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(54) Title: ANTI-INFLAMMATORY AGENT

(57) Abstract: The present invention relates to agents that modulate the effect of a Toll-IL-1 receptor regulator polypeptide on Toll-IL-1 receptor function, to methods for the identification of such agents and to the use of such agents in the modulation of inflammatory responses and innate immune responses.



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ANTI-INFLAMMATORY AGENT

The invention relates to the use of a cell surface polypeptide in the identification of agents that modulate Toll-IL-1 receptor function and to the use of such agents in the modulation of inflammatory responses and innate immune responses.

Members of the Toll-IL-1 receptor superfamily are defined by the presence of an evolutionary conserved intracellular Toll-IL-1 receptor (TIR) domain, a stretch of 200 amino acids. Members of the Toll-IL-1 receptor family are important in mediating inflammation and immune responses. This superfamily can be divided into two main subgroups, based on the extracellular domains; the Ig domain (Ig)-containing and Leucine-rich repeat (LRR) motif-containing receptors. The Ig domain subgroup includes IL-1R1, IL-18R, T1/ST2, and SIGIRR (Rock et al. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 588-593; Mitcham et al. (1996) *J. Biol. Chem.* **271**, 5777-5783; Parnet et al. (1996) *J. Biol. Chem.* **271**, 3967-3970; Lovenberg et al. (1996) *J. Neuroimmunol.* **70**, 113-122; Thomassen et al., (1999) *Cytokine* **11**, 389-399).

IL-1 has been demonstrated to be one of the key orchestrators of the immune response, eliciting a wide range of biological responses, including fever, lymphocyte activation, and leukocyte infusion to the site of injury and infection (Dinarello, C. A. (1996) *Blood* **87**, 2095-2147). IL-18 promotes T_H1 cell differentiation and NK cell activation, whereas T1/ST2 has important functions in developing T_H2 cell responses. The LRR subgroup consists of at least 10 human Toll-like receptors (TLR), which are important in the recognition of pathogens (Rock et al (1998) *Proc. Natl. Acad. Sci. USA* **95**, 588-593; Medzhitov et al (1997) *Nature* **388**, 394-397; Takeuchi et al (1999) *Gene* **231**, 59-65; Chuang & Ulevitch (2000) *Eur. Cytokine Network* **11**, 372-378; Hemmi et al (2000) *Nature* **408**, 740-745). An individual TLR recognizes its own specific pathogen-associated molecular patterns (PAMP).

Whereas TLR4 has been genetically identified as a signaling molecule essential for the responses to LPS, a component of Gram-negative bacteria (Poltorak et al (1998) *Science* **282**, 2085-2088) TLR2 responds to mycobacteria, yeast, and Gram-positive

bacteria (Takeuchi et al (1999) *Immunity* **11**, 443-451; Takeuchi et al (1999) *J.Immunol* **164**, 554-557; Underhill et al (1999) *Nature* **401**, 811-815; Underhill et al (1999) *Proc. Natl. Acad. Sci. USA* **96**, 14459-14463) TLR6 associates with TLR2 and recognizes lipoproteins from microplasma. Whereas TLR5 mediates the
5 induction of the immune response by bacterial flagellins (Hayashi et al (2001) *Nature* **410**, 1099-1103) TLR9 has been shown to recognize bacterial DNA (Hemmi et al (2000) *Nature* **408**, 740-745) and TLR3 recognizes double-stranded RNA (dsRNA) (Alexopoulou et al (2001) *Nature* **413**, 732-738). The natural ligands for TLR7, TLR8, and TLR10 are not known, although a synthetic compound (imidazoquinoline
10 compound R848) with antiviral activity has now been described as a ligand for TLR7 and TLR8 (Hemmi et al (2002) *Nat. Immunol* **3**, 196-200; Jurk et al (2002) *Nat. Immunol* **3**, 499).

Because Toll-IL-1 receptors share sequence similarities in their intracellular domain,
15 most of them can activate transcription factor NF- κ B through a common signaling pathway, which has been studied extensively for the IL-1 receptor. On the binding of IL-1 to the receptor, the cytosolic adapters MyD88 (Lord et al (1990) *Oncogene* **5**, 1095-1097; Wesche et al (1997) *Immunity* **7**, 837-847; Adacei et al (1998) *Immunity* **9**, 143-150) and Tollip (Burns et al (2000) *Nat.Cell Biol.* **2**, 346-351) are rapidly
20 recruited to the receptor complex (IL-1R/IL-1R-Acp), which then recruits serine-threonine kinases IRAK4 (IL-1 receptor-associated kinase 4) (Suzuki et al (2002) *Nature* **416**, 750-756; Li et al (2002) *Proc. Natl. Acad. Sci. USA* **99**, 5567-5572) and IRAK (Cao et al (1996) *Nature* **383**, 443-446). IRAK is phosphorylated and mediates the recruitment of TRAF6 to the receptor (Li et al (2002) *Proc. Natl. Acad.*
25 *Sci. USA* **99**, 5567-5572; Cao et al (1996) *Nature* **383**, 443-446; Jiang et al (2002) *Mol. Cell. Biol.* **22**, 7158-7167). The IRAK-TRAF6 then forms a complex with another adapter protein, Pellino 1, and leaves the receptor to interact with TAK1 (TGF β -activated kinase 1), a member of the mitogen-activated protein (MAP) kinase kinase kinase family (MAPKKK), on the membrane (Jiang et al (2002) *Mol. Cell.*
30 *Biol.* **22**, 7158-7167). TAK1 is phosphorylated on the membrane, but activated in the cytosol (Jiang et al (2002) *Mol. Cell. Biol.* **22**, 7158-7167). The activation of TAK1

- eventually leads to the activation of I κ B kinase (IKK), which in turn leads to the phosphorylation and degradation of I κ B proteins, and liberation of NF- κ B to activate transcription in the nucleus (Mercurio et al (1997) *Science* **278**, 860-866; Regnier et al (1997) *Cell* **90**, 373-383; Woronicz et al (1997) *Science* **278**, 866-869; Zandi et al (1997) *Cell* **91**, 243-252). Activated TAK1 has also been implicated in the IL-1-induced activation of MKK6 and c-Jun N-terminal kinase (JNK) (Ninomiya-Tsuji et al (1999) *Nature* **398**, 252-256) leading to the activation of other transcription factors, including ATF and AP1, thereby also activating gene transcription.
- IL-1 responses are regulated through a receptor complex to which an increasing number of components have been found to belong. The receptor uses a well conserved mechanism for regulating immune and inflammatory responses (Letsou et al., EMBO J, 12: 3449-3458 (1993); Grosshans et al., Nature, 372: 563-566 (1994); Galindo et al, Development, 121:2209-2218 (1995); Norris and Manley, Genes Dev. 10: 862-872 (1996); Cao et al., Science, 271: 1128-1131 (1996)) but which is still not completely understood. Initiation of the signal occurs through a heterodimeric receptor complex, composed of the interleukin type I receptor (IL-1RI) (Dower et al. J. Exp. Med. 162:501—515 (1985), Sims et al., Science, 241: 585-589 (1988)) and the accessory protein (AcP) (Greenfeder et al., J. Biol. Chem. 270: 13757-13765(1995)), both 80-90 kDa glycoproteins. The type I receptor complex is present on T cells and connective tissue derived cells (Sims et al., Science, 241: 585-589 (1988); Sims et al., Proc. Natl. Acad. Sci. USA, 86:8946-8950 (1989)) characteristically at low levels (Dower et al., J. Exp. Med. 162:501—515 (1985)). The receptor binds both forms of IL-1 (Dower et al., Nature, 324: 266-268 (1986); Eisenberg et al., Nature, 343: 341-346 (1990)), with active forms of 17 kDa, and is responsible for IL-1 induced activation of both NF- κ B and stress kinase pathways (Bomsztyk et al., J. Biol. Chem. 265: 9413-9417 (1990); Liou and Baltimore, Curr. Opin. Cell Biol. 5: 477-487 (1993); Qwarnstrom et al., J. Biol. Chem. 269: 30765-30768 (1994); Freshney et al., Cell, 78: 1039-1049 (1994)). In addition, a 67-kDa, nonsignaling receptor (type II) is expressed on B cells, monocytes, and T cells (Horuk et al., J. Biol. Chem. 262: 16275-16278 (1987); Bomsztyk et al., Proc. Natl.

Acad. Sci. USA, 86: 80034-8038 (1989); Chizzonite et al., Proc. Natl. Acad. Sci. USA, 86: 8029-8033 (1989); Spriggs et al., J. Biol. Chem. 265: 22499-224505 (1990); McMahan et al., EMBO J, 10: 2821-2832 (1991)). The receptor proximal stages of signal activation involve the adapter proteins such as MyD88 (Muzio et al.,
5 Science, 278: 1612-1615 (1997); Wesche et al., Immunity, 7: 837-847 (1997); Burns et al., J. Biol. Chem. 273: 12203-12209 (1998)), kinases, IRAKs 1-4 (Muzio et al., Science, 278: 1612-1615 (1997), Cao et al., Science, 271: 1128-1131 (1996), signalling components TRAF 6 (Cao et al., Nature, 383: 443-446 (1996)) and Tollip (Burns et al. Nature Cell Biol. 2: 346-51 (2000)).

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In adherent cells, such as fibroblasts, IL-1 receptors are located at focal adhesions (Qwarnstrom et al., J. Biol. Chem. 263: 8261-8269 (1988); Dower et al., J. Invest. Dermatol. 94: 68S-73S (1990)), and IL-1 binding to the type I receptor has rapid effects on cell structure (Qwarnstrom et al., Proc. Natl. Acad. Sci. USA, 88:1232-
15 1236 (1991)). IL-1 signal transduction is regulated by cell attachment and spreading (Zhu et al., Biochem J. 330: 975-981 (1998)), and fibronectin attachment is permissive in IL-1 responses in adherent cells (Ostberg et al., FEBS Lett. 367: 93-97 (1995)).

20 We describe the isolation of a human DNA sequence encoding a Toll-IL-1 receptor regulator protein the expression of which is closely correlated with Toll-IL-1 receptor function in response to IL-1 and/or pathogens.

According to a first aspect of the invention there is provided an agent that modulates
25 the effect of a cell surface polypeptide on Toll-IL-1 receptor function wherein said cell surface polypeptide is selected from the group consisting of:

- i) a polypeptide, or variant thereof, encoded by a nucleic acid molecule consisting of a nucleic acid sequence as represented by Figure 1 or a sequence complementary thereto;

ii) a polypeptide encoded by a nucleic acid molecule which hybridises to a nucleic acid molecule as defined in (i) above and which modulates Toll-IL-1 receptor function; and

5 iii) a polypeptide comprising a nucleic acid which is degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) and (ii), for use as a medicament.

As used herein the "Toll-IL-1 receptor" relates to any receptor having a TIR domain and includes Toll-like receptors and IL-1 receptor.

10

As used herein "Toll-IL-1 receptor function" includes, but is not limited to, Toll-IL-1 receptor activity in binding to IL-1 or to one or more pathogens as defined herein. Moreover, Toll-IL-1 receptor function includes the regulation of the transcription factor, NF- κ B, or the regulation of inflammatory responses or innate immunity.

15

As used herein "a nucleic acid sequence as represented by Figure 1" includes a nucleic acid sequence represented by Figure 1A or Figure 1B. Preferably, the nucleic acid sequence as represented by Figure 1 is that shown in Figure 1B.

20 In a preferred aspect of the invention, said polypeptide comprises an amino acid sequence as represented in Figure 2 or a variant polypeptide thereof wherein said variant is modified by addition, deletion or substitution of at least one amino acid residue of the amino acid sequence represented in Figure 2 wherein said variant polypeptide modulates Toll-IL-1 receptor function. Such polypeptides will be
25 referred to herein as "Toll-IL-1 receptor regulators (TILRRs)".

As used herein "an amino acid sequence as represented by Figure 2" includes an amino acid sequence represented by Figure 2A or Figure 2B. Preferably, the amino acid sequence as represented in Figure 2 is that shown in Figure 2B.

30

A variant polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations which may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and asparatic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan.

The nucleic acid molecule according to the first aspect of the invention may anneal under stringent hybridisation conditions to the nucleic acid sequence shown in Figure 1 or to its complementary strand.

Stringent hybridisation/washing conditions are well known in the art. For example, nucleic acid hybrids that are stable after washing in 0.1xSSC, 0.1% SDS at 60°C. It is well known in the art that optimal hybridisation conditions can be calculated if the sequences of the nucleic acid is known. For example, hybridisation conditions can be determined by the GC content of the nucleic acid subject to hybridisation. Please see Sambrook *et al* (1989) Molecular Cloning; A Laboratory Approach. A common formula for calculating the stringency conditions required to achieve hybridisation between nucleic acid molecules of a specified homology is:

$$T_m = 81.5^{\circ} \text{C} + 16.6 \text{ Log } [\text{Na}^+] + 0.41 [\% \text{ G} + \text{C}] - 0.63 (\% \text{ formamide}).$$

The nucleic acid molecule of the first aspect of the invention may comprise the sequence set out in Figure 1 or a sequence which is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, for example 98%, or 99%, identical to the nucleic acid sequence set out in Figure 1 respectively at the nucleic acid residue level.

“Identity”, as known in the art, is the relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the

sequences. In the art, identity also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. Identity can be readily calculated (*Computational Molecular Biology*, Lesk, A.M. ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A.M., AND Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or two polypeptide sequences, the term is well-known to skilled artisans (*Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., *SIAM J. Applied Math.*, 48: 1073 (1988). Methods commonly employed to determine identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., *SIAM J. Applied Math.*, 48: 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity are codified in computer programs. Preferred computer program methods to determine identity between two sequences include, but are not limited to, GCG program package (Devereux, J., et al., *Nucleic Acids Research* 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F. et al., *J. Molec. Biol.* 215: 403 (1990)).

As used herein, the term "polypeptide" means, in general terms, a plurality of amino acid residues joined together by peptide bonds. It is used interchangeably and means the same as peptide, protein, oligopeptide, or oligomer. The term "polypeptide" is also intended to include fragments, variants including alternate slice variants or isoforms, analogues and derivatives of a polypeptide wherein the fragment, variant, analogue or derivative retains essentially the same biological activity or function as a reference protein.

As used herein "fragment" may include any contiguous 10 residue sequence, or greater, such as 15, 20, 30, 50 or 100 residue sequence. Preferably, fragments of nucleotide or polypeptide sequences share one or more functional characteristics with
5 TILRR or its gene.

The complementary sequences of the nucleic acid sequences according to the first aspect of the invention may be useful as probes or primers, or in the regulation of the gene(s) encoding the TILRRs. They may be useful in the identification and/or
10 treatment of individuals having diseases relating to a defective or deficient TILRR. These sequences are preferably isolated.

Preferably the agent modulates the effect of the cell surface polypeptide on Toll-IL-1 receptor function by modulating the association of the cell surface polypeptide with
15 the Toll-IL-1 receptor or by modulating the expression of the cell surface polypeptide. Typically the agent interacts with the Toll-IL-1 receptor but is not the primary or natural ligand of the Toll-IL-1 receptor.

The agent may be an agonist or antagonist of Toll-IL-1 receptor function.
20

The agent may be a polypeptide. The polypeptide may be an antibody or an active binding part of an antibody, for example a monoclonal antibody or active binding part thereof. The antibody, or binding fragment thereof, may be a chimeric antibody. In an alternative embodiment of the invention said antibody, or binding fragment
25 thereof, is a humanised antibody. Preferably, the antibody is specific to TILRR.

Antibodies or immunoglobulins (Ig) are a class of structurally related proteins consisting of two pairs of polypeptide chains, one pair of light (L) (low molecular weight) chain (κ or λ), and one pair of heavy (H) chains (γ , α , μ , δ and ϵ), all four
30 linked together by disulphide bonds. Both H and L chains have regions that contribute to the binding of antigen and that are highly variable from one Ig molecule

to another. In addition, H and L chains contain regions that are non-variable or constant. The L chains consist of two domains. The carboxy-terminal domain is essentially identical among L chains of a given type and is referred to as the "constant" (C) region. The amino terminal domain varies from L chain to L chain and contributes to the binding site of the antibody. Because of its variability, it is referred to as the "variable" (V) region. The variable region contains complementarity determining regions or CDR's which form an antigen binding pocket. The binding pockets comprise H and L variable regions which contribute to antigen recognition. It is possible to create single variable regions, so called single chain antibody variable region fragments (scFv's). If a hybridoma exists for a specific monoclonal antibody it is well within the knowledge of the skilled person to isolate scFv's from mRNA extracted from said hybridoma via RT PCR. Alternatively, phage display screening can be undertaken to identify clones expressing scFv's.

Thus the agent may be an antibody fragment for example a fragment selected from the group consisting of Fab, Fab₂, F(ab')₂, Fv, Fc and scFvs. Alternatively the antibody fragment may be a "domain antibody fragment". Domain antibodies are the smallest part of an antibody (approximately 13kDa). Examples of this technology are disclosed in US6248516, US6291158 and US6127197.

In a preferred aspect of the invention said antibody fragment is a single chain antibody variable region fragment.

A fragment of an antibody or immunoglobulin can also have bispecific function binding two different epitopes of two different antigens.

Preferably said chimeric/humanised monoclonal antibody to said polypeptide is produced as a fusion polypeptide in an expression vector suitably adapted for transfection or transformation of prokaryotic or eukaryotic cells.

A chimeric antibody is produced by recombinant methods to contain the variable region of an antibody with an invariant or constant region of a human antibody. A humanised antibody is produced by recombinant methods to combine the CDR's of an antibody with both the constant regions and the framework regions from the
5 variable regions of a human antibody.

Antibodies from non-human animals provoke an immune response to the foreign antibody and its removal from the circulation. Both chimeric and humanised antibodies have reduced antigenicity when injected to a human subject because there
10 is a reduced amount of rodent (i.e. foreign) antibody within the recombinant hybrid antibody, while the human antibody regions do not elicit an immune response. This results in a weaker immune response and a decrease in the clearance of the antibody. This is clearly desirable when using therapeutic antibodies in the treatment of human diseases. Humanised antibodies are designed to have less "foreign" antibody regions
15 and are therefore thought to be less immunogenic than chimeric antibodies.

In a preferred aspect of the invention said antibodies are opsonic antibodies.

Phagocytosis is mediated by macrophages and polymorphic leukocytes and involves
20 the ingestion and digestion of micro-organisms, damaged or dead cells, cell debris, insoluble particles and activated clotting factors. Opsonins are agents which facilitate the phagocytosis of the above foreign bodies. Opsonic antibodies are therefore antibodies which provide the same function. Examples of opsonins are the Fc portion of an antibody or complement C3.

25

Preferably, said antibody is provided with a marker including a conventional label or tag, for example a radioactive and/or fluorescent and/or epitope label or tag.

In an alternative preferred embodiment of the invention said antibody, or antibody
30 fragment has associated therewith or crosslinked thereto a therapeutic agent. Preferably said therapeutic agent is an anti-inflammatory agent.

In a further preferred aspect of the invention the agent is a peptide such as a modified peptide. The peptide may consist of an amino acid sequence selected from the group consisting of amino acid residues 280 to 380 of the amino acid sequence presented in Figure 2, or amino acid residues 600 to 716 of the amino acid sequence presented in Figure 2, amino acid residue 1 to 6 or amino acid residues 77 and/or 102 and/or 219 and/or 245 and/or 398 and/or 477 and/or 548 of the amino acid sequence presented in Figure 2, or a variant peptide wherein said variant polypeptide sequence has been altered by addition, substitution or deletion of at least one amino acid residue and wherein the variant modulates Toll-IL1 receptor function.

In a further preferred aspect of the invention the agent is a polypeptide comprising an amino acid sequence as represented in Figure 2 or a variant polypeptide thereof wherein said variant is modified by addition, deletion or substitution of at least one amino acid residue of the amino acid sequence represented in Figure 2 wherein said variant polypeptide modulates Toll-IL1 receptor function, for example increases Toll-IL1 receptor activity.

It will be apparent to one skilled in the art that modifications to the amino acid sequence of peptides which modulate Toll-IL-1 receptor function could enhance the binding and/or stability of the peptide with respect to its target sequence. In addition, modification of the peptide may also increase the *in vivo* stability of the peptide thereby reducing the effective amount of peptide necessary to inhibit an interaction. This would advantageously reduce undesirable side effects which may result *in vivo*. Modifications include, by example and not by way of limitation, acetylation and amidation. Thus the peptide of the invention may be acetylated, for example by acetylation to the amino terminus of said peptide. The peptide of the invention may be amidated, for example by amidation to the carboxyl-terminus of said peptide. The peptide of the invention may be modified by both acetylation and amidation.

