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(54) Title: CSF-1 IMMUNOMODULATION

(57) Abstract: Methods and compositions are provided for modulating immune responses by modulating the activity of CSF-1. In particular, the methods and compositions reduce CSF-1 activity and thereby suppress immune responses to bacterial lipopolysaccharides while enhancing immune responses to immunostimulatory DNA. The methods and compositions of the invention may be particularly useful in suppressing immune responses associated with bacterially-induced septic shock. Also provided are methods of identifying CSF-1 antagonists by measuring the effect of candidate molecules on CSF-1 mediated immune responses to bacterial lipopolysaccharides and/or immunostimulatory DNA.

CSF-1 IMMUNOMODULATION

FIELD OF THE INVENTION

THIS INVENTION relates to methods and compositions for modulating immune responses. More particularly, this invention relates to modulating the activity of CSF-1 to thereby affect CSF-1-dependent immune responses. A particular aspect of the invention is to reduce CSF-1 activity as a means for suppressing immune responses to bacterial lipopolysaccharides while enhancing immune responses to immunostimulatory DNA. This invention also provides methods of identifying CSF-1 antagonists by measuring the effect of candidate molecules on CSF-1 mediated immune responses to bacterial lipopolysaccharides and/or immunostimulatory DNA.

BACKGROUND OF THE INVENTION

The ability of the host to respond to a bacterial challenge is conferred by cells of the innate immune system which detect bacterial products such as LPS, CpG DNA, peptidoglycan and bacterial lipoproteins (1). Recognition of these products by macrophages results in secretion of cytokines and small molecules that mediate the inflammatory response. Both the site of challenge and the magnitude of the response dictate outcome; local infections trigger a controlled response in which the infection is contained and resolved whilst systemic infection can lead to dysregulated cytokine production that can ultimately result in multiple organ failure and mortality. Although the effects of LPS and CpG DNA on macrophages are very similar, their toxicities in mice differ greatly; LPS is highly toxic (2, 3) whilst CpG DNA alone is not toxic (4), unless administered to D-galactosamine sensitised mice (5). This important difference has a major implication for the therapeutic potential of CpG DNA. However, the reason for this difference is still unclear.

Toll-like receptors (TLRs)[3] are an evolutionarily conserved family that share homology with the IL-1 receptor family in the cytoplasmic domain. Mammalian TLRs are critical in instigating responses to bacterial products. C3H/HeJ LPS non-responder mice contain an inactivating point mutation in the TLR4 gene (6, 7) and TLR4 deficient mice do not produce inflammatory cytokines in response to LPS (8). TLR2 deficient mice are still LPS-responsive, but fail to respond to bacterial lipoproteins or

peptidoglycan (9) and TLR9 deficient mice are incapable of responding to CpG-containing DNA (10). Engagement of TLRs triggers signaling through at least NF-kB and the mitogen-activated protein kinase (MAPK) family members, extracellular signal-related kinase (ERK)-1 and -2, p38 and c-Jun N-terminal kinase and results in transcription of pro-inflammatory genes (1, 11).

The macrophage response to bacterial products is also regulated by a variety of endogenous cytokines; both IFN-γ and GM-CSF (primarily T cell products) can prime the inflammatory response whilst IL-4, IL-10 and TGF-β are able to suppress macrophage activation. Colony stimulating factor-1 (CSF-1), a cytokine that regulates growth, differentiation and function of macrophages, is detectable in peripheral blood in the steady state and is further induced *in vivo* after infection (12) or challenge with LPS (13).

SUMMARY OF THE INVENTION

The mechanism whereby CSF-1 affects macrophage responsiveness to LPS and CpG DNA has remained elusive. The present inventors disclose herein that CSF-1 suppresses macrophage responses to CpG DNA while enhancing macrophage responses to LPS.

Therefore, the present invention is broadly directed to modulating CSF-1 activity as a means of modulating immune responses.

In one aspect, the invention provides a method of modulating an immune response, said method including the step of modulating CSF-1 activity in an animal, to thereby modulate the immune response of said animal.

In another aspect, the invention provides a method of immunizing an animal, said method including the step of administering a modulator of CSF-1 activity to said animal, in combination with an immunogen, to thereby elicit a modified immune response to said immunogen.

The aforementioned aspects of the invention also extend to administering a modulator of CSF-1 activity to one or more cells derived from an animal, inclusive of cell lines, to thereby modulate the immune response of said cells.

In yet another aspect, the invention provides a pharmaceutical composition comprising a modulator of CSF-1 activity and a pharmaceutically-acceptable carrier, diluent or excipient.

In a particular embodiment, the pharmaceutical composition is an immunotherapeutic composition that further comprises an immunogen.

The vaccine may still further comprise immunostimulatory DNA, such as in the form of one or more immunostimulatory oligonucleotides, for example phosphorothioate CpG DNA.

Preferably, the immune response is that which is directed by said animal, or said one or more cells derived therefrom, to bacteria or products derived from said bacteria such as bacterial nucleic acids, proteins, polysaccharides and lipopolysaccharides, oligosaccharides proteoglycans, lipoproteins and glycoproteins although without limitation thereto.

In one embodiment, the invention provides modulation of an immune response by enhancing, augmenting, priming or otherwise improving the immune response to immunostimulatory DNA.

In another embodiment, the method provides modulation of an immune response by suppressing, preventing limiting, delaying or otherwise attenuating said immune response to LPS.

Preferably, modulation of the immune response is achieved through modulating the activity of cells of the monocyte/macrophage lineage.

In a further aspect, the invention provides a method of identifying a CSF-1 antagonist by measuring an immune response to immunostimulatory DNA and/or an immune response to bacterial lipopolysaccharide (LPS).

According to this aspect, reduced CSF-1 activity is measured as:

(a) an enhanced immune response to immunostimulatory DNA; and/or

(b) a reduced immune response to bacterial lipopolysaccharide (LPS);

In one embodiment, a candidate molecule is administered to an animal and CSF-1 activity measured.

In another embodiment, said candidate molecule is administered to one or more animal cells.

Preferably, said animal cells are monocyte or macrophage cells.

In a particular embodiment, said animal cells are RAW264 cells that express green fluorescent protein (GFP) in response to CSF-1, wherein reduced CSF-1 activity is measured as reduced GFP expression.

Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. Effect of CSF-1 on BMM responses to LPS and CpG DNA. BMM were pretreated with CSF-1 (10^4 U/ml) or were left untreated overnight. The following morning cells were treated with medium, LPS (100 ng/ml), AO-1 (3 μM) or NAO-1 (3 μM) for 24 h. IL-12, IL-6 and TNF-α levels in supernatants were assayed by ELISA. Data are mean of triplicates ± SD. IL-6 was not detected in supernatants from control or NAO-1 treated cells. Similar results were obtained in 6 independent experiments.

FIGURE 2. IFN-γ does not overcome the inhibitory effect of CSF-1 on CpG responses. BMM were pretreated with CSF-1 (10^4 U/ml) or were left untreated overnight. The following morning, appropriate wells were pretreated with IFN-γ (50 U/ml) for 30 min before stimulation with LPS (100 ng/ml), AO-1 (3 μM) or NAO-1 (3 μM). Supernatants were collected after 24 h and IL-6 and IL-12 levels were estimated by ELISA (values are mean of triplicates ± SD). IL-6 and IL-12 were not detected in supernatants from control or NAO-1 treated cells.

FIGURE 3. Comparison of the effects of CSF-1, IL-3 and PMA on IL-6 production in response to LPS and CpG DNA. BMM were pretreated with CSF-1 (10^5 U/ml), IL-3 (1000 U/ml), PMA (100 ng/ml) or were left untreated overnight. The following morning cells were treated with medium, LPS (100 ng/ml), or AO-1 (3 μM) for 24 h. IL-6 levels in supernatants were assayed by ELISA. Results (mean of triplicates ± SD) were expressed as fold increase compared to IL-6 levels in unprimed supernatants treated with LPS (1125+/-52 pg/ml) or AO-1 (331+/-49 pg/ml). IL-6 was not detectable in

supernatants of cells primed with medium alone, CSF-1, IL-3 or PMA and triggered with medium only (data not shown). Similar results were obtained in 2 experiments.

FIGURE 4. Effect of CSF-1 pretreatment on LPS and CpG DNA-induced IL-12 (p40), IL-12 (p35) and TNF-α mRNAs. BMM, pretreated overnight with CSF-1 (10^4 U/ml) or left untreated, were stimulated for 4 h with LPS (100 ng/ml), AO-1 (3 μM) or medium. Total cellular RNA was isolated, cDNAs were prepared and levels of IL-12 (p40), IL-12 (p35) and TNF-α mRNA relative to HPRT were estimated by quantitative PCR (n=3 ± SD). Similar results were obtained in 2 independent experiments.

FIGURE 5. Time course of CpG DNA-induced p38 phosphