments containing any portion of the protein down to the identified size would probably be functional, as would be fusion constructs containing at least the functional core of the protein.

To generate variants that incorporate one or more amino acid changes in the encoding sequence, the skilled reader can change particular nucleotides or codons and retest for activity. Optionally, the user may be guided in her site-specific mutagenesis by known homology data. For example, to make variants of MP8, the user may wish to avoid making mutations in regions conserved amongst vertebrates (SEQ. ID NO:89) or amongst mammals (SEQ. ID NO:90). Regions that show considerable variation (a plurality of x's in SEQ. ID NOs:89 or 90) may more likely accommodate deletions or additions. Adopting this strategy, the user would obtain a homolog identifiable by a degree of sequence identity (or an ability of the gene sequence to hybridize with the prototype sequence). A functional MP8 variant may contain one or several of the conserved motifs (SEQ. ID NOs:91-102); particularly those in the core protein (SEQ. ID NOs:97-102), which this region of the molecule is associated with receptor releasing activity.

Unless particular changes are desired, there is no need to target the mutations to particular positions in the sequence. An effective way to generate a large collection of functional variants is to use a random mutation strategy. The standard texts Protocols in Molecular Biology (Ausubel et al. eds.) and Molecular Cloning: A Laboratory Manual (Sambrook et al. eds.) describe techniques employing chemical mutagenesis, cassette mutagenesis, degenerate oligonucleotides, mutually priming oligonucleotides, linker-scanning mutagenesis, alanine-scanning mutagenesis, and error-prone PCR. Other efficient methods include the E. coli mutator strains of Stratagene (Greener et al., Methods Mol. Biol. 57:375, 1996) and the DNA shuffling technique of Maxygen (Patten et al., Curr. Opin. Biotechnol. 8:724, 1997; Harayama, Trends Biotechnol. 16:76, 1998; U.S. Patents 5,605,793 and 6,132,970). To the extent that the user may wish to test variants near the outer limit of variability in the claims (i.e., only ~90% identical to SEQ ID NO:2), they may subject the representative sequence to successive cycles of mutation and functional testing — or choose a mutation strategy that generate more abrupt changes, such as the DNA shuffling technique.

There are several commercially available services and kits available to the skilled reader to use in obtaining variants of the claimed proteins. By way of illustration, enclosed with this Amendment is information regarding for several marketed systems specifically designed for mutagenesis projects of this kind (Exhibit 1): the GeneTailor™ Site-Directed Mutagenesis System sold by InVitrogen™ Life Technologies (Exhibit 1); the BD Diversify™ PCR Random Mutagenesis Kit™, sold by BD Biosciences/Clontech; the Template Generation System™, sold by MJ Research Inc., the XL1-Red™ mutator strain of E. coli, sold by Stratagene; and the GeneMorph® Random Mutagenesis Kit, also sold by Stratagene . By employing any of these types of systems in conjunction with a suitable functional assay such as those described in Examples 1 and 4, variants can be generated and tested in a high throughput manner.

After each iteration of mutagenesis, the user can screen the resultant variants for biological activity as already described, selecting the clones retaining the ability to cause cytokine receptor release.

Optionally, the selected clones can be subject to further rounds of mutagenesis, until the desired degree of variation from the original sequence has been achieved.

Extended sequences

One of the important discoveries of this disclosure is the finding that extending the sequence of MP8 beyond the core protein can improve production and recovery of the protein as much as 10-fold (Example 15). Thus, one aspect of the invention is the use of the full length sequence (SEQ. ID NO:53) for purposes previously contemplated only for the core sequence (SEQ. ID NO:41, and functional subfragments thereof). The user may readily make intermediate sized fragments and variants of the full-length MP8 sequence that are longer than the core fragment, but less than the complete open reading frame. Many of these will share with SEQ. ID NO:53 the property of being much easier to produce than the core protein.

To find such useful intermediate sized fragments, two tests would be involved. First, a candidate recombinant fragment would be tested to determine whether it retained the ability to cause cytokine receptor release. The user would also determine whether the candidate fragment was suitable for large-scale production in *E. coli, Pichia pastoris,* and other expression systems according to the strategy outlined in Example 15. It is generally not necessary to do a complete purification protocol to determine what recombinant proteins are suitable: more simply, the encoding sequence is cloned into the system, expression is induced, and a soluble extract is prepared as appropriate for the system being used. The extract can then immediately be immunoassayed for the amount of target protein in the extract using an appropriately specific antibody.

Similarly, variants of SEQ. ID NO:53 extending beyond the core protein can be generated using the mutation strategies described in the previous section, and tested for both biological activity and ease of expression. The variant proteins would contain or consist of an amino acid sequence having a degree of identity with SEQ. ID NO:53 (e.g., 70%, 80%, or 90% identical), but be longer (one, 10, or 50 amino acids or more) and not have the same degree of identity with SEQ. ID NO:41.

Alternatively or in addition, the variant may be constructed as a fusion protein, for example, containing the sequence of another protein towards the N-terminal end of SEQ. ID NO:41. The second sequence could take the place of the N-terminal 100 residues in SEQ. ID NO:53, promoting production of the protein as a soluble and recoverable protein in a suitable expression system. Candidate second sequences can be taken from innocuous human proteins known to be soluble, easily producible in common expression systems, and unlikely to interfere with the biological activity of MP8: for example, albumin, or other serum proteins. Other candidate sequences may promote accumulation of the fusion protein near the target tissue: for example, TNF ligand, ligand for a neighboring cell surface receptor, or a single chain antibody. Suitable protein sequences for inclusion in the fusion protein can be determined empirically using the testing systems already described.

Antibodies

Polyclonal antibodies can be prepared against the proteins of this invention by injecting a vertebrate with a polypeptide of this invention in an immunogenic form. Immunogenicity of a polypeptide can be enhanced by linking to a carrier such as KLH, or combining with an adjuvant, such as Freund's adjuvant. If desired, the specific antibody activity can be further purified by a combination of techniques, which may include protein A chromatography, ion exchange chromatography, and immunoaffinity chromatography.

Monoclonal antibodies can be prepared according to such standard references as Harrow & Lane (1988), U.S. Patent Nos. 4,491,632, 4,472,500 and 4,444,887, and *Methods in Enzymology* 73B:3 (1981). Briefly, a mammal is immunized, and antibody-producing cells (usually splenocytes) are harvested. Cells are immortalized by fusion with a non-producing myeloma, transfecting with Epstein Barr Virus, or transforming with oncogenic DNA. The treated cells are cloned and cultured, and the clones are selected that produce antibody of the desired specificity.

Antibody can be produced for particular regions of a molecule by using fragment, and if necessary, selecting against or absorbing out unwanted activity. For example, antibody against core MP8 can be raised by using a protein consisting of SEQ. ID NO:41 or a portion thereof as immunogen. Antibody against full-length can be raised by immunizing with the portion of SEQ. ID NO:53 that does not contain SEQ. ID NO:41; or else by immunizing with the complete SEQ. ID NO:53 and selecting against or absorbing unwanted activity with SEQ. ID NO:41.

Other methods of obtaining specific antibody molecules (optimally in the form of single-chain variable regions) involve contacting a library of immunocompetent cells or viral particles with the target antigen, and growing out positively selected clones. Immunocompetent phage can be constructed to express immunoglobulin variable region segments on their surface. See Marks et al., *New Eng. J. Med.* 335:730, 1996, International Patent Applications WO 9413804, WO 9201047, WO 90 02809, and McGuiness et al., *Nature Biotechnol.* 14:1449, 1996.

The antibodies of this invention are can be used in immunoassays for proteins believed to cause receptor release. General techniques of immunoassay can be found in "The Immunoassay Handbook", Stockton Press NY, 1994; and "Methods of Immunological Analysis", Weinheim: VCH Verlags gesellschaft mbH, 1993). The antibody is combined with a test sample under conditions where the antibody will bind specifically to any modulator that might be present, but not any other proteins liable to be in the sample. The complex formed can be measured in situ (U.S. Patent Nos. 4,208,479 and 4,708,929), or by physically separating it from unreacted reagents (U.S. Patent No. 3,646,346). Separation assays typically involve labeled target reagent (competition assay), or labeled antibody (sandwich assay) to facilitate detection and quantitation of the complex. Suitable labels are radioisotopes such as $^{125}I$, enzymes such as β-galactosidase, and fluorescent labels such as fluorescein. Antibodies of this invention can also be used to detect molecules that cause receptor release in fixed tissue sections by immunohistology. The antibody is contacted with the tissue,

unreacted antibody is washed away, and then bound antibody is detected — typically using a labeled anti-immunoglobulin reagent. Immunohistology will show not only whether the modulator is present, but where it is located in the tissue.

Detection of molecules that cause release of cytokine receptors is of interest for research purposes, and for clinical use. High expression of receptor cleaving enzymes may correlate with progression of cancer. Diagnostic tests are also of use in monitoring substances causing receptor release that are administered in the course of therapy.

Antibodies of this invention can also be used for preparation of medicaments. Antibodies with therapeutic potential include those that affect receptor releasing activity — either by promoting clearance of a receptor protease, or by blocking its physiological action. Antibodies can be screened for desirable activity according to assays described in the next section.

Screening assays

This invention provides screening methods for selecting and developing products that modulate the activity of receptor releasing compounds of this invention, and thus affect cytokine signaling.

A screening method embodied in this invention is a method for screening substances that interfere with the action of a receptor protease at the protein level. The method involves incubating cells expressing cytokine receptor (such as C75R or THP cells) with a polypeptide of this invention having receptor releasing activity. There are two options for supplying the molecule with receptor releasing activity in this assay. In one option, the polypeptide is added to the medium of the cells as a reagent, along with the substance to be tested. In another option, the cells are genetically altered to express the molecule at a high level, and the assay requires only that the test substance be contacted with the cells. This option allows for high throughput screening of a number of test compounds.

Either way, the rate of receptor release is compared in the presence and absence of the test substance, to identify compounds that enhance or diminish receptor releasing activity. Parallel experiments should be conducted in which the activity of the substance on receptor shedding is tested in the absence of added polypeptide (using cells that don't express the polypeptide). This will determine whether the activity of the test substance occurs via an effect on the receptor releasing molecule being added, or through some other mechanism.

Another screening method of this invention involves an assay in which a cytokine receptor cleaving enzyme is combined with a short peptide spanning the receptor cleavage site. Enzyme activity can be measured, for example, by change in molecular weight of the peptide (detectable by mass spectroscopy), or labeling the peptide with a fluorescent quench pair. The test compound is then added to this system to determine whether it inhibits the rate of cleavage of the peptide by the enzyme. An illustration of such an assay is provided below in Example 4.

Screening assays are useful for high throughput screening of small molecule compounds that have the ability to affect the level of cytokine receptors on a cell, by way of its influence on receptor

release. Small molecule compounds that have the desired activity have beneficial properties in the making pharmaceutical compositions, such as being more stable and less expensive to produce.

Medicaments and their use

As described earlier, a utility of certain products embodied in this invention is to affect signal transduction from cytokines. Products that promote receptor release have the effect of decreasing cytokine receptors on the surface of cells, which decrease signal transduction. Conversely, products that inhibit receptor release prevent cleavage of cytokine receptors, increasing signal transduction.

The ability to affect signal transduction is of considerable interest in the management of clinical conditions in which cytokine signaling contributes to the pathology of the condition. Such conditions include:

- Heart failure. IL-1β and TNF are believed to be central mediators for perpetuating the inflammatory process, recruiting and activating inflammatory cells. The inflammation depress cardiac function in congestive heart failure, transplant rejection, myocarditis, sepsis, and burn shock.
- Cachexia. The general weight loss and wasting occurring in the course of chronic diseases, such as cancer. Cytokines are believed to affect appetite, energy expenditure, and metabolic rate.
- Crohn's disease. The inflammatory process mediated by multiple cytokines leads to thickening of the intestinal wall, ensuing from lymphedema and lymphocytic infiltration.
- Endotoxic shock. The shock induced by release of endotoxins from gram-negative bacteria, such as E. coli, involves cytokine mediated inflammation
- Arthritis. TNF, IL-6, IL-1 and other cytokines promote expression of nitric oxide synthetase, believed to be involved in disease pathogenesis of rheumatoid arthritis and other arthritis sub-types.

Other conditions of interest include those conditions where part of the pathology is caused by inflammation, or a cross-over between inflammation and other biological systems. Non-limiting examples are multiple sclerosis, ankylosing spondylitis, psoriasis, psoriatic arthritis, osteoarthritis, arteriosclerosis, sepsis, ulcerative colitis, arteriosclerosis, inflammation brought on by microbial infection, and diseases that have an autoimmune etiology, such as Type I Diabetes, myasthenia gravis, and systemic lupus erythematosis.

Polypeptides of this invention that promote receptor cleavage activity can be administered with the objective of decreasing or normalizing cytokine signal transduction. For example, in congestive heart failure or Crohn's disease, the polypeptide is given at regular intervals to lessen the inflammatory sequelae. The treatment is optionally in combination with small-molecule anti-inflammatory agents (such as methyltrexate), or with other agents that affect signal transduction (such as cytokine blockers

like Enbrel®, or receptor antagonists like Kineret®) or that lessen the extent of inflammation in other ways.

Polynucleotides of this invention can also be used to promote cytokine receptor cleavage by gene therapy. The encoding sequence is operatively linked to control elements for transcription and translation in human cells. It is then provided in a form that will promote entry and expression of the encoding sequence in cells at the disease site. Forms suitable for local injection include naked DNA, polynucleotides packaged with cationic lipids, and polynucleotides in the form of viral vectors (such as adenovirus and AAV constructs). Methods of gene therapy known to the practitioner skilled in the art will include those outlined in U.S. Patent Nos. 5,399,346, 5,827,703, and 5,866,696.

The ability to affect cytokine signal transduction is also of interest where a cytokine is thought to play a beneficial role in resolving the disease. In particular, TNF and other cytokines play a beneficial role in the necrotizing of solid tumors. Accordingly, products of this invention can be administered to cancer patients to inhibit receptor release, thereby increasing cytokine signal transduction and improve the beneficial effect.

Embodiments of the invention that inhibit receptor release include antisense polynucleotides. A method of conferring long-standing inhibitory activity is to administer antisense gene therapy. A genetic construct is designed that will express RNA inside the cell which in turn will decrease the transcription of the target gene (U.S. Patent No. 5,759,829). In humans, a more frequent form of antisense therapy is to administer the effector antisense molecule directly, in the form of a short stable polynucleotide fragment that is complementary to a segment of the target mRNA (U.S. Patent Nos. 5,135,917 and 5,789,573) — in this case, the transcript that encodes the receptor releasing molecule. Another embodiment of the invention that inhibits receptor release are ribozymes, constructed as described in an earlier section. The function of ribozymes in inhibiting mRNA translation is described in U.S. Patent Nos. 4,987,071 and 5,591,610.

Once a product of this invention is found to have suitable receptor releasing activity in the in vitro assays described in this disclosure, it is preferable to also test its effectiveness in an animal model of a cytokine mediated disease process. The Examples below provide animal models for sepsis, arthritis, multiple sclerosis, edema, and asthma. Those skilled in the art will know of other animal models for testing effects on cytokine signal transduction or inflammation: for example, the cardiac ischemia reperfusion models of Weyrich et al. (*J. Clin. Invest.* 91:2620, 1993) and Garcia-Criado et al. (*J. Am. Coll. Surg.* 181:327, 1995); the pulmonary ischemia reperfusion model of Steinberg et al. (*J. Heart Lung Transplant.* 13:306, 1994), the lung inflammation model of International Patent Application WO 9635418; the bacterial peritonitis model of Sharar et al. (*J. Immunol.* 151:4982, 1993), and the colitis model of Meenan et al. (*Scand. J. Gastroenterol.* 31:786, 1996).

For use as an active ingredient in a pharmaceutical preparation, a polypeptide, polynucleotide, or antibody of this invention is generally purified away from other reactive or potentially immunogenic components present in the mixture in which they are prepared. Typically, each active ingredient is

provided in at least about 90% homogeneity, and more preferably 95% or 99% homogeneity, as determined by functional assay, chromatography, or SDS polyacrylamide gel electrophoresis. The active ingredient is then compounded into a medicament in accordance with generally accepted procedures for the preparation of pharmaceutical preparations, as described in standard textbooks on the subject. Steps in the compounding or formulating of the medicament depend in part on the intended use and mode of administration, and may include sterilizing, mixing with appropriate non-toxic and non-interfering excipients, buffers and other carriers, lyophilizing or freezing, dividing into dose units, and enclosing in a delivery device. The medicament will typically be packaged in a suitable container accompanied by or associated with written information about its intended use, such as the inflammatory disease to be treated, and aspects of dosing and administration.

Mode of administration will depend on the nature of the condition being treated. For conditions that are expected to require moderate dosing and that are at well perfused sites (such as cardiac failure), systemic administration is acceptable. For example, the medicament may be formulated for intravenous administration, intramuscular injection, or absorption sublingually or intranasally. Sometimes it is possible to administer the active ingredient locally to the disease site (such as near an inflamed joint), in order to enhance the concentration of the active ingredient, and minimize effects on cytokine receptors on other tissues not involved in the disease process. Alternatively, the pharmaceutical composition may be formulated to enhance accumulation of the active ingredient at the disease site. For example, the active ingredient can be encapsulated in a liposome or other matrix structure that displays an antibody or ligand capable of binding a cell surface protein on the target cell. Suitable targeting agents include antibodies or ligands for tissue-specific receptors (e.g., serotonin for pulmonary targeting). For compositions that decrease cytokine signaling, an appropriate targeting molecule may be the cytokine itself, since the target tissue may likely display an unusually high density of the corresponding receptor.

Effective amounts of the compositions of the present invention are those that alter receptor releasing activity by at least about 10%, typically by at least about 25%, more preferably by about 50%, 75%, or even 90%. Where increase of receptor release is desirable, preferred compositions increase receptor release by at least 2-fold. A minimum effective amount of the active compound will depend on the disease being treated, which of the effector molecules is selected for use, and whether the administration will be systemic or local. Effective doses can be estimated from the animal test results indicated below, scaling appropriately for the subject being treated.

The following examples provided as a further guide to the reader, and are not intended to limit the invention.

## EXAMPLES

### Example 1: Isolation of naturally occurring receptor cleaving enzyme activity

Receptor cleaving activity present in inflammatory cells was first isolated using an assay system using cells transfected to express cytokine receptors on their surface.

cDNA of human p75 TNF receptor was cloned from a λgt10 cDNA library from human monocytic U-937 cells, covering positions 58-2380 of the reported p75 TNF-R sequence, which encompasses the full length of the p75 TNF-R-coding sequence from positions 90-1475. The 2.3 kb p75 TNF-R cDNA was then subcloned into the pCDNA3 eukaryotic expression vector, and verified by restriction endonuclease mapping. The transfected cell line was designated C75R. The level of p75 TNF-R expression was assessed using $^{125}$I-labeled human recombinant TNF, and estimated to be 60,000-70,000 receptors per cell with an affinity of $5.6 \times 10^{-10}$ M.

Receptor cleaving activity was obtained from THP-1 cells (ATCC 45503) as follows. $1 \times 10^6$ cells/mL in RPMI-1640 plus 1% FCS were stimulated with $10^{-6}$ M phorbol myristal acetate (PMA) for 30 min at 37°C. Other stimulating agents can be used as an alternative, such as IL-10 and epinephrine. The cells were washed, and cultured in fresh medium for 2 h, and the cell-free supernatant was collected. Specific binding of $^{125}$I-TNF to the C75R cells was decreased by 87% after preincubating with the THP-1 supernatant. Soluble p75 TNF-R released into the supernatant was measured by ELISA. One unit of receptor cleaving activity was defined as 1 pg of net soluble p75 TNF-R release. The protease was found to release both p55 and p75 receptors from the surface of THP-1 cells.

Native receptor cleaving activity harvested from stimulated THP-1 cells was purified as follows. First, protein from the medium was concentrated by 100% saturated ammonium sulfate precipitation, resuspended in PBS, and dialyzed into 10 mM Tris-HCl, 60 mM NaCl, pH 7.0. This sample was loaded on an anion-exchange chromatography, DEAE-Sephadex® A-25 column. Receptor cleaving activity was eluted with a linear gradient of 60 to 250 mM NaCl in 50 mM Tris-HCl, pH 8.0.

DEAE fractions showing activity in the C75R cleavage assay can be purified further (WO 98/02140). The fractions were concentrated to 500 µL, and applied to electrophoresis on 6% polyacrylamide gels under non-denaturing conditions. The gel was sliced horizontally into 5 mm strips, which were each eluted into PBS.

**Figure 1** shows the results. In the top panel, receptor cleaving activity (measured using C75R cells) eluted from the DEAE column at lower ionic strength than the bulk of the protein in the extract ($A_{280}$), consistent with a relatively electropositive pI. In the lower two panels, receptor activity had a defined mobility on native gels. The purified preparations were then analyzed for physicochemical and functional characteristics of the receptor cleaving enzyme.

Example 2: Gene cloning of receptor cleaving activity

Different inflammatory cells have been found to express high levels of receptor cleaving activity, including the cell lines designated THP-1, U-937, HL-60, ME-180, MRC-5, Raji, K-562. Jurkat cells have cleaving activity of 850 U/mL following stimulation with $10^{-2}$ M PMA). In this example, the expression library of the Jurkat T cell (ATCC #TIB-152) was obtained and used to clone out genes involved in regulating cytokine receptor release.

Sequences were selected from the library by repeated cycles of transfection into COS-1 cells, followed by assaying of the supernatant for proteolytic activity as in Example 1. Briefly, the DNA of $10^6$ Jurkat cells was extracted using an InVitrogen™ plasmid extraction kit according to manufacturer's directions. cDNA was inserted in the ZAP Express™/Eco*RI* vector (cat. no. 938201, Stratagene, LaJolla CA. The library was divided into 48 groups of DNA and transformed into COS-1 cells using the CaCl transfection method. Once the cells were grown out, the C75R assay was performed, and five positive groups were selected. DNA from each of these five groups was obtained, and transfected into *E. coli*, with 15 plates per group. DNA was prepared from these cells and then transfected into COS-1 cells once more. The cells were grown out, and cleavage activity was tested again. Two positive groups were selected and transfected into *E. coli,* yielding 98 colonies. DNA was prepared from 96 of these colonies and transfected into COS-1 cells. The C75R assay was performed again, and nine clones were found to substantially increase receptor cleavage activity in the assay. These clones were designated MP1 to MP9, and then sequenced by DyeDeoxy sequencing techniques.

TABLE 1: Cloned Genes Associated with Cytokine Receptor Releasing Activity

| Clone designation | Length (bp) | Nucleotide SEQ. ID NO: | Open reading frame | Protein SEQ. ID NO: |
|---|---|---|---|---|
| MP1 | 4,047 | 1 | 482 amino acids | 30 |
| " | | 1 | 163 amino acids | 31 |
| MP2 | 739 | 2 | (partial sequence) | |
| " | 233 | 3 | (partial sequence) | |
| MP3 | 2,998 | 4 | 482 amino acids | 32 |
| " | | 4 | 368 amino acids | 33 |
| " | | 4 | 363 amino acids | 34 |
| " | | 4 | 254 amino acids | 35 |
| MP4 | 4,152 | 5 | 1022 amino acids | 36 |
| MP5 | 3,117 | 6 | 1028 amino acids | 37 |
| MP6 | 3,306 | 7 | 849 amino acids | 38 |
| " | | 7 | 208 amino acids | 39 |
| MP7 | 4,218 | 8 | 869 amino acids | 40 |
| MP8 | 1,187 | 9 | 281 amino acids | 41 |
| MP9 | 3,306 | 10 | 768 amino acids | 42 |

Example 3: Characterization of the cloned sequences

The sequences have been compared with the UniGene compilation of expressed human genes. SEQ. ID NOs:11 to 25 were analyzed in March of 2003, using UniGene *homo sapiens* build #159, dated January 25, 2003. The database contained 4056423 sequences in 108944 assemblies.

TABLE 2: UniGene Match of Clone Sequences

| Clone | No. of Amino acids in open reading frame | GenBank Accession | Description | Nucleotide SEQ. ID NO: | Protein SEQ. ID NO: |
|---|---|---|---|---|---|
| MP1 | 482 | | delangin isoform B | 79 | 80 |
| " | 163 | NM_058189 | EST | 11 | 43 |
| MP2 | | | *(no match)* | | |
| MP3 | 482 | | *(no match)* | | |
| " | 368 | NM_002819 | Polypyrimidine Tract Binding Protein | 12 | 44 |
| " | 363 | NM_012402 | Carboxylase Protein | 13 | 45 |
| " | 254 | NM_006452 | RAC-1 binding protein (Arfaptin) | 14 | 46 |
| MP4 | 1022 | NM_014718 | Calsyntenin3 | 15 | 47 |
| " | | | Eukaryotic Initiation Factor 5A | 16 | 48 |
| MP5 | 1038 | NM_001970 | image clone 4328688 | 17 | 49 |
| MP6 | 849 | NM_000876 | IGF2 Receptor (Mannose-6-phosphate receptor) | 18 | 50 |
| " | 208 | NM_007006 | pre-mRNA cleavage factor "Im" | 19 | 51 |
| MP7 | 869 | AK091534 | EST1 | 20 | 52 |
| " | | AK074035 | EST2 | 21 | |
| MP8 | 281 | AF110322 | CDK5 Associated Protein | 22 | 53 |
| MP9 | 768 | NM_007040 | E1B-55kDa-associated protein 5 isoform a | 23 | 54 |
| " | | AB075864 | EST1 | 24 | 55 |
| " | | AF318367 | EST2 | 25 | 56 |

The following species orthologs and full-length image clones have been identified (percent identity and conserved residues calculated by the *blastp* BLOSUM62 algorithm at the NCBI Blast website, with a gap cost of 11 (Extension 1).

TABLE 3: MP8 Related Sequences

| Species | GenBank | % Identical | % Conserved | Nucleotide SEQ. ID NO: | Protein SEQ. ID NO: |
|---|---|---|---|---|---|
| Human | AF110322 | (100%) | (100%) | 22 | 53 |
| Mouse | BC002318 | 87% | 93% | 26 | 57 |
| Rat | AAH81793 | 87% | 94% | | 81 |
| Danio rerio (zebrafish) | AAH71504 | 62% | 81% | | 82 |
| Tetraodon nigroviridis (Spotted Green Pufferfish) | CAG00652 | 61% | 78% | | 83 |
| Xenopus laevis (African clawed frog) | AAH77996 | 57% | 76% | | 84 |
| Anopheles gambiae (mosquito) | XP_318917 | 39% | 59% | | 85 |
| Caenorhabditis briggsae (soil nematode) | CAE67421 | 38% | 56% | | 86 |
| Drosophila melanogaster (fruit fly) | AAL28291 | 36% | 54% | | 87 |
| Arabidopsis thaliana (thale cress) | NP_196301 | 29% | 47% | | 88 |
| Human MP8 | Image clone 4130677 | | | 28 | 59 |
| Mouse MP8 | Image clone 3593792 | | | 29 | 60 |

**Figure 2** is an alignment of full-length human MP8 (SEQ. ID NO:53) compared with species orthologs (SEQ. ID NOs:57 and 81-88). Dots indicate residues that are identical with the human sequence on the top line. As reflected in Table 3, the orthologs are remarkably similar, sharing a good deal of identity especially between vertebrates (SEQ. ID NO:89) and mammals (SEQ. ID NO:90) — particularly towards the N- and C-terminals. Motifs are identified (SEQ. ID NOs:91-102) that are shared throughout the family.

Example 4: Measuring receptor cleaving activity by fluorescence resonance energy transfer

Cytokine-specific proteolytic activity can rapidly be quantified by Fluorescence Resonance Energy Transfer (FRET). Peptides having the amino acid sequence of the TNF Receptor or other protein substrates are labeled at opposite ends with a fluorescence emitter and a fluorescence

quencher. The peptide is then incubated with a source of receptor cleaving enzyme, and fluorescence is measured. The quenching group normally absorbs fluorescence from the emitter. But enzymatic cleavage of the peptide decouples the quenching group, and fluorescence emission increases proportionally.

For Examples 4 through 14, MP8 was produced under research conditions on a small scale. The MP8 cDNA sequence (SEQ. ID NO:9) was cloned into an expression vector behind an N-terminal His Tag sequence followed by a thrombin site. The protein extract was chromatographed on Q-Sepharose®, the peak was purified by fast-flow chromatography on Ni-NTA, and endotoxin levels was reduced on a Q-Sepharose® (HiTrap) column. Endotoxin level was measured using a chromogenic LAL assay.

**Figure 2** shows Western analysis of a typical MP8 preparation. Using either anti-His or anti-MP8, the product had an apparent molecular weight in the 45-50 kDa range.

The peptides used as substrates in the cleavage assay were taken from the known sites of proteolytic cleavage of the whole protein. The peptides were labeled with the fluorescence emitter (Edans-•) at the C-terminal, and the quenching hapten (Dabcyl-▲) at the N-terminal.

The assay is conducted in the presence of the metal cations $Zn^{++}$ (0.1 mM) and $Ca^{++}$ (2 mM), and corrected for cleavage in the presence of EDTA (20 mM). Dependence on divalent cations confirms that the enzyme activity measured in this assay is a metalloprotease. The assay mixture also contains a cocktail of protease inhibitors and bovine albumin. Cleavage is measured as the net change in fluorescence emission after incubating the enzyme with the peptide at 37°C for 3 hours.

| Cytokine receptor peptides: | | SEQ. ID NO: |
|---|---|---|
| p55 TNF Receptor | ▲-N-V-K-G-T-E-D-S-G-• | 71 |
| p55 TNF Receptor (peptide 2) | ▲-K-G-T-E-D-S-G-T-T-• | 72 |
| p75 TNF Receptor | ▲-C-T-S-T-S-P-T-R-• | 73 |
| IL-6 Receptor | ▲-A-N-A-T-S-L-P-• | 74 |
| IL-1 Type I Receptor | ▲-T-H-G-I-D-A-A-Y-I-Q-• | 75 |
| IL-1 Type II Receptor | ▲-Q-T-K-R-T-T-V-K-E-A-• | 76 |

| Other substrate peptides: | | SEQ. ID NO: |
|---|---|---|
| pro TNF (TACE substrate) | ▲-L-A-Q-A-V-R-S-S-S-R-● | 77 |
| TNO-211 (matrix metalloprotease substrate) | ▲-γ-Abu-P-Q-G-L-E(●)-A-K-$NH_2$ | 78 |

**Figure 4(A)** shows results of a typical FRET assay for the purified clone MP8. Enzyme activity is calculated as Fluorescence Units per mL, the activity that causes increase in fluorescence at 460 nm in the presence of $Zn^{++}$ and $Ca^{++}$, corrected for the activity measured in the presence of EDTA.

MP8 cleaves the peptides from both the p55 and p75 TNF receptors (TNF-R1 and TNF-R2). These peptides span the extracellular region believed to be cleaved when TNF receptor is released from cells in vivo. MP8 also cleaves the IL-6 receptor peptide with high activity. Data published elsewhere indicate that metalloproteases ADAM-10 and MDC-9 do not efficiently cleave either the p55 or p75 TNF receptor.

This is the standard assay method used in the following Examples for quantitation of receptor cleaving activity.

**Figure 4(B)** is taken from an experiment measuring cleavage of peptides to determine the ability of MP8 to cause release of human IL-1 type1 and type 2 receptors.

**Figure 4(C)** is a compilation of data from experiments with different peptide substrates, showing relative cleavage activity standardized to the p55 TNF receptor peptide. Clone MP8 shows specificity for several receptors involved in the inflammatory pathway (TNF-R p55 and p75 isoforms, IL-6 receptor and IL-1 receptors) in comparison with control substrates spanning known cleavage sites of other proteases. MMP-1 is fibroblast collagenase, MMP-2 is stromalysin, MMP-3 is gelatinase A, MMP-11 is collagenase III, renin is an aspartate protease; malaria is a cysteine protease, and CMV is a serine protease.

**Figure 4(D)** is taken from an experiment illustrating the use of the FRET assay to assess potential inhibitors or activators. The MP8 protein was combined with the inhibitor, added to the FRET peptide, and cleavage (increased fluorescence) was measured after 3 hours.

Inhibitors were as follows: 1,10 phenanthroline at 40 mM; phospharamidon at 500 μM; hydroxamate (Pharmingen) at 2 mM; TIMP-1 (Chemicon) at 2.5 μg/mL; TIMP-2 (Chemicon) at 2.5 μg/mL. Hydroxamate is a small molecule metalloprotease inhibitor. The TIMPs are naturally occurring tissue metalloprotease inhibitors. .

Example 5: MP8 causes TNF receptor release in vivo

To demonstrate that clone MP8 is a protease capable of cleaving receptors from cell surfaces, MP8 was incubated with THP-1 cells. This cell line expresses both the p55 TNF receptor and the p75 TNF receptor. After incubating for 45 minutes at 37°C, the cells were washed, immunostained for cell surface receptor, and counted by flow cytometry.

**Figure 5** shows that MP8 cleaves both the p55 and p75 TNF receptors when presented on the surface of cells. .

To determine if the enzyme cleaves TNF receptors *in vivo*, 100,000 FU of purified MP8 was injected subcutaneously as a 500 μL bolus into female Balb/c mice. Control mice were injected with saline. Serum was sampled periodically, and tested for cytokine levels using ELISA kits from R&D System.

**Figure 6** shows that the cloned enzyme causes shedding of TNF receptor to an extent that causes it to accumulate in the circulation.

**Figure 7** is a compilation of data from two experiments, showing the kinetics of receptor release over a 48 hour period. These data have several important implications:

- Both the p55 and p75 TNF receptors are cleaved *in vivo* — meaning that signal transduction through either receptor will be affected.
- Cloned MP8 causes an increase in the level of circulating TNF receptor by about 100-fold. The enzyme should affect TNF signal transduction in two ways — by removing TNF receptor from the surface of inflammatory cells at the affected site, and creating an extracellular sink for TNF ligand.
- The effect of MP8 persists for 48 hours after administration — meaning that frequent dosing is not required.
- Human receptor cleaving enzyme is evidently capable of cleaving TNF receptors of other species. This validates use of the mouse as a model for studying the effects of this enzyme family as therapeutic agents.

Example 6: MP8 protects against septic shock

A classic model for determining effectiveness of agents against cytokine-mediated inflammation is endotoxin-induced septic shock (Morrison et al., J Infect Dis 162:1063, 1990). When tested in this model, the cloned MP8 enzyme was found to be completely protective against septic shock in a dose-dependent fashion, whether given simultaneously with the LPS challenge, or 3 hours in advance.

In order to test effectiveness in the septic shock model, MP8 enzyme was prepared under contract by Alliance Protein Laboratories from source material produced at Biosource International. The enzyme was purified using Nickel NTA column chromatography and Q-Sepharose® analytical column chromatography. Endotoxin level was reduced using Q-Sepharose®. Enzymatic activity was

determined in the FRET assay, and endotoxin contamination was determined in a chromogenic LAL assay. Endotoxin was 0.16 μg per 300,000 fluorescence units of enzyme activity.

The septic shock experiment was conducted as follows. Female Balb/c mice were randomized by weight into 6 treatment groups. Sepsis was induced by injecting 10 μg LPS and 7 mg galactosamine in the lateral or dorsal caudal vein. Some of the groups were pretreated with 50,000 FU of MP8 simultaneously or 3 hours in advance of the LPS challenge to determine whether there was a protective effect.

**Figure 8** is a Kaplan-Meier survival curve, showing the mortality in each of the treatment groups in the study. Without any treatment or challenge, there is no effect on the animals, and the line stays at the top. Life-compromising challenge causes the line to fall to the bottom of the graph in a step-wise fashion as each animal succumbs. Treatment that is protective against the challenge maintains the line near the top of the graph.

The results of this experiment show that MP8 is fully protective against LPS-induced septic shock at a dose of 50,000 — whether it is given simultaneously with the LPS challenge, or 3 hours in advance.

**Figure 9** shows an experiment in which the amount of MP8 was titrated out to determine the minimum effective dose. The enzyme was administered in a volume of 110 μL at one hour before the LPS challenge.

The data show that the protective effect of MP8 is dose-dependent. The LPS/galactosamine challenge was invariably fatal in mice treated only with saline control. At a single dose of 30,000 FU, MP8 was able to protect a proportion of the challenged subjects. At 300,000 FU, MP8 was completely protective.

Example 7: Stability of receptor cleaving activity upon storage

The data provided in this example show that MP8 retains its full activity for more than a month's storage both in terms of its proteolytic activity in the FRET assay, and its protective effect in the septic shock model. It is able to protect mice against LPS challenge at least 3 days after administration — indicating that frequent dosing with receptor releasing enzyme is not required for it to have a therapeutically important effect.

The stability of MP8 was determined by storing aliquots of MP8 at 4°C or -70°. Aliquots were taken out periodically to determine enzyme cleaving activity in the fluorescence resonance energy transfer peptide cleavage assay.

**Figure 10** shows that in its purified form, MP8 is as stable in a standard refrigerator as it is in deep freeze. There was no detectable loss of activity after a month of storage.

Stability of the clinical effect of MP8 was determined in the septic shock model. Female Balb/c mice were injected with 300,000 FU of MP8 that had been stored for 4 days at -70°C, or 7 days at 4°C. One hour later, the mice were challenged with 10 μg LPS and 7 mg galactosamine as before.

**Figure 11** shows the results. The purified enzyme has a stable shelf life, and can be frozen without causing a loss in clinical efficacy.

Example 8: Persistence of receptor release in vivo

In this example, durability of receptor cleavage *in vivo* was determined by treating animals subcutaneously up to three days in advance with 300,000 FU of MP8. The mice were then challenged with 10 μg LPS and 7 mg galactosamine in the usual fashion.

**Figure 12(A)** shows the clearance of MP8 protein from the circulation of mice, determined by dot-blot analysis. The first clearance half-time is ~39 hours.

**Figure 12(B)** shows that the cloned enzyme is able to protect most mice up to 3 days after administration. This is either because the enzyme continues to cleave TNF receptor for at least this long, or because an effect of the treatment (such as release of TNF receptor) persists in such a manner that TNF signal transduction is modulated for several days. Either way, this result means that frequent dosing with the enzyme is not required for clinical efficacy.

Tissue distribution of MP8 expression was determined by Northern analysis. mRNA preparations from various human tissues were probed using labeled oligonucleotides based on the MP8 sequence.

Substantial expression was observed in peripheral blood, and in other tissues relatively rich in macrophage-like cells and other leukocytes (liver, spleen, small intestine). There is a degree of MP8 expression in all tissues, which is consistent with the view that the enzyme acts as a down-regulator of inflammation on an ongoing basis.

Example 10: MP8 treats experimentally induced arthritis

The potential clinical effectiveness of receptor cleaving enzyme was assessed in collagen-induced arthritis, an animal model for rheumatoid arthritis. In this example, the animals were treated daily with 300,000 FU of MP8, simultaneously with administration of the disease agent. Eight out of 9 control mice were affected, but MP8 treated mice showed no joint swelling or other signs of arthritis. The treatment was at least as effective as a scaled dose of Enbrel® (etanercept).

Collagen-induced arthritis is a standard model for evaluating potential therapeutic agents for rheumatoid arthritis (Courtenay et al., Nature 283:666, 1980; Williams et al., Proc Natl Acad Sci USA 89:9784, 1992; Gerlag et al., J Immunol 165:1652, 2000). Arthritis was induced in 7-9 week old female DBA/1LacJ mice by immunization with collagen. On day 0, the mice were injected at the base of the tail with 100 μg bovine type II collagen in complete Freund's adjuvant. On day 7, mice were boosted with an intraperitoneal injection of 100 μg collagen. To enhance and synchronize synovitis, the mice were injected subcutaneously on day 14 with 100 μg of lipopolysaccharide (LPS). Joint swelling was monitored in a blinded fashion by measuring the diameter in all 4 paws and both ankles using a constant

pressure gauge. Arthritis Index was also determined on the following scale, and summed for all extremities. 0 ≡ normal; 1 ≡ one digit swollen; 2 ≡ more than one digit swollen; 3 ≡ joint distortion; 4 ≡ ankylosis. The arthritis experiments were conducted under contract by Calvert Preclinical Services, Inc., Olyphant PA.

**Figure 13** shows results of an experiment in which mice were treated with a daily dose of 300,000 FU of MP8, a scaled dose of *Enbrel* ®, or saline control, starting 3 days before the first collagen injection. As is typical in this model, not all animals respond to the collagen challenge. In this experiment, 8 out of the 9 control animals showed signs of arthritis. However, none of the animals treated with MP8 were affected.

**Figure 14** shows the average increase in joint swelling and arthritis index measured for the animals in each group. When MP8 was given prophylactically, it prevented the animals from showing any measurable signs of the disease.

Example 11: MP8 prevents progression of established arthritis

In this example, clone MP8 was tested for its ability to treat established disease. This protocol is closer to the clinical situation in rheumatoid arthritis, where patients are treated after the onset of inflammatory synovitis.

Mice were immunized with collagen on days 0, and 7, and then boosted with LPS on day 14. Treatment with MP8 was initiated on day 22 when arthritis was well established. At that time, animals with arthritis were randomized into three groups, and unaffected animals were excluded. The three affected groups were then treated for 18 consecutive days with saline control, or with MP8 at either of two different doses.

**Figure 15** shows the results. The results show a highly significant reduction in swelling in the affected joints of the two MP8-treated groups compared with control ($n = 10$ in each group; $p < 0.001$ at the end of the experiment, 1-tailed Student's *t*-test). The control animals had little change in joint swelling after daily treatment was commenced at day 22. In contrast, the animals treated with MP8 at either dose showed substantial regression of disease.

**Figure 16** shows the level of antibodies to type II collagen in the serum of the mice, as determined by ELISA. The groups treated with low or high doses of MP8 had levels of pathogenic antibody that were almost half the control (both $p < 0.02$). The group treated at the higher dose of MP8 also had lower severity of inflammatory synovitis as determined by histopathology at the end of the experiment. No toxicity was detected in the MP8 treated groups.

These experiments show that systemic administration of receptor cleaving enzyme is both safe and effective in the treatment of experimentally induced arthritis — even in established disease. MP8 reduces the level of circulating autoantibody, and prevents or reverses joint swelling.

Example 12: MP8 inhibits carrageenan-induced edema

Further experiments were performed to determine whether the beneficial effects of cytokine receptor releasing enzyme extend beyond arthritis. In the carrageenan-induced paw edema model, systemically administered MP8 was at least as effective as orally administered indomethacin in protecting against cytokine-mediated fluid accumulation.

Based on the standard model (Winter et al., Proc Soc Exp Biol Med 111:544, 1962; Hansra et al., Inflammation 24:141, 2000), the experiment was performed as follows. Male Sprague Dawley rats were randomized by weight into three treatment groups. The animals were administered MP8 or control solution subcutaneously, or indomethacin by mouth. One hour later, they were injected in the left hind paw with a sterile solution of 1% carrageenan suspension in water, in order to induce swelling. Three hours later, the volume of the injected paw was measured by water displacement in a water plethysmograph (mean ± SEM; 10 animals per group).

Figure 17 shows the results. MP8 inhibited edema formation by 62% ($p \leq 0.05$). In comparison, indomethacin (a small-molecule nonsteroidal anti-inflammatory agent) inhibited edema formation by only 49%.

Example 13: MP8 prevents development of Multiple Sclerosis

In Experimental Autoimmune Encephalomyelitis, an animal model for Multiple Sclerosis, cytokine receptor releasing enzyme was found to delay emergence of symptoms from 12 days to 18 days, and lowered disease severity by about 3-fold.

The EAE model (Brown et al., Lab. Invest. 45:278, 1981) was conducted as follows. Female SJL/J mice (6 weeks old) were randomized into 3 groups of 10. 2 mL of Myelin Proteolipid Peptide (PLP) was emulsified in 3 mL of Complete Freund's Adjuvant containing an additional 20 mg of *M. tuberculosis* H37Ra. On day 0, mice were immunized subcutaneously in the base of the tail and footpad with a total of 60 μg PLP. They were also given 400 mg pertussis toxin i.p. on days 0 and 2. MP8 or saline control was administered s.c. every day from day –3 to day 20. Progression of the disease was measured up to day 21 on the following scale: 0 ≡ normal; 1 ≡ limp tail or hind limb weakness; 2 ≡ both limp tail and hind limb weakness; 3 ≡ partial hind limb paralysis; 4 ≡ complete hind limb paralysis; 5 ≡ moribund or sacrificed. **Figure 18** shows the results. MP8 had four clinically important effects.

- It completely prevented the disease from appearing in a proportion of animals
- In the animals that were affected, MP8 substantially delayed the onset of symptoms
- It reduced the severity of the disease by over 3-fold
- Treated animals continued to show normal weight gain

TABLE 4: Effect of MP8 on Experimental Autoimmune Encephalomyelitis

| Treatment | Incidence of Disease | Day of Onset (± SEM) | Peak Clinical Score (± SEM) |
|---|---|---|---|
| Saline Control | 7 out of 7 | 12.7 ± 0.6 | 3.2 ± 0.4 |
| MP8 - 100,000 FU | 5 out of 7 | 18.0 ± 0.8 [a] | 2.4 ± 0.7 |
| MP8 - 300,000 FU | 3 out of 7 | 18.0 ± 2.5 [a] | 1.0 ± 0.5 [b] |
| | | [a] $p < 0.001$ | [b] $p < 0.01$ |

Example 14: MP8 limits cellular involvement in experimentally-induced Asthma

In a further animal model for inflammation, clone MP8 was tested for its ability to modulate the pathology associated with experimentally induced Asthma.

Mice were sensitized on Days 0, 7, and 14 with 10 µg ovalbumin in 1% aluminum hydroxide. On Day 21, the mice were challenged with the allergen in aerosol form (5% wt/vol in saline). Treatment with MP8 or control was administered 1 h before the aerosol challenge, and 24 h and 48 h afterwards. On Day 24 (72 h after the challenge), lungs were lavaged under anesthesia with 2 x 0.5 mL buffer to recover cells in the alveolar fluid. **Figure 19** shows the results. MP8 reduced the number of the white blood cells migrating into the alveolar fluid. The proportion of eosinophils was also substantially reduced. These results indicate that MP8 reduced inflammatory and allergenic sequelae of an intrabronchial assault.

Example 15: Unexpected improvement in yield using full-length MP8

Process development was undertaken to scale up the production of MP8 for clinical testing. MP8 was produced in *E. coli* strain MP87 (HMS174(DE3)/pMP87). Only a small amount of the protein is expressed in a soluble form; the rest accumulated as inclusion bodies. While the amount of soluble protein produced in this manner may be adequate for research use, it is inadequate for clinical or commercial scale production.

Accordingly, a process was developed to refold the MP8 in the inclusion bodies. MP8 was recovered from the insoluble fraction obtained after centrifugation of crude extracts by dissolving the protein in buffered 8 M urea. The denatured product was fractionated by Ni affinity chromatography to separate the protein by way of an N-terminal poly-His sequence (HIS tag). After the affinity purification step, 1.8-2.5 mg of MP8 was recovered per gram of cells (65 to 90 mg per L of culture). The affinity purified protein was diluted in 50 mM sodium phosphate buffer at pH 8.0 supplemented with 1 mM EDTA. The protein was added to chilled (4°C) buffer at the rate of 1 ml/min and held for 1 to 2 hours on ice with gentle stirring. The MP8 was then captured on a Q-Sepharose™ anion exchange column and eluted with a linear salt gradient. One preparation of MP8 made using this procedure, designated MP87-086-20, was active in the murine sepsis model. However, this and subsequent preparations gave poor recoveries and in most cases showed evidence of extensive degradation. There was negligible improvement obtained by adding a cocktail of protease inhibitors to the buffers, or by any other strategies employed in an extensive series of experiments. After considerable process development, a typical recovery from the soluble and insoluble fractions combined was ~7.5 mg MP8 (3.3 g total protein) per liter of fermentation. The product was designated **MP8(7)**, and would be equivalent in terms of the active agent to the material used in previous testing.

As an alternative production process, the MP8 encoding sequence was cloned into the yeast *Pichia pastoris*. However, virtually no MP8 protein was recovered using expression systems which accumulate the protein in the cytoplasm or extracellularly. The evidence to date suggested that the protein was being degraded as fast as it was being synthesized.

The story turned out to be completely different for expression of the full-length protein. The human Image™ clone 4130677 (Table 3) was obtained, and ligated into plasmid pQE81 behind the T5 promoter and a HIS tag. At least 50% of the synthesized full-length protein (MP8-FL1) was produced as soluble protein, and could be purified in a straight forward manner. The supernatant of a crude extract was bound and eluted from a nickel affinity column followed by an anion exchange column, and then concentrated by diafiltration. The product was ~90% pure, and 75 mg MP8-FL1 was recovered per liter of fermentation.

Because of this remarkable improvement in yield, the decision was made to switch to full length MP8 for clinical testing. To produce MP8FL for clinical purposes, the His tag was removed, the *bla* gene replaced with *nptII*, and the resulting plasmid introduced into *E. coli* strain HMS174 to create strain MP820 (HMS174/pMP820) (**Figure 20**). Current production protocol is as follows: One mL working

stock is inoculated into 50 mL Turbo Prime Broth™ supplemented with 50 μg/mL kanamycin and incubated for 8 hours at 37°C. The 50 mL culture is used to inoculate 500 mL Turbo Prime Broth™ supplemented with 50 μg/mL kanamycin which is incubated overnight at 37°C. When the culture density reaches 2.3-2.8 $OD_{600}$, the temperature is lowered to 30°C and expression is induced by adding IPTG to 1 mM . Maximum accumulation of soluble protein occurs at 3 h after induction.

The current purification protocol begins by extracting the cells in a buffer containing Triton X-100™ and guanidine HCl at pH 7.8 on ice for 2 h. The clarified extract is subject to anion exchange chromatography using StreamLine™ QXL or Q-Sepharose™ FF resin. The bound MP8-FL is eluted, ~~and~~ further purified hydrophobic interaction chromatography on a Toyopearl™ Phenyl 650M column, and subject to final polishing (e.g., another anion exchange step). The protein produced from strain MP820 by this process has been designated MP8-FL2. Even though purification of the protein without the HIS tag takes more steps to purify, the yield is still considerably higher than the original MP8 clone (**Figure 21**). About 50 mg of MP8-FL2 is recovered per liter of fermentation, at a purity level of ~85 to 90% (**Figure 22**). Other properties of MP8-FL2 are shown in Table 5:

Table 5: Properties of MP8-FL2

| | | |
|---|---|---|
| Apparent Mass — | Calculated from amino acid sequence: | 57,305 |
| | SDS-PAGE: | 64,000 |
| | TOF mass spectroscopy: | 57,005 |
| | Native SEC-HPLC: | 120,000 |
| Isoelectric Point — | Theoretical: | 4.68 |
| | By isoelectric focusing: | 5.6 |

Other products have also been produced as control proteins for MP8 biological activity. One is the N-terminal fragment of the full length protein (SEQ. ID NO:53), purified by way of a HIS tag. Another is fibronectin polyprotein, the I27 domain of human fibronectin duplicated 8 times to produce a protein of 92,000 mol. wt. The synthetic fibronectin polyprotein is also purified by Nickel affinity chromatography followed by anion exchange chromatography. A third control preparation is an extract from the isogenic parent of the MP8-FL2 *E. coli* construct, subject to anion exchange chromatography in a similar manner to MP8-FL2.

Example 16: Full length MP8 is an effective anti-inflammatory agent

MP8-FL2 has been tested both in vitro and in vivo to determine whether it has biological activity suitable for treating inflammation.