Alternatively, or preferably, said modification includes the use of modified amino acids in the production of recombinant or synthetic forms of peptides. It will be apparent to one skilled in the art that modified amino acids include, by way of example and not by way of limitation, 4-hydroxyproline, 5-hydroxylysine, N⁶-acetyllysine, N⁶-methyllysine, N⁶,N⁶-dimethyllysine, N⁶,N⁶,N⁶-trimethyllysine, cyclohexylalanine, D-amino acids, ornithine. Other modifications include amino acids with a C₂, C₃ or C₄ alkyl R group optionally substituted by 1, 2 or 3 substituents selected from halo (e.g. F, Br, I), hydroxy or C₁-C₄ alkoxy.

10 Alternatively, peptides could be modified by, for example, cyclisation. Cyclisation is known in the art, (see Scott et al Chem Biol (2001), 8:801-815; Gellerman et al J. Peptide Res (2001), 57: 277-291; Dutta et al J. Peptide Res (2000), 8: 398-412; Ngoka and Gross J Amer Soc Mass Spec (1999), 10:360-363. Thus the peptide of the invention may be modified by cyclisation.

15

In a further preferred aspect of the invention the agent is a nucleic acid molecule, for example an aptamer, a small interfering RNA (RNAi) or an antisense nucleic acid.

Nucleic acids have both linear sequence structure and a three dimensional structure which in part is determined by the linear sequence and also the environment in which these molecules are located. Conventional therapeutic molecules are small molecules, for example, peptides, polypeptides, or antibodies, which bind target molecules to produce an agonistic or antagonistic effect. It has become apparent that nucleic acid molecules also have potential with respect to providing agents with the requisite binding properties which may have therapeutic utility. These nucleic acid molecules are typically referred to as aptamers. Aptamers are small, usually stabilised, nucleic acid molecules which comprise a binding domain for a target molecule, in the present invention a polypeptide comprising a scavenger receptor cysteine rich domain. A screening method to identify aptamers is described in US 5,270,163 which is incorporated by reference. Aptamers are typically

oligonucleotides which may be single stranded oligodeoxynucleotides, oligoribonucleotides, or modified oligodeoxynucleotide or oligoribonucleotides.

The term "modified" encompasses nucleotides with a covalently modified base and/or sugar. For example, modified nucleotides include nucleotides having sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleotides may also include 2' substituted sugars such as 2'-O-methyl-; 2-O-alkyl; 2-O-allyl; 2'-S-alkyl; 2'-S-allyl; 2'- fluoro-; 2'-halo or 2;azido-ribose, carbocyclic sugar analogues a-anomeric sugars; epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, and sedoheptulose.

Modified nucleotides are known in the art and include by example and not by way of limitation; alkylated purines and/or pyrimidines; acylated purines and/or pyrimidines; or other heterocycles. These classes of pyrimidines and purines are known in the art and include, pseudoisocytosine; N4, N4-ethanocytosine; 8-hydroxy-N6-methyladenine; 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil; 5-fluorouracil; 5-bromouracil; 5-carboxymethylaminomethyl-2-thiouracil; 5-carboxymethylaminomethyl uracil; dihydrouracil; inosine; N6-isopentyl-adenine; 1-methyladenine; 1-methylpseudouracil; 1-methylguanine; 2,2-dimethylguanine; 2-methyladenine; 2-methylguanine; 3-methylcytosine; 5-methylcytosine; N6-methyladenine; 7-methylguanine; 5- methylaminomethyl uracil; 5-methoxy amino methyl-2-thiouracil; β -D-mannosylqueosine; 5-methoxycarbonylmethyluracil; 5-methoxyuracil; 2 methylthio-N6-isopentenyladenine; uracil-5-oxyacetic acid methyl ester; psueouracil; 2-thiocytosine; 5-methyl-2 thiouracil, 2-thiouracil; 4-thiouracil; 5-methyluracil; N-uracil-5-oxyacetic acid methylester; uracil 5—oxyacetic acid; queosine; 2-thiocytosine; 5-propyluracil; 5-propylcytosine; 5-ethyluracil; 5-ethylcytosine; 5-butyluracil; 5-pentyluracil; 5-pentylcytosine; and 2,6,-diaminopurine; methylpsuedouracil; 1-methylguanine; 1-methylcytosine.

30

The aptamers of the invention may be synthesized using conventional phosphodiester linked nucleotides and synthesized using standard solid or solution phase synthesis techniques which are known in the art. Linkages between nucleotides may use alternative linking molecules. For example, linking groups of the formula P(O)S, (thioate); P(S)S, (dithioate); P(O)NR'²; P(O)R'; P(O)OR₆; CO; or CONR'² wherein R is H (or a salt) or alkyl (1-12C) and R₆ is alkyl (1-9C) is joined to adjacent nucleotides through -O- or -S-. The binding of aptamers to a target polypeptide is readily tested by assays hereindisclosed.

10 Preferably the RNAi molecule is derived from the nucleic acid molecule according to the first aspect of the invention. The RNAi molecule may have a length of between 10 nucleotide bases (nb) -1000nb. The RNAi molecule may have a length of 10nb; 20nb; 30nb; 40nb; 50nb; 60nb; 70nb; 80nb; 90nb; or 100bp. The RNAi molecule may be 21-29nb in length.

15

Preferably the RNAi molecule comprises a nucleic acid sequence selected from the group consisting of a sequence as shown in Table 1 for example:

5' CUCUCUCCACAGGUCUUUG 3'

20

5' CCAGACUGUAACAUCAUCC 3'

5' ACCGUAUACAAGAGAGUGC 3'

25 Preferably the antisense oligonucleotide is capable of hybridising to the nucleic acid molecule according to the first aspect of the invention.

As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and thereby, inhibits the transcription of that gene and/or the

translation of that mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence.

The invention further provides a pharmaceutical composition comprising an agent as described herein in combination with a pharmaceutically acceptable adjuvant, carrier or diluent.

According to a further aspect of the invention there is provided a composition comprising a nucleic acid molecule selected from the group consisting of:

- i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 1;
- ii) a nucleic acid molecule that hybridises under stringent hybridisation conditions to the nucleic acid molecule in (i) above and which encodes a polypeptide that modulates Toll IL-1 receptor function, for use as a vaccine.

According to a further aspect of the invention there is provided a vaccine composition comprising a polypeptide comprising an amino acid sequence as represented in Figure 2, or a variant polypeptide wherein said variant is modified by addition, deletion or substitution of at least one amino acid residue of the amino acid sequence presented in Figure 2, for use as a vaccine.

In a preferred embodiment of the invention said composition includes an adjuvant and/or a carrier.

An adjuvant is a substance or procedure that augments specific immune responses to antigens by modulating the activity of immune cells. Examples of adjuvants include, by example only, Freund's adjuvant, muramyl dipeptides, liposomes. A carrier is an

immunogenic molecule which, when bound to a second molecule, augments immune responses to the latter. Some antigens are not intrinsically immunogenic yet may be capable of generating antibody responses when associated with a foreign protein molecule such as keyhole-limpet haemocyanin or tetanus toxoid. Such antigens contain B-cell epitopes but no T cell epitopes. The protein moiety of such a conjugate (the "carrier" protein) provides T-cell epitopes which stimulate helper T-cells that in turn stimulate antigen-specific B-cells to differentiate into plasma cells and produce antibody against the antigen. Helper T-cells can also stimulate other immune cells such as cytotoxic T-cells, and a carrier can fulfil an analogous role in generating cell-mediated immunity as well as antibodies.

When administered, the therapeutic compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents (for example, cisplatin; carboplatin; cyclophosphamide; melphalan; carmustine; methotrexate; 5-fluorouracil; cytarabine; mercaptopurine; daunorubicin; doxorubicin; epirubicin; vinblastine; vincristine; dactinomycin; mitomycin C; taxol; L-asparaginase; G-CSF; etoposide; colchicine; derferoxamine mesylate; and camptothecin.

The therapeutics of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal. When antibodies are used therapeutically, a preferred route of administration is by pulmonary aerosol. Techniques for preparing aerosol delivery systems containing antibodies are well known to those of skill in the art. Generally, such systems should utilize components which will not significantly impair the biological properties of the antibodies, such as the paratope binding capacity (see, for example, Sciarra and Cutie, "Aerosols," in Remington's Pharmaceutical Sciences, 18th edition, 1990, pp 1694-1712; incorporated by

reference). Those of skill in the art can readily determine the various parameters and conditions for producing antibody aerosols without resort to undue experimentation.

When administered, the compositions of the invention are applied in
5 pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When
10 used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric,
15 hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

20 Pharmaceutical compositions may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active
25 ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

30

According to a further aspect of the invention there is provided a vector which is adapted for the expression of a humanised or chimeric antibodies according to the invention.

- 5 According to a further aspect of the invention there is provided a cell which has been transformed or transfected with the vector encoding a humanised or chimeric antibody according to the invention

According to a further aspect of the invention there is provided a method for the
10 production of a humanised or chimeric antibody according to the invention comprising:

- (i) providing a cell transformed or transfected with a vector which comprises a nucleic acid molecule encoding the humanised or chimeric antibody according to the invention;
- 15 (ii) growing said cell in conditions conducive to the manufacture of said antibody; and
- (iii) purifying said antibody from said cell, or its growth environment.

In a yet further aspect of the invention there is provided a hybridoma cell line which
20 produces a monoclonal antibody as hereinbefore described.

In a further aspect of the invention there is provided a method of producing monoclonal antibodies according to the invention using hybridoma cell lines according to the invention.

25

In a further aspect of the invention there is provided a method for preparing a hybridoma cell-line producing monoclonal antibodies according to the invention comprising the steps of:

- i) immunising an immunocompetent mammal with an immunogen
30 comprising at least one polypeptide having the amino acid sequence as represented in Figure 2, or fragments thereof;

- ii) fusing lymphocytes of the immunised immunocompetent mammal with myeloma cells to form hybridoma cells;
- iii) screening monoclonal antibodies produced by the hybridoma cells of step (ii) for binding activity to the polypeptide of (i);
- 5 iv) culturing the hybridoma cells to proliferate and/or to secrete said monoclonal antibody; and
- v) recovering the monoclonal antibody from the culture supernatant.

Preferably, the said immunocompetent mammal is a mouse. Alternatively, said
10 immunocompetent mammal is a rat.

The production of monoclonal antibodies using hybridoma cells is well-known in the art. The methods used to produce monoclonal antibodies are disclosed by Kohler and Milstein in Nature 256, 495-497 (1975) and also by Donillard and Hoffman, "Basic
15 Facts about Hybridomas" in Compendium of Immunology V.II ed. by Schwartz, 1981, which are incorporated by reference.

According to a further aspect of the invention there is provided a kit comprising a binding agent specifically reactive with a nucleic acid molecule that encodes a
20 polypeptide comprising an amino acid sequence as represented in Figure 2, or an agent specifically reactive with a polypeptide comprising an amino acid sequence as represented in Figure 2.

In a preferred embodiment of the invention said kit further comprises an
25 oligonucleotide or antibody specifically reactive with said nucleic acid molecule or said polypeptide.

Preferably said kit comprises a thermostable DNA polymerase and components required for conducting the amplification of nucleic acid. Preferably said kit includes
30 a set of instructions for conducting said polymerase chain reaction and control nucleic acid.

In an alternative preferred embodiment of the invention said kit comprises an antibody specifically reactive with a polypeptide comprising an amino acid sequence as represented in Figure 2.

- 5 Preferably said kit comprises components required for conducting an immunoassay including, for example, a secondary antibody specifically reactive with a primary antibody that specifically binds said polypeptide(s) and enzyme reagents required to detect the binding of said secondary antibody with said primary antibody.
- 10 In a further aspect of the invention there is provided a method for the identification of agents which modulate Toll-IL-1 receptor function the method comprising the steps of
- i) forming a preparation comprising a polypeptide and a Toll-IL-1 receptor wherein the polypeptide is selected from the group consisting of:
 - 15 i) a polypeptide, or fragment or variant thereof, encoded by a nucleic acid molecule consisting of a nucleic acid sequence as represented by Figure 1;
 - ii) a polypeptide encoded by a nucleic acid molecule which hybridises to a nucleic acid molecule as defined in (i) above and which modulates Toll-IL-1 receptor function;
 - 20 iii) a polypeptide comprising a nucleic acid which is degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) and (ii); and
 - ii) adding at least one candidate agent to be tested;
 - 25 iii) determining the effect, or not, of said agent on the function of the Toll-IL-1 receptor.

The method of the invention enables the identification of agents which modulate (for example inhibit, reduce, block or promote) Toll-IL-1 receptor function. Agents may modulate Toll-IL-1 receptor function by modulating the association of TILRR protein to a Toll-IL-1 receptor. An agent may be as described herein or the agent

30

may be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics (e.g., peptoids), amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

10

In a preferred method of the invention the polypeptide (TILRR) and/or the Toll-IL-1 receptor is expressed by a cell. The cell may be a mammalian cell including HEK293, CHO, HeLa, or COS cells, smooth muscle cells, fibroblasts, lymphocytes, macrophages and other cell types carrying Toll and/or IL-1 receptors. The cell may be part of a transgenic animal wherein the genome of said animal has been modified to include nucleic acid molecules which encode the polypeptide (TILRR) and/or a Toll-IL-1 receptor.

15

In a preferred method of the invention said agent is an antagonist. In an alternative preferred method of the invention said agent is an agonist.

20

Agents identified by the screening method of the invention include, antibodies, siRNA, aptamers, small organic molecules, (for example peptides, cyclic peptides), dominant negative variants of the polypeptides herein disclosed.

25

As mentioned above, the invention also provides, in certain embodiments, "dominant negative" polypeptides derived from the polypeptides hereindisclosed. A dominant negative polypeptide is an inactive variant of a protein, which, by interacting with the cellular machinery, displaces an active protein from its interaction with the cellular machinery, displaces an active protein from its interaction with the cellular machinery or competes with the active protein, thereby reducing the effect of the active protein. For example, a dominant negative receptor which binds a ligand but

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does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand. Likewise, a dominant negative catalytically-inactive kinase which interacts normally with target proteins but does not phosphorylate the target proteins can reduce phosphorylation of the target proteins in response to a cellular signal. Similarly, a dominant negative transcription factor which binds to another transcription factor or to a promoter site in the control region of a gene but does not increase gene transcription can reduce the effect of a normal transcription factor by occupying promoter binding sites without increasing transcription.

10

The agent according to the invention may be used in the prevention or treatment of a disorder relating to Toll-IL-1 receptor function in a subject. Preferably the subject is human.

15 Examples of disorders which relate to Toll-IL-1 receptor function include inflammatory disorders and disorders of the immune system. The inflammatory disorder may be selected from the group consisting of atherosclerosis, rheumatoid arthritis, osteoarthritis, polyarthritis, gout, lupus erythematosus, scleroderma, Sjorgen's syndrome, poly- and dermatomyositis, vasculitis, tendonitis, synovitis, bacterial endocarditis, osteomyelitis, psoriasis, pneumonia, fibrosing alveolitis, chronic bronchitis, bronchiectasis, emphysema, silicosis, pneumoconiosis, tuberculosis, ulcerative colitis, Crohn's disease, chronic inflammatory demyelinating polyradiculoneuropathy, chronic inflammatory demyelinating polyneuropathy, multiple sclerosis, Guillan-Barre Syndrome and myasthenia gravis, mastitis, 20 laminitis, laryngitis, chronic cholecystitis, Hashimoto's thyroiditis, and inflammatory breast disease. The inflammatory disorder may be the result of tissue or organ rejection after transplantation.

Disorders of the immune system include microbial infections such bacterial, viral, 30 fungal, yeast parasitic or protozoan infections. Examples of microbial infections include mucosal infections, for example, gastrointestinal, urogenital or respiratory

infections. The agents may also be useful in the treatment or prevention of, inter alia, wounds, ulcers and lesions for example, cutaneous wounds such cuts or burns, and conditions associated therewith. The agents may also be useful in the treatment of immunosuppression induced by infectious agents (eg HIV) or by treatments such as radiation or chemotherapy.

Patients who are immuno-suppressed, for example patients undergoing anti-cancer treatments such as chemotherapy, may benefit from a treatment in which inflammation is induced. Thus the agent according to the invention may also be used to induce inflammation in a subject. Preferably the subject is human.

In a yet further aspect, the invention provides the use of a polypeptide in the identification of agents which modulate Toll-IL-1 receptor function wherein the polypeptide is selected from the group consisting of:

- i) a polypeptide, or fragment or variant thereof, encoded by a nucleic acid molecule consisting of a nucleic acid sequence as represented by Figure 1, or a sequence complementary thereto, or a fragment thereof;
- ii) a polypeptide encoded by a nucleic acid molecule which hybridises to a nucleic acid molecule as defined in (i) above and which modulates Toll-IL-1 receptor function; and
- iii) a polypeptide comprising a nucleic acid which is degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) and (ii).

A yet further aspect of the invention provides the use of a Toll-IL-1 receptor in the identification of agents which modulate the interaction of said receptor with a TILRR polypeptide selected from the group consisting of:

- i) a polypeptide, or fragment or variant thereof, encoded by a nucleic acid molecule consisting of a nucleic acid sequence as represented

by Figure 1 or a sequence complementary thereto, or a fragment thereof;

- ii) a polypeptide encoded by a nucleic acid molecule which hybridises to a nucleic acid molecule as defined in (i) above and which modulates Toll-IL-1 receptor function; and
- iii) a polypeptide comprising a nucleic acid which is degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) and (ii).

The TILRR polypeptides can be used for structure-based design of TILRR inhibitors or of molecules which modulate Toll-IL-1 receptor function such as though the modulation of TILRR binding to a Toll-IL-1 receptor. Such "structure based design" is also known as "rational drug design". The TILRR polypeptides can be three-dimensionally analysed by, for example, X-ray crystallography, nuclear magnetic resonance or homology modelling, all of which are well-known methods. The use of TILRR structural information in molecular modelling software systems is also encompassed by the invention. Such computer-assisted modelling and drug design may utilise information such as chemical conformational analysis, electrostatic potential of the molecules, protein folding etc. One particular method of the invention may comprise analysing the three-dimensional structure of TILRR for likely binding sites of targets, synthesising a new molecule that incorporates a predictive reactive site, and assaying the new molecule as described above.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are

to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

Throughout the description and claims of this specification, the words “comprise”
5 and “contain” and variations of the words, for example “comprising” and “comprises”, mean “including but not limited to”, and are not intended to (and do not) exclude other moieties, additives, components, integers or steps.

The invention will now be described, by way of example only, with reference to the
10 following figures:

Figure 1 (A) Partial cDNA sequence of human TILRR; (B) Full-length cDNA sequence of human TILRR;

15 Figure 2 (A) Amino acid sequence encoded by the cDNA sequence of Figure 1(A); (B) Amino acid sequence encoded by the cDNA sequence of Figure 1(B);

Figure 3 DNA and amino acid sequence of mouse TILRR;

20 Figure 4 Features of the amino-acid sequence of TILRR;

Figure 5 ^{125}I -IL-1 binding to cells transfected with TILRR-specific siRNAs (squares) or with a random/control siRNA (diamonds), demonstrating a 50% reduction in saturated, specific IL-1 binding after inhibition of TILRR;

25

Figure 6

A. Confocal images of cells transfected with I κ B α GFP together with random siRNA used as control or with a siRNA specific for the Toll/IL-1 receptor regulating HSPG (TILRR) and stimulated with IL-1 for the indicated times demonstrating reduction in
30 cytoplasmic I κ B α during IL-1 induction in control cells. In contrast, the cytoplasmic

levels of I κ B α are unchanged during IL-1 stimulation of cells containing the TILRR-specific siRNAs, demonstrating inhibition of signalling activity.

B. Quantitation of numerous readings such as demonstrated in A. Cells transfected with I κ B α EGFP and co-transfected with a random, control siRNA (diamonds) or
5 non-transfected cells (triangles) and stimulated with IL-1 show successive reduction in I κ B α levels, reflecting activation of the NF- κ B pathway. In contrast, cells transfected with I κ B α EGFP together with a TILRR-specific siRNA (squares) demonstrate no significant IL-1 induced degradation of I κ B α , reflecting lack of
10 pathway activation. The same effect was induced using a series of TILRR-specific siRNAs.

C. Quantitation of experiments as in A and B using TLR4 induced activity through LPS stimulation, demonstrating that similarly to IL-1 induced responses, Toll receptor mediated NF- κ B activities are potently blocked by a TILRR-specific
15 siRNA (squares) with no effect in controls transfected with a random siRNA (triangles).

EXAMPLES

Materials and Methods

20 Characterisation of the cell surface protein, TILRR

Tissue culture

C12756, a transformed high IL-1 receptor type I expressing mouse mammary epithelial cell line, HeLa cells or human gingival fibroblasts, or smooth muscle cells were used.
25 Cells were propagated in monolayer cultures under standard conditions in DMEM containing 5mM sodium pyruvate, 100 μ g/ml penicillin and streptomycin, and 10% heat inactivated foetal bovine serum (Life Technologies) in humidified atmosphere (95% air/5% CO₂) at 37°C. Cells were detached using EDTA (5mM, Sigma). For sequence analysis of TILRR, cells were plated on coated plates (FN, 10 μ g/ml, Life Sciences) for 4
30 hours or on bare plastic and grown for 48-72 hours. Incubation with IL-1 (1nM), and with

LPS (1µg/ml) was carried out to stimulate activities through the IL-1 receptor type I and TLR4, respectively.

Crosslinking and Immunoprecipitation:

- 5 For immunoprecipitation, of the IL-1 receptor complex, cells were incubated with IL-1α (1-6nM) in the cold for 2 hours to obtain saturated IL-1 binding. Following extensive washing to remove unbound IL-1, as described (Valles et al., 1999), cells were incubated with crosslinker (BS³, 0.1mg/ml, 30 min rt) according to Valles et al., 1999 or using SDST crosslinker (gelecysteines reduced/protected using DTT/IAM). Subsequently, cells
- 10 were harvested using EDTA and extracted at 4°C using triton-X, (50µl/10⁶cells), repeated vortexing and by passaging through a 21gauge needle (Valles et al., 2002) and centrifuged (10 min, 13000g). For preclearing the samples, supernatant was removed and cell extracts were incubated with 2nd ab (anti-sheep IGg, Abcam, 5µl/ml cell extract) together with precleared beads (Protein G Dynabeads, Dynal Biotech, 20µl/1ml extraction buffer)
- 15 for 30 min at 4°C, during gentle rotation. Subsequently, samples were cleared using Dynal magnets (Dynal Biotech, 4°C). The supernatant for each sample was transferred to new tubes and incubated with the primary antibody (10µl/mlsheep anti-human IL1alpha polyclonal, kind gift by Steve Poole, NIBSC) (60 minutes, 4°C). After collecting the beads using the Dynal Magnets as above, and removal of the supernatant, beads were
- 20 resuspended (4X washed) in RIPA buffer containing per 50mls:150mM NaC, 50mM Tris-HCl pH7, 0.5% Deoxycholate, 0.5%, NP-40, 0.1% SDS. Subsequently, SDS sample buffer was added and beads with precipitates were boiled for 5-10 min prior to separation by SDS PAGE gel electrophoresis and chomassie blue staining, using conventional procedures as described in Valles et al., 1999, 2002). Controls included samples
- 25 immunoprecipitated in the absence of the primary antibody or in the absence of both primary and secondary antibodies.

MALDI TOF and Mass spec Analysis:

Preparation of Samples

- 30 For sequence analysis, the high molecular weight component of about 300-350kDa (Valles et al., 1999, 2002) from immunoprecipitated samples, separated using SDS gel

electrophoresis were cut out and subjected to cysteine alkylation by Tributylphosphine (reduction.T 7567) and Iodoacetamide (alkylation A 3221).using the Sigma ProteoPrep Reduction and Alkylation Kit (Cat. No. PROTRA) using siliconised tubes (Sigma, T4691-500EA). Subsequently, samples were subjected to Trypsin digest using the
5 Trypsin Profile IGD Kit from Sigma (Cat. No. PP0100), and according to manufacturers instructions.

MS Analysis

These samples were subsequently subjected to Surface Enhanced Laser Desorption
10 Ionisation (SELDI) / Matrix Assisted Laser Desorption Ionisation (MALDI) as described (Ashcroft *Nat.Prod. Rep.*, **20**, 202-215). Briefly, an aliquot (1 μ L) of the sample in solution (1:1 v/v aqueous methanol, with 0.1% trifluoroacetic acid) was placed on the sample target and treated with an aliquot (1 μ L) of the matrix, α -cyano-4-hydroxy cinnamic acid (CHCA), in solution (4.5 mg/ml in 1:1 v/v aqueous acetonitrile, with 0.05%
15 trifluoroacetic acid). The mixture was allowed to dry at room temperature and then analysed by MALDI-MS on the SELDI ProteinChip mass spectrometer (Ciphergen, USA; purchased with funds from the Wellcome Trust) using a laser intensity of 190 and a detector sensitivity of 9. Approximately 100 laser shots were accumulated and an external calibration applied to the resulting spectrum using ions from a standard peptide mixture
20 (Arg-8-vasopressin, 1084.2 Da; somatostatin, 1637.9 Da; bovine insulin B chain, 3495.9 Da; human insulin, 5807.7 Da; hiruden, 7033.6 Da). A mass accuracy of $\pm$ 1 Da is expected.

Sequence analysis:

25 Peptide maps were analysed using peptide mass map fingerprinting programmes ProFound at PROWL site (prowl.rockefeller.edu/cgi-bin/ProFound) and the MASCOT, MOWSE and PeptIdent sites. Further, database (www.ensembl.org) and BLAST searching of the obtained sequence revealed a human protein which is the product of a unique gene, which GeneScan predictions suggests gives rise to several alternately spliced
30 mRNAs, encoding polypeptides of the sizes reported by Valles et al 1999.

Analysis of biological effects*Plasmids*

Specific siRNA's designed based on the obtained sequence from TILRR obtained from (Eurogentec) are shown in Table 1 below:

SiRNA 1	5'	CUCUCUCCACAGGUCUUUG	dTdT	3'
	3' dTdT	GAGAGAGGUGUCCAGAAAC		5'
scrambled		UCCUACGGUUCUUCAGUCC		
SiRNA2	5'	CCAGACUGUAACAUCAUCC	dTdT	3'
	3' dTdT	GGUCUGACAUUGUAGUAGG		5'
scrambled		CCAAACCUGAAUCCUGCAU		
SiRNA3	5'	ACCGUAUACAAGAGAGUGC	dTdT	3'
	3' dTdT	UGGCAUAUGUUCUCUCACG		5'
scrambled		AAAGAUGCCGGAGACCAUU		

5

and random controls (pGL2, Eurogentec) were used at concentrations of 10-100pM.

The plasmid *pIkappaBalpaEGFP* encodes a red shifted variant of green fluorescent protein fused to the carboxy terminus of IkB α constructed as described (Carlotti et al., 1999, Yang et al., 2003) by cloning the IkB α cDNA from *pIkappaBalpa* ctag (kind gift from Ronald Hay), digested with *Bam*HI plus *Eco*RI, into *pEGFP-N2* (Clontech) and with *Bgl*III plus *Eco*RI, and infilling of a *Sma*I plus *Eco*RI digest for alignment of the reading frame. The plasmid containing human TLR4 was induced through the pUNO promoter and coding a 5.5kbp fragment (Invivogen) used at 10 and 100pM concentration. The sequences of all constructs were confirmed by sequence analysis.

15

Transfection methods:

For receptor binding analyses including radioreceptor assays and SDS-PAGE gel electrophoresis, cells were grown in 10 cm or 48 well plates, and 24 hour prior to analyses were transfected at 70% confluency with siRNAs (10pM) using 1.5 μ l Jetsi

20

transfection agent (Eurogentec) per well of a 48 well plate according to the manufacturer's instructions. For confocal analysis calcium phosphate method was used as previously (Carotti et al., 1999; Yang et al., 2003). Briefly, Cells were plated 24 hours prior to transfection and transiently transfected using calcium phosphate co-precipitation with glycerol shock (60 seconds, 15% glycerol in PBS) four hours after transfection.

Receptor binding:

Cells were transfected with random siRNA or specific HSPG blocking siRNA, using JetSi as above. Twenty-four hours after transfection IL-1 receptor binding was carried out using radiolabeled IL-1 α , as previously (Valles et al., 1999, 2002).

Radiolabeling of human IL-1

Recombinant human IL-1 α (kind gift of Immunex Corp) was radiolabeled with ^{125}I by a modified chloramine-T method as described previously (Dower et al., 1985). Briefly, 30ng of IL-1 α (1.71nmol) in 10 μl of PBS were incubated with 5mCi (2.0nmol) of sodium ^{125}I -iodide (New England Nuclear, Boston, MA) in 25 μl of 0.5M sodium phosphate, pH7, and 30 μl of $1.4 \times 10^{-4}\text{M}$ chloramine-T (4.2nmol) for 30 min on ice. The reaction mixture was fractionated and the reaction terminated by rapid filtration on a 1ml bed volume Biogel P6 column (BioRad) preblocked with BSA (1%w/v in PBS). Following elution with PBS, fractions 2-4 (100 μl ea.) containing the labeled protein, were pooled. The labeled protein had a specific activity in the range $3\text{-}6 \times 10^{15}$ dpm/mmol.

Receptor binding

For binding experiments monolayer cultures plated on fibronectin coated plates or on bare plastic, as described above, were washed twice with fresh medium. Subsequently, 2 ml of binding medium (RPMI 1640, with 1% BSA, 20mM Hepes, pH 7.2 and 0.1% sodium azide) containing the appropriate concentration of ^{125}I -IL-1, was added to the wells. Non-specific binding was measured using 100 fold higher concentration of unlabelled IL-1 α . Cultures were incubated at 4°C in the presence of sodium azide for 2 hours using a gyratory shaker to ensure continuous mixing of the supernatant. The incubation time

chosen (2 hours) was based on previous kinetic studies (Qwarnstrom et al., 1988 JBC 263: 8261-69).

Association kinetics experiments were performed at four IL-1 concentrations (0.1, 0.75, 1, 1.5 nM). Following incubation, 60µl of supernatant was withdrawn to measure free ¹²⁵I-IL-1 concentrations. The monolayers were washed rapidly with 5 ml of ice-cold binding medium to remove unbound ligand and harvested following incubation at 37°C for 15 minutes in 1 ml trypsin EDTA. Bound ¹²⁵I-IL-1 was measured in a gamma- or beta-counter.

10

Non-specific binding data were analyzed by curve fitting using the equation:

$$\text{Bound (molecules/cell)} = A \times C$$

where C is the free IL-1 concentration (M) and A (molecules/cell/M) either measured directly for each concentration, or determined using one or two IL-1 concentrations, is the slope of a line passing through the origin. Non-specific binding for all experimental points was subsequently calculated by interpolation and subtracted from the specific binding data. Molecules/cell was calculated using the specific activity of the radiolabeled ligand using the formula

$$\text{molecules bound/cell} = \frac{\text{cpm} \times A}{B \times C}$$

20

Where A is Avogadros number, B is the specific activity of the radiolabeled IL-1 (cpm/mmol) and C is the number of cells determined in duplicate by haemocytometer.

Values for specific binding (molecules/cell) were analyzed by non-linear least squares fitting using the equation:

25

$$\text{Bound (molecules/cell)} = \frac{R_0 \times K \times C}{1 + K \times C}$$

where K (M⁻¹) is the affinity of IL-1 for its receptor, R₀ is the total receptor concentration (sites/cell) and C (M) is the IL-1 concentration. Parameter values were estimated by non-linear least squares fitting of this equation to equilibrium binding data. Association

30

kinetics data were analyzed using a pseudo first order treatment as described. All calculations were done using MLAB for Macintosh (Civilized Software, Silver Spring, MD). For illustration purposes, in some cases the data were converted to Scatchard format.

5

Confocal Microscopy:

Cells transfected with siRNAs (control and IL-1RIII, specific) or with a construct containing TLR4 or with empty vector, together with IkappaBalphaEGFP . The
10 IkappaBalphaEGFP fusion protein was visualized using a Molecular Dynamics confocal laser scanning microscope fitted with a 37°C stage incubator and coupled to a Nikon Diaphot 300 microscope and a Silicon Graphics workstation. Laser power was set to 10 mW, band selection to 488 nm, PMT voltage to 750 and varying laser attenuation to maintain pixel density below 200, within the linear range of the instrument. Emission
15 scans were done with a 60x Plan Apo oil immersion objective (NA 1.4) and a 50µm aperture generating an optical section of 0.54 micro-m, and using a 530nm band pass filter. To quantitate the cytoplasmic and the nuclear fusion protein level, transfected cells were scanned horizontally through the nucleus and images analyzed using NIH image. Relative fluorescence was calculated by measuring mean intensity of representative areas
20 of nucleus or cytoplasm and normalized by dividing by the attenuation, and further by 2.41 for consistency with previous results obtained with a PMT of 666 V (Carlotti et al., 1999).

Quantitation of levels of fluorescence by correlation to levels of endogenous protein was
25 done by immunocytochemical staining, as describe previously (Carlotti et al, 1999; Yang et al., 2003). Briefly, incubation with a rabbit polyclonal IkappaBalpha antisera (1µg/ml, 1 hr, rt; Santa Cruz Biotechnology) of cells transfected with IkappaBalphaEGFP was carried out following fixation in methanol (-20°C, 5 min) and incubation in blocking serum (5%, 1 hr), and prior to incubation with biotinylated secondary antibody (2 µg/ml,
30 ON 4°C, Santa Cruz Biotechnology) and streptavidin/Texas Red (0.2µg/ml, 15 min, Molecular Probes). Data (red fluorescence) was acquired using 750V with excitation at

568nm and emission at 590nm. Excitation and emission of green fluorescence from the fusion protein was done at 488 and 530 nm respectively, as above. Green and red fluorescence was plotted for individual cells over a range of transfection levels. The level of GFP fluorescence was corrected for minor reduction in intensity (15%) induced by methanol fixation.

Results

Demonstrated earlier, structural agonists induces association of a high molecular weight component with the IL-1 receptor type 1, which appears at a level around 350 kDa published in Valles et al., 1999. Direct association of the complex with the IL-1 receptor type I was demonstrated by immuno-precipitation using an anti-IL-1 type I receptor antibody following IL-1 stimulation with radiolabeled ligand. Cell surface labelling an subsequent heparinase treatment demonstrates degradation of the high molecular weight species and the appearance of a 90 kDa and a 150kDa species, published in Valles et al., 1999. Independent ligand binding to the TILRR was demonstrated by inhibiting IL-1 receptor type using a blocking antibody and demonstrating a 50% remaining IL-1 binding under conditions of increased TILRR expression, published in Valles et al., 2002.

Maldi-tof analysis of the trypsin digests demonstrated a series of larger peaks in the range of 1,200-3,500kDa and a couple of peaks in the range of 5,000 kDa. These data were subsequently used for predicting the protein sequence using the analysis programmes ProFound and the MASCOT, MOWSE and PeptIdent sites, leading to the identification of a series of fragments and sequences which Database (www.ensembl.org) and BLAST searching revealed to correspond to a human and a mouse protein (Figures 1 and 2).

GeneScan predictions suggested the genes to give rise to several alternately spliced mRNAs, encoding polypeptides of the sizes of 90 and 150 kDa respectively. The sequences of the long forms of the distinct human and mouse proteins has a core protein of 716 aa (Figures 1 and 3). The protein has no cytoplasmic domain but

contains a c-terminal GPI attachment site in its lectin domain at residue 710 (Figure 4). The lectin domain extends from residue 600 until the c-terminal end of the protein at residue 600. A 100 residue segment starting at residue 280 contains an integrin Ca binding domain. The protein contains a series of N-linked sites
5 throughout, starting at residue 77 and including (residue 102, 219, 245, 398, 477, 548). Finally, the sequence analysis demonstrates a GAG attachment site, located in the N terminal portion of the protein residues 4-9.

To assess the biological effects of this protein in relation to IL-1 signalling and thus
10 confirm the initial biological studies siRNAs were designed based on the protein sequence and as outlined in material and methods. Radioreceptor assay using cells transfected of the protein specific siRNAs as described in Materials and Methods, demonstrated a 50% reduction in specific ¹²⁵I-IL-1 binding compared to non transfected cells and to cells transfected with a random control siRNA (Figure 5). In
15 addition scatchard analysis demonstrated effects on ligand receptor affinity (K_a).

Effects on NF-κB signalling was demonstrated using a single cell assay and determining effects on degradation of the NF-κB inhibitor IκBα over time during IL-1 stimulation of cells transfected with TILRR specific siRNA and random siRNA as
20 above, respectively (Figure 6A). Quantitation of these types of data and averaging of numerous single cell readouts showed that reduction in IκBαEGFP degradation in the presence of the random siRNA was the same as that induced in the absence of siRNA, demonstrating, as previously (Yang et al., JBC 2003) showed a successive reduction of about 40-50% during the first 30 minutes of IL-1 stimulation and a
25 reduction corresponding to 55% of initial levels after 60 minutes (Figure 6B). In contrast, co-transfection with specific siRNA, caused a total inhibition of NF-κB activation as determined by IκBα degradation, demonstrating near initial values throughout stimulation.

30 To determine potential general effects on TIR regulated receptors, similar experiments were carried out using cells co-transfected with the toll like receptor 4

(TLR4) and TILRR specific or random siRNAs and stimulated with LPS (Figure 6C). These experiments demonstrated that the effect of TLR4 activity in control cultures (transfected with random siRNA) was less rapid compared to that induced by IL-1, demonstrating reduction of 40% over 2 hours of LPS stimulation, and corresponding
 5 to 50% after 8 hours of stimulation. Similarly to the effect observed on IL-1 mediated stimulation (Figure 6B), activation of TLR4 by LPS was inhibited by TILRR specific siRNA (Figure 6C). Thus, co-transfection with specific siRNA, caused a total inhibition of NF- κ B activation as determined by I κ B α degradation, demonstrating near initial values throughout stimulation.

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- 20

CLAIMS

1. An agent that modulates the effect of a cell surface polypeptide on Toll-IL-1 receptor function wherein said cell surface polypeptide is selected from the group consisting of:
- 5 i) a polypeptide, or variant thereof, encoded by a nucleic acid molecule consisting of a nucleic acid sequence as represented by Figure 1 or a sequence complementary thereto;
- ii) a polypeptide encoded by a nucleic acid molecule which hybridises to a nucleic acid molecule as defined in (i) above and which modulates Toll-IL-1
10 receptor function; and
- iii) a polypeptide comprising a nucleic acid which is degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) and (ii), for use as a medicament.
- 15 2. An agent as claimed in claim 1 wherein the nucleic acid sequence is as represented by Figure 1B.
3. An agent as claimed in claim 1 wherein said agent is an agonist.
- 20 4. An agent as claimed in claim 1 wherein said agent is an antagonist.
5. An agent that modulates the effect of a cell surface polypeptide on Toll-IL-1 receptor function wherein said polypeptide comprises an amino acid sequence as represented in Figure 2 or a variant polypeptide thereof wherein said variant is
25 modified by addition, deletion or substitution of at least one amino acid residue of the amino acid sequence represented in Figure 2 and said variant polypeptide modulates Toll-IL-1 receptor function.
6. An agent as claimed in claim 5 wherein said agent is a polypeptide.
- 30

7. An agent as claimed in claim 6 wherein said polypeptide is an antibody or an active binding part of an antibody.
8. An agent as claimed in claim 7 wherein said antibody is a monoclonal antibody or active binding part thereof.
9. An agent as claimed in claim 7 or 8 wherein said antibody is a chimeric antibody or a humanised antibody.
10. An agent as claimed in any of claims 7 to 9 wherein said agent is an antibody fragment.
11. An agent as claimed in claim 10 wherein said fragment is selected from the group consisting of Fab, Fab₂, F(ab')₂, Fv, Fc and scFvs.
12. An agent as claimed in claim 11 wherein said fragment is a single chain antibody variable region fragment.
13. An agent as claimed in any of claims 7 to 12 wherein said antibody is provided with a marker including a conventional label or tag.
14. An agent as claimed in claim 6 wherein said agent is a peptide consisting of an amino acid sequence selected from the group consisting of amino acid residues 280 to 380 of the amino acid sequence presented in Figure 2, amino acid residues 600 to 716 of the amino acid sequence presented in Figure 2, amino acid residues 1 to 6, amino acid residues 77 and/or 102 and/or 219 and/or 245 and/or 398 and/or 477 and/or 548 of the amino acid sequence presented in Figure 2, or a variant peptide wherein said variant polypeptide sequence has been altered by addition, substitution or deletion of at least one amino acid residue and wherein the variant modulates Toll-IL1 receptor function.

15. An agent as claimed in any of claims 1 to 5 wherein said agent is a nucleic acid molecule, for example an aptamer, a small interfering RNA (RNAi) or an antisense nucleic acid.

- 5 16. An agent as claimed in claim 15 wherein said RNAi molecule comprises a nucleic acid sequence selected from the group consisting of:

CUCUCUCCACAGGUCUUUG

10 CCAGACUGUAACAUCAUCC

ACCGUAUACAAGAGAGUGC

17. A pharmaceutical composition comprising an agent as claimed in any of
15 claims 1 to 16 in combination with a pharmaceutically acceptable adjuvant, carrier or diluent.

18. A composition comprising a nucleic acid molecule selected from the group consisting of:

- 20 i) a nucleic acid molecule consisting of a nucleic acid sequence as represented in Figure 1;
- ii) a nucleic acid molecule that hybridises under stringent hybridisation conditions to the nucleic acid molecule in (i) above and which encodes a polypeptide that modulates Toll IL-1 receptor function, for use as a vaccine.

25

19. A composition comprising a polypeptide comprising an amino acid sequence as represented in Figure 2, or a variant polypeptide wherein said variant is modified by addition, deletion or substitution of at least one amino acid residue of the amino acid sequence presented in Figure 2, for use as a vaccine.

30

20. A vector which is adapted for the expression of a humanised or chimeric antibody or an antibody fragment as claimed in any of claims 7 to 12.

21. A cell which has been transformed or transfected with the vector of claim 20 encoding a humanised or chimeric antibody or an antibody fragment
- 5 22. A method for the production of a humanised or chimeric antibody or antibody fragment comprising:
- i) providing a cell transformed or transfected with a vector which comprises a nucleic acid molecule encoding the humanised or chimeric antibody as claimed in any of claims 9 to 13;
 - 10 ii) growing said cell in conditions conducive to the manufacture of said antibody; and
 - iii) purifying said antibody from said cell, or its growth environment.
23. A hybridoma cell line which produces a monoclonal antibody as claimed in
15 claim 8.
24. A method of producing a monoclonal antibody as claimed in claim 8 comprising using a hybridoma cell line as claimed in claim 23.
- 20 25. A method for preparing a hybridoma cell-line producing a monoclonal antibody comprising the steps of:
- i) immunising an immunocompetent mammal with an immunogen comprising at least one polypeptide having the amino acid sequence as represented in Figure 2, or fragments thereof;
 - 25 ii) fusing lymphocytes of the immunised immunocompetent mammal with myeloma cells to form hybridoma cells;
 - iii) screening monoclonal antibodies produced by the hybridoma cells of step (ii) for binding activity to the polypeptide of (i);
 - iv) culturing the hybridoma cells to proliferate and/or to secrete said
30 monoclonal antibody; and
 - v) recovering the monoclonal antibody from the culture supernatant.

26. A kit comprising a binding agent specifically reactive with a nucleic acid molecule that encodes a polypeptide comprising an amino acid sequence as represented in Figure 2, or an agent specifically reactive with a polypeptide
5 comprising an amino acid sequence as represented in Figure 2.

27. A kit as claimed in claim 26 wherein said kit further comprises an oligonucleotide or antibody specifically reactive with said nucleic acid molecule or said polypeptide.

10

28. A kit as claimed in claim 27 wherein said kit comprises an antibody specifically reactive with a polypeptide comprising an amino acid sequence as represented in Figure 2.

15 29. A method for the identification of agents which modulate Toll-IL-1 receptor function the method comprising the steps of

i) forming a preparation comprising a polypeptide and a Toll-IL-1 receptor wherein the polypeptide is selected from the group consisting of:

20 i) a polypeptide, or fragment or variant thereof, encoded by a nucleic acid molecule consisting of a nucleic acid sequence as represented by Figure 1;

ii) a polypeptide encoded by a nucleic acid molecule which hybridises to a nucleic acid molecule as defined in (i) above and which modulates Toll-IL-1 receptor function;

25 iii) a polypeptide comprising a nucleic acid which is degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) and (ii); and

i) adding at least one candidate agent to be tested;

30 ii) determining the effect, or not, of said agent on the function of the Toll-IL-1 receptor.

30. A method as claimed in claim 29 wherein said agent is an antagonist.
31. A method as claimed in claim 29 wherein said agent is an agonist.
- 5 32. A method to treat a disorder relating to Toll-IL-1 receptor function in a subject comprising administering a therapeutically effective amount of an agent as claimed in any of claims 1 to 16 or a composition as claimed in any of claims 17 to 19.
- 10 33. A method as claimed in claim 32 wherein the disorder which relates to Toll-IL-1 receptor function is an inflammatory disorder or a disorder of the immune system.
- 15 34. A method as claimed in claim 33 wherein the inflammatory disorder is selected from the group consisting of atherosclerosis, rheumatoid arthritis, osteoarthritis, polyarthritis, gout, lupus erythematosus, scleroderma, Sjorgen's syndrome, poly- and dermatomyositis, vasculitis, tendonitis, synovitis, bacterial endocarditis, osteomyelitis, psoriasis, pneumonia, fibrosing alveolitis, chronic bronchitis, bronchiectasis, emphysema, silicosis, pneumoconiosis, tuberculosis, ulcerative colitis, Crohn's disease, chronic inflammatory demyelinating
- 20 polyradiculoneuropathy, chronic inflammatory demyelinating polyneuropathy, multiple sclerosis, Guillan-Barre Syndrome and myasthenia gravis, mastitis, laminitis, laryngitis, chronic cholecystitis, Hashimoto's thyroiditis, and inflammatory breast disease.
- 25 35. A method as claimed in claim 33 wherein the disorder of the immune system is a microbial infection.
- 30 36. A method as claimed in claim 33 wherein the microbial infection is a bacterial, viral, fungal, yeast parasitic or protozoan infections.

37. A method as claimed in claim 33 wherein the microbial infection is a mucosal infection.

38. A method as claimed in claim 37 wherein the mucosal infection is a
5 gastrointestinal, urogenital or respiratory infection.

39. Use of a polypeptide in the identification of agents which modulate Toll-IL-1 receptor function wherein the polypeptide is selected from the group consisting of:

- 10 i) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence as represented by Figure 1;
- ii) a polypeptide encoded by a nucleic acid molecule which hybridises to a nucleic acid molecule as defined in (i) above and which modulates Toll-IL-1 receptor function; and
- 15 iii) a polypeptide comprising a nucleic acid which is degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) and (ii).

40. Use of a Toll-IL-1 receptor in the identification of agents which modulate the interaction of said receptor with a TILRR polypeptide selected from the group
20 consisting of:

- i) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence as represented by Figure 1;
- 25 ii) a polypeptide encoded by a nucleic acid molecule which hybridises to a nucleic acid molecule as defined in (i) above and which modulates Toll-IL-1 receptor function; and
- iii) a polypeptide comprising a nucleic acid which is degenerate as a result of the genetic code to the nucleic acid sequence defined in (i) and (ii).

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Figure 1

(A)

ATGAACTCTCTGAGTTGGGGGGC
TGCGAATGCCGTGCTGCTGCTCCTCCTGGCCTGGGCCAGCCCCACCTTCATCAGCATCAACCGCGG
GTGAGGGTGATGAAGGCCACTCTGCCTTCTGTGTCAGGAGATGACCTGAAGTTGCCATCCCTAAAGAGA
AAGATGCCTGCAAAGTGAAGTTGTGATGAATGAGCCAATAACCCAGAGGGTTGGGAACTCACTCCACA
GGTCTTTGACTGCCATTTCTTCCCAACGAAGTCAAGTATGTTCACAATGGTTGCCAATTTCTGATGAA
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TGGCTTGTCCCAAGCGATTGATAAAAATCTGCTCAGATTGATTATGATAGGATGGCTAGCCTGGAATGT
ACCGTCAGCCTGGACACTGCGAGAAGTCCGGCTGCCAGCCCATGGCCAGATGGTTCTCGGGGAGCCTCGAC
CAGAAGAACCTCGTGGAGATCAGCCACACAGTTTTCCTCAGAGTCCCAACTGAGAGCAAACTGAAGTG
TCCAGGTGGGAGCTGTACCCAGGATTAAGAAAATAGGAAGCCTCAAAGTGAGCTGTGAGGAGTTCTCTG
CTGATGGGCCTTCGTTATCAGCATCTGGATCCCCCTTCACCCAACATTGATTATATCTCCATCCAAGTGG
ACCTCACAGATACCAGGAGCAAAATGTGTACAAGTCAGAGAGTGGCTGGCTGCCTGTCTATATCAGAGC
TGGAATTCGAATCAGATTCCAAAGGCTGCATTCTATGGCCGTGTTTATTCTGGAAGTGGATCAGTTTCATC
CTGACCTCCTTGACTACATCAGTTCTGGACTGTGAAGAAGATGAGACCCCTAAACCTTGTGGTGTTCAT
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CACCTGGAAGATCTCAGTGACATGCAGATCGCCTATCAGCCACCAACAGCAGCCATTCTGAGAGGAGA
CATGATGAGGTAGAATTGGAGGTATACGACTTCTTCTTTGAAAGGAGTGCACCTATGACAGTCCACATCT
CCATCAGAACAGCAGATACAAATGCCCCCGTGTATCCTGGAATACAGGTCTGAGTCTCCTTGAGGGGCA
GTCTCGAGCCATCACTTGGGAACAGTTTCAGGTTGTGACAATGACGACATTGGTGTCTCCGGCTAGTC
ACCGTTGGTGGCCTGCAGCATGGATGGCTGACTTTAAGAGGGGGGAAAGGGTTTCTCTTCACCGTGGCTG
ACCTCCAGGCTGGAGTTGTTGCTATCATCATGATGACAGCGACTCCACCAAGACTTCGTGGTCTTCCG
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GGGACAGCCATGAACCTCCAAATCTTTAGTGCCACAGGTGGCAACAATCCATATAACTCCAGTGGATGA
CCAGCTTCCAAAGAGGCTCCTGGAGTTTCTCGGCATTTGGTTGTCAAGGAACTGAGGTGGCCTATATA
ACTAAGAAACAGCTACATTTTATAGATTGAGAATCATATGACAGGGAGCTGGTCTACACAATACTACTC
CTCCATTTTCTCCTTCAGTCACAGACACTTGGATGCTGGGAAATTTATTTATGGTGGACAGCATACCAAA
AGTAGTTAAGAATCCTACGGCCCTGGAGCTGAGGTCACTCAGCATGCTGTGAATATATGAAAGTG
GCCTACATGCCCCCATGCAAGACATTGGTCCCCATGTCAGAGATGTCCAGTTCACATTTTCTGTGAGTA
ACCAACATGGCGGTACTTTGCATGGGATCTGCTTTAACATTACAATCCTCCCAGTGGACAATCAAGTTCC
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TAGGGGGTTTAGCAAGACTTCTCTGAGAATAAGCAGCCAGCCAACCTCACCAGAAACATGACAGTGT
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CATCCCAGTTAATGATGAAAAACCAATGCTGAGCAAGAAGGCTGAAATTGCAATGAATATGGGTGAAACT
CGTATTATTTCCAGTGCTATTTCTTTACGCCATAGATGAAGACTCACCCAGGGAGAGATTTACTATGTAT
TTGAAAGGCTTCCCCAAAATGGGCAACTTCAGCTTAAGGAATGTCAACTAAGATGTGGAATATAG

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(B)

ATGAACTCTCTGAGTTGGGGGGCTGCGAATGCCGTGCTGCTGCTGCTCCTCCTGGCCTGGGCCAGCCCCA
CCTTCATCAGCATCAACCGCGGGGTGAGGGTGATGAAGGGCCACTCTGCCTTCTGTGTCAGGAGATGACCT
GAAGTTTGCCATCCCTAAAGAGAAAAGATGCCTGCAAAGTGGAAGTTGTGATGAATGAGCCAATAACCCAG
AGGGTTGGGAACTCACTCCACAGGTCTTTGACTGCCATTTCTTCCCAACGAAGTCAAGTATGTTTACA
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GTGCTGGAGGTGCCTGAATTCATCTGAGCTTGTCCCAAGCGATTGATAAAAAATCTGCTCAGATTCGATTATG
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GATGGTTCTCGGGGAGCCTCGACCAGAAGAACCTCGTGGAGATCAGCCACACAGTTTTTTCCAGAGTCC
CAACTGAGAGCAAACTGAAGTGTCCAGGTGGGAGCTGTACCCCAGGATTAAAGAAAATAGGAAGCCTCA
AAGTGAGCTGTGAGGAGTTCCTGCTGATGGGCCTTCGTTATCAGCATCTGGATCCCCCTTCACCCAACAT
TGATTATATCTCCATCCAACCTGGACCTCACAGATACCAGGAGCAAAATTTGTGTACAAGTCAGAGATGCG
TGGCTGCCTGTCTATATCAGAGCTGGAATTCGGAATCAGATTCCAAAGGCTGCATTCATGGCCGTGTTTA
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TGCACCTATGACAGTCCACATCTCCATCAGAACAGCAGATACAAATGCCCCCGTGTATCCTGGAATACA
GGTCTGAGTCTCCTTGAGGGGCAGTCTCGAGCCATCACTTGGGAACAGTTTCAGCTTGTGCAATGACG
ACATTGGTGTCTGTCCGGCTAGTCACCGTTGGTGGCTGCAGCATGGATGGCTGACTTTAAGAGGGGGAA
AGGGTTTCTCTTACCGTGGCTGACCTCCAGGCTGGAGTTGTTTCGCTATCATCATGACAGCGACTCC
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TCTTGCCCAAAGATGATAGTCCCCCGTTCTCATAACCAATGTTGTGATTGAACTGGAGGAGGGGCAGAC
CATCCTGATCCAGGGATCCATGCTGCGAGCTTCAGATGTGGACGCCAGTGATGACTACATCTTCTTCAAT
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ATCCATATAACTCCAGTGGATGACAGCTTCCAAAAGAGGCTCCTGGAGTTTCTCGCATTTGCTTGTCA
AGGAAACTGAGGTGGCTATATAACTAAGAAAGCTACATTTTATAGATTGAGAAATCATATGACAGGGA
GCTGGTCTACACAATAACTACTCCTCCATTTTCTCCTTCAGTCACAGACACTTGGATGCTGGGAAATTA
TTTATGGTGGACAGCATAACCAAAGTAGTTAAGAATCCTACGGCCCTGGAGCTGAGGTCAATCACTCAGC
ATGCTGTGAATATATGAAAGTGGCCTACATGCCCCCATGCAAGACATTGGTCCCCATTCAGAGATGT
CCAGTTCACATTTTCTGTGAGTAAACCAACATGGCGGTACTTTGCATGGGATCTGCTTTAACATTACAATC
CTCCCAGTGGACAATCAAGTTCCTGAGGCGTTACCAACCCCTCTGAAAGTGAAGTGGAGGAGGTCAAAGCA
TCATCAGCAGAGACATTAATTTCTGATGCAGATACCAAGCTGGACAATATTGACCTTCTCGCGG
GGAATTGCCTCTGACGGAAGGGTGGAGCTGAATGGATTTCTCCTCTAAATTCAGGGGGCACATTTTCTTGG
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TCTGAATACATTTTGTACTGATGTGGACAGCGATAACTTGAAGTTGATGTTTGTGATTGCTCGCGAAC
CTCAGCATGGGGTGGTGGAGAGAGCTGGAGTCACAGTGGATCAGTTCTCTCAGAGAGATGTTATCTCAGA
GGCCGTGACATACAAACACAGGTGGCGAGATTGGCCTGATGCCTTGTTTTGACACCATTAACATTGGTG
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Figure 2

(A)

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(B)

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 241 llmglyqhl dppspnidy siqlldttr skivyksesa wlpvyiragi pnqipkaafn
 301 avfilevdqf iltsltsvl dceedetpkp llvfnitkap lqgyvthld htrpissftw
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 1981 ikvaelpqad kvesttdshf prqdqlpsf knctlelkg lfhfeegiql yqcngiawka
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Figure 3**Mouse coding region**

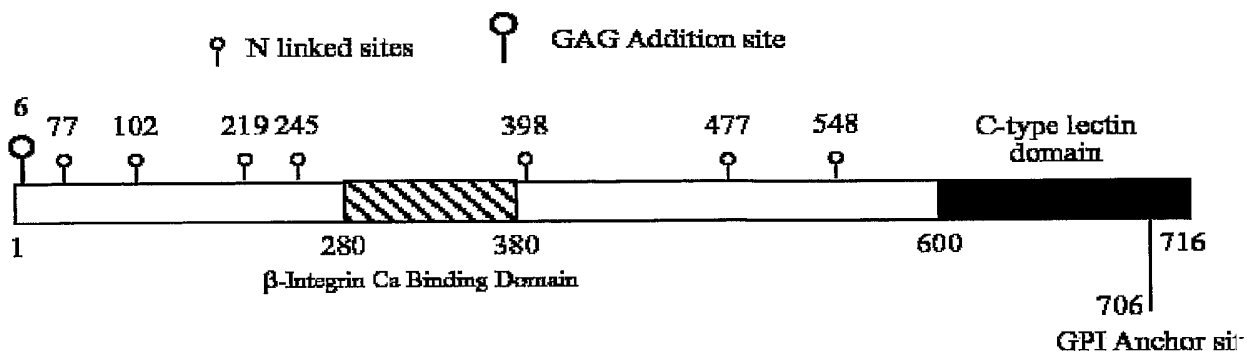
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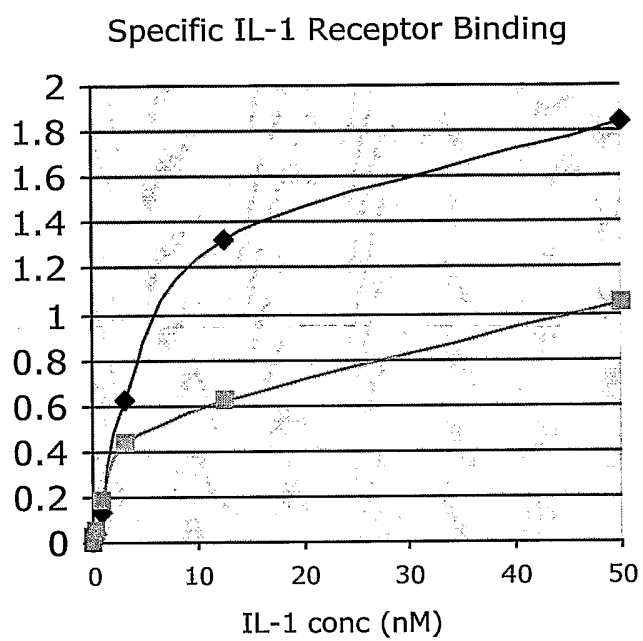
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Figure 4



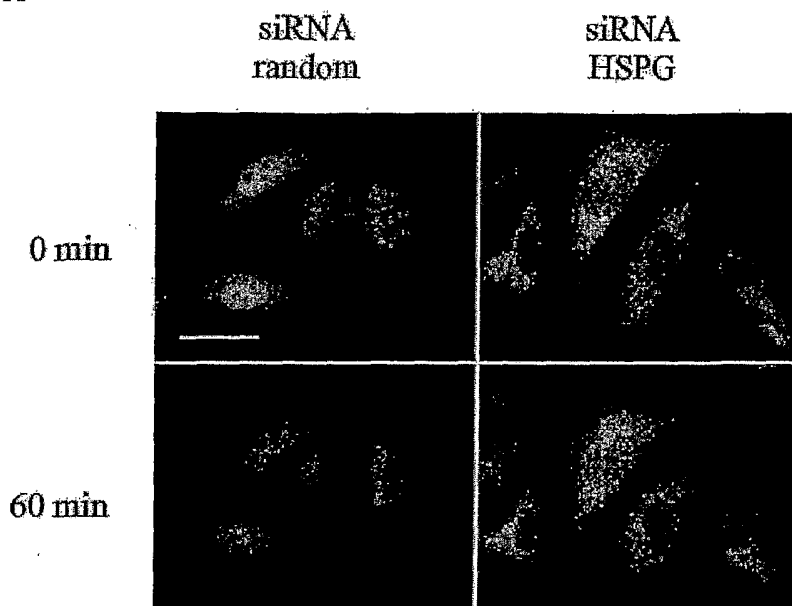
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Figure 5

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Figure 6

A

Bar=10 μ

B