**Figure 23** shows that MP8-FL2 causes release of cytokines from the cell surface. To determine cell surface cleavage activity, 25 μg of MP8-FL2 was combined with $2.5 \times 10^6$ cells. Release of both TNF receptor isoforms was determined by enzyme immunoassay as (treated - untreated) / untreated. The Bottom Panel shows that both the original MP8 core protein [I believe the MP8 in this picture is from the material made before Athena's involvement and is not MP87. Dropping the MP87 reference in the beginning would clarify this.] and MP8-FL2 both cause cleavage and release of TNF receptor, with preference for the R1 (p55) isoform. The same preparation of MP8-FL2 caused cleavage of both R1 (Dabcyl-N-V-K-G-T-E-D-S-G-Edans; SEQ. ID NO:71) and R2 (Dabcyl-C-T-S-T-S-P-T-R-Edans; SEQ. ID NO:73), whereas the matched synthetic fibronectin polyprotein control protein expressed and purified in the same manner had no activity.

**Figure 24** shows that MP8-FL2 reduces carrageenan-induced edema in an animal model. Male Sprague Dawley rats were administered with test protein or indomethacin (anti-inflammatory control), and then challenged in a hind footpad with carrageenan, as in Example 12. MP8-FL2 significantly prevented paw swelling, whereas the synthetic fibronectin polyprotein control protein did not.

**Figure 25** shows that MP8-FL2 is prophylactic against septic shock. Sepsis was induced in Balb/c mice by administering 10 μg LPS and 15 mg galactosamine, as in Example 3. Randomized groups were pretreated with up to 40 μg MP8-FL2 one hours in advance of the LPS challenge. The data show that MP8-FL2 protected the mice in a dose-dependent fashion.

These experiments demonstrate that MP8-FL2 has anti-inflammatory activity, and is suitable for use as a therapeutic agent for clinical use.

## SEQUENCE INFORMATION

TABLE 6: Sequences Listed in this Disclosure

| SEQ. ID NO: | Description | | Reference |
|---|---|---|---|
| 1 to 10 | Cloned genes that increase cytokine receptor releasing activity | DNA | This invention |
| 11 to 25 | UniGene match of cloned genes | DNA | Table 2 |
| 26 & 27 | MP8 species orthologs | DNA | Table 3 |
| 28 | Full length MP8 image clone (human) | DNA | Table 3 |
| 29 | Full length MP8 image clone (mouse) | DNA | Table 3 |
| 30 to 42 | Selected open reading frames of cloned genes | amino acid | This invention |
| 43 to 56 | UniGene match of cloned genes | amino acid | Table 2 |
| 26 & 27 | MP8 species orthologs | DNA | Table 3 |
| 28 | Full length MP8 image clone (human) | DNA | Table 3 |
| 29 | Full length MP8 image clone (mouse) | DNA | Table 3 |
| 57 & 58 | MP8 species orthologs | amino acid | Table 3; Figure 3 |
| 59 | Full length MP8 image clone (human) | amino acid | Table 3 |
| 60 | Full length MP8 image clone (mouse) | amino acid | Table 3 |
| 61 | human p55 TNF receptor | DNA | GenBank M58286 |
| 62 | | amino acid | |
| 63 | human p75 TNF receptor | DNA | GenBank NM_001066 |
| 64 | | amino acid | |
| 65 | human IL-6 receptor | DNA | GenBank NM_000565 |
| 66 | | amino acid | |
| 67 | human IL-1 type I receptor | DNA | GenBank AAH67508 |
| 68 | | amino acid | |
| 69 | human IL-1 type II receptor | DNA | GenBank NM_173343 |
| 70 | | amino acid | |
| 71 to 78 | receptor cleavage assay peptides | amino acid | This invention |
| 79 | GenBank match of MP1 | DNA | Table 2 |
| 80 | GenBank match of MP1 | amino acid | Table 2 |

TABLE 6: Sequences Listed in this Disclosure

| SEQ. ID NO: | Description | | Reference |
|---|---|---|---|
| 81-88 | More MP8 species orthologs | amino acid | Table 3; Figure 3 |
| 89 | Vertebrate consensus sequence | amino acid | Figure 3 |
| 90 | Mammal consensus sequence | amino acid | Figure 3 |
| 91-102 | Motifs | amino acid | Figure 3 |

*Implementation of the invention in a particular context may entail further optimization, which the skilled reader can accomplish as a matter of routine experimentation, without departing from the claimed invention and its equivalents*

## CLAIMS

The invention claimed is:

1. A method for preparing a pharmaceutical composition, comprising formulating a polypeptide or nucleic acid encoding said polypeptide as a pharmaceutical product,
   wherein the polypeptide comprises SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41, and causes a cytokine receptor to be released from the surface of a cell.

2. A method for causing a cytokine receptor to be released from the surface of a cell, comprising contacting the cell with a polypeptide or nucleic acid encoding said polypeptide,
   wherein the polypeptide comprises SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41.

3. A method for inhibiting signal transduction from a cytokine receptor into a cell, comprising contacting the cell with a polypeptide or nucleic acid encoding said polypeptide,
   wherein the polypeptide comprises SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41.

4. A method for screening a substance for its ability to inhibit release of a cytokine receptor from a cell, comprising:
   a) combining the substance with a polypeptide comprising SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41;
   b) contacting the polypeptide with a cell; and then
   c) determining whether the substance affects release of a cytokine receptor from the cell caused by the polypeptide;
   wherein the polypeptide comprises SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41.

5. A method for producing a protein that causes a cytokine receptor to be released from the surface of a cell, comprising expressing in *E. coli* a DNA that encodes SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41.

6. A method for treating inflammation in a subject, comprising administering to the subject a polypeptide or nucleic acid encoding said polypeptide,

wherein the polypeptide comprises SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41.

7. The method of any of claims 1-6, wherein the polypeptide comprises an amino acid sequence at least 80% identical to a sequence contained in SEQ. ID NO:53, but not in SEQ. ID NO:41.

8. The method of any of claims 1-6, wherein the polypeptide comprises an amino acid sequence at least 90% identical to a sequence contained in SEQ. ID NO:53, but not in SEQ. ID NO:41.

9. The method of any of claims 1-6, wherein the polypeptide is encoded by DNA that hybridizes under stringent conditions to DNA consisting of SEQ. ID NO:22 , but not to a DNA consisting of SEQ. ID NO:9.

10. The method of any of claims 1-6, wherein the polypeptide is encoded by DNA that hybridizes to DNA consisting of SEQ. ID NO:22 , but not to a DNA consisting of SEQ. ID NO:9 at 30°C in 6x SSC containing 50% formamide.

11. The method of any of claims 1-6, wherein the polypeptide contains one or more of the motifs listed in SEQ. ID NOs:97-102.

12. The method of claim 2 or 3, wherein the cytokine receptor is the p55 or p75 TNF receptor.

13. The method of claim 2 or 3, wherein the cytokine receptor is the IL-1 Type I receptor, the IL-1 Type II receptor, the IL-6 receptor, the IL-13 receptor, the IL-15 receptor α-chain, the IL-17 receptor, or the IL-18 receptor.

14. The method of claim 6, which is a method for treating rheumatoid arthritis.

15. The method of claim 6, which is a method for treating ankylosing spondylitis, psoriasis, psoriatic arthritis, osteoarthritis, cardiac insufficiency, arteriosclerosis, asthma, myasthenia gravis, septic shock, ulcerative colitis, or Crohn's disease.

16. An isolated protein comprising SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41, that causes a cytokine receptor to be released from the surface of a cell; with the proviso that the sequence of said polypeptide is not contained in any of SEQ. ID NOs:53, 57, and 81 to 88.

17. An isolated chimeric protein comprising SEQ. ID NO:53, or a fragment or variant thereof that causes a cytokine receptor to be released from the surface of a cell, fused to an amino acid sequence of another protein that promotes production of the chimeric protein in *E. coli* as soluble protein.

18. The protein of claim 11 or 12, comprising an amino acid sequence at least 80% identical to a sequence contained in SEQ. ID NO:53, but not in SEQ. ID NO:41.

19. A nucleic acid encoding the protein of any of claims 11-13.

20. A nucleic acid comprising SEQ. ID NO:22, or fragment or variant thereof encoding a protein that causes a cytokine receptor to be released from the surface of a cell, wherein said nucleic acid hybridizes under stringent conditions to SEQ. ID NO:22 but not SEQ. ID NO:9, with the proviso that the nucleic acid encodes a protein not contained in any of SEQ. ID NOs:53, 57, and 81 to 88.

21. An immunoassay method for deterring whether a sample contains a protein that causes release of a cytokine receptor from the surface of a cell, comprising:

a) combining the sample with an antibody specific for SEQ. ID NO:53 but not SEQ. ID NO:41 under conditions where the antibody will complex with SEQ. ID NO:53 if present in the sample; and

b) determining that the sample contains a protein that causes release of a cytokine receptor from the surface of a cell if a complex is formed in step a).

22. A hybridization method for deterring whether a sample contains a nucleic acid encoding a protein that causes release of a cytokine receptor from the surface of a cell, comprising:

a) combining the sample with a nucleic acid probe containing at least 30 consecutive nucleic acids in SEQ. ID NO:22 but not in SEQ. ID NO:9 under conditions where the probe will specifically hybridize to a nucleic acid comprising SEQ. ID NO:22 is present in the sample; and

b) determining that the sample contains a nucleic acid encoding a protein that causes release of a cytokine receptor from the surface of a cell if a hybridization complex is formed in step a).

23. A PCR method for deterring whether a sample contains a nucleic acid encoding a protein that causes release of a cytokine receptor from the surface of a cell, comprising:

a) combining the sample with an amplification primer containing at least 20 consecutive nucleic acids in SEQ. ID NO:22 but not in SEQ. ID NO:9 under conditions where the primer will specifically amplify a nucleic acid comprising SEQ. ID NO:22 is present in the sample; and

b) determining that the sample contains a nucleic acid encoding a protein that causes release of a cytokine receptor from the surface of a cell if a complex is formed in step a).

24. A kit for determining whether a sample contains a protein that causes release of a cytokine receptor from the surface of a cell, comprising an antibody specific for SEQ. ID NO:53 but not SEQ. ID NO:41.

25. A kit for determining whether a sample contains a nucleic acid encoding a protein that causes release of a cytokine receptor from the surface of a cell, comprising a nucleic acid probe or amplification primer containing at least 30 consecutive nucleic acids in SEQ. ID NO:22 but not in SEQ. ID NO:9.

26. A polypeptide comprising SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41, or a nucleic acid encoding said polypeptide, for use as a pharmaceutical medicament.

27. Use of a polypeptide comprising SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41, or a nucleic acid encoding said polypeptide, in the preparation of a medicament for treating inflammation.

28. Use of a polypeptide comprising SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41, or a nucleic acid encoding said polypeptide, in the preparation of a medicament for treating rheumatoid arthritis.

29. Use of a polypeptide comprising SEQ. ID NO:53, or a fragment or variant thereof not contained in SEQ. ID NO:41, or a nucleic acid encoding said polypeptide, in the preparation of a medicament for treating ankylosing spondylitis, psoriasis, psoriatic arthritis, osteoarthritis, cardiac insufficiency, arteriosclerosis, asthma, myasthenia gravis, septic shock, ulcerative colitis, or Crohn's disease

30. The protein, nucleic acid, or use of claims 21-24, wherein the protein or polypeptide comprises an amino acid sequence at least 80% identical to a sequence contained in SEQ. ID NO:53, but not in SEQ. ID NO:41.

31. A method for causing IL-1 Type I receptor, IL-1 Type II receptor or IL-6 receptor to be cleaved, comprising contacting the receptor with a composition comprising one of the following:

a) a protein recombinantly expressed from an encoding sequence selected from SEQ. ID NOs:1 to 29, or by a sequence that is at least 90% identical to any one of SEQ. ID NOs:1 to 29, or fragment thereof;

b) a recombinantly produced protein containing an amino acid sequence that is at least 90% identical to any one of SEQ. ID NOs:30 to 59 and 80, or fragment thereof; or

c) a recombinant nucleic acid comprising a sequence that is at least 90% identical to any one of SEQ. ID NOs:1 to 29; or

d) a recombinant nucleic acid encoding an amino acid sequence that is at least 90% identical to any one of SEQ. ID NOs:30 to 59 and 80;

wherein said composition causes IL-1 Type I receptor, IL-1 Type II receptor or IL-6 receptor to be released from the cell.

32. The method of claim 31, wherein the composition contains a protein comprising an amino acid sequence that is at least 90% identical to a sequence contained in any one of SEQ. ID NOs:30 to 42.

33. The method of claim 31, wherein the composition contains a nucleic acid that encoding an amino acid sequence that is at least 90% identical to a sequence contained in any one of SEQ. ID NOs:30 to 42.

34. The method of claim 31 wherein the composition contains a protein comprising an amino acid sequence that is at least 90% identical to a sequence contained in any one of SEQ. ID NOs:43 to 59 and 80, or fragment thereof, but not in SEQ. ID NOs:30 to 42 or fragment thereof.

35. The method of claim 31 wherein the composition contains a nucleic acid that encoding an amino acid sequence that is at least 90% identical to a sequence contained in any one of SEQ. ID NOs:43 to 59 and 80, or fragment thereof, but not in SEQ. ID NOs:30 to 42 or fragment thereof.

36. The method of claims 31-35, wherein the cytokine receptor peptide is a human IL-6 receptor recombinantly expressed by a mammalian host cell.

37. The method of claims 31-35, wherein the cytokine receptor peptide is an IL-6 receptor expressed endogenously on the surface of a human cell.

38. The method of claims 31-35, wherein the cytokine receptor peptide consists essentially of 7 to 20 consecutive amino acids of the human IL-6 receptor.

39. The method of claims 31-35, wherein the cytokine receptor peptide is a human IL-1 receptor recombinantly expressed by a mammalian host cell.

40. The method of claims 31-35, wherein the cytokine receptor peptide is an IL-1 receptor expressed endogenously on the surface of a human cell.

41. The method of claims 31-35, wherein the cytokine receptor peptide consists essentially of 7 to 20 consecutive amino acids of a human IL-1 receptor.

42. A method for reducing inflammation, comprising cleaving IL-6 receptor from the surface of an inflammatory cell with a metalloprotease.

43. A method for reducing inflammation, comprising cleaving IL-1 Type I or IL-1 Type II receptor from the surface of an inflammatory cell with a metalloprotease.

44. The method of claim 42 or 43, wherein the metalloprotease contains an amino acid sequence that is at least 90% identical to either SEQ. ID NO:40 or 52 (MP7), or any one of SEQ. ID NOs:41, 53, and 57-60 (MP8), or fragment thereof that causes human cytokine receptor to be cleaved.

45. Use of an IL-6 receptor protease in the manufacture of a medicament for treating inflammation.

46. Use of an IL-1 receptor protease in the manufacture of a medicament for treating inflammation.

47 The use according to claim 45 or 46, wherein the medicament is for treating rheumatoid arthritis, ankylosing spondylitis, psoriasis, psoriatic arthritis, osteoarthritis, cardiac insufficiency, arteriosclerosis, asthma, myasthenia gravis, septic shock, ulcerative colitis, or Crohn's disease.

48. A method for reducing inflammation, comprising contacting an inflammatory cell with an IL-6 receptor protease.

49. A method for reducing inflammation, comprising contacting an inflammatory cell with an IL-1 receptor protease.

50. The method according to claim 48 or 49, wherein the medicament is for treating rheumatoid arthritis, ankylosing spondylitis, psoriasis, psoriatic arthritis, osteoarthritis, cardiac insufficiency, arteriosclerosis, asthma, myasthenia gravis, septic shock, ulcerative colitis, or Crohn's disease.

51. A method for determining cytokine receptor releasing enzyme activity in a solution; comprising:
a) combining the solution with a peptide consisting essentially of 8 to 20 consecutive amino acids of a human cytokine receptor, selected from p55 TNF receptor, p75 TNF receptor, IL-6 receptor, IL-1 type I receptor or IL-1 type II receptor, under conditions where the enzyme (if present in the solution) cleaves the peptide;
b) measuring cleavage of the peptide (if it occurs); and
c) determining the enzyme activity from the cleavage measured in b).

52. The method of claim 51, wherein cleavage of the peptide is measured by mass spectroscopy.

53. The method of claim 51, wherein the peptide is labeled with a fluorescence emitter and a fluorescence quencher, and cleavage is measured by measuring change in fluorescence of the labeled peptide.

54. The method of claims 51-23, further comprising measuring cleavage of the peptide (if it occurs) in the absence of $Zn^{++}$ (or in the presence of a $Zn^{++}$ chelator), and subtracting the cleavage in the absence of $Zn^{++}$ from cleavage in the presence of $Zn^{++}$ in order to determine the cytokine receptor releasing enzyme activity.

55. The method of any of claims 51-54, further comprising determining between which amino acids the peptide is cleaved.

56. The method of claims 51-55, where cleavage of p55 TNF receptor is measured between the sequence NVKG and the sequence TEDS.

57. The method of claims 51-55, wherein cleavage of p75 TNF receptor is measured between the sequence DAVC and the sequence TSTS.

58. The method of claims 51-55, wherein cleavage of IL-6 receptor is measured between the sequence DSAN and the sequence ATSL

59. The method of claims 51-58, which is a method for determining whether a compound can modulate cytokine receptor releasing activity, wherein the compound is added to the solution before step b).

60. The method of claim 59, comprising determining whether the compound inhibits or enhances cleavage of a TNF receptor, IL-6 receptor, or IL-1 receptor peptide.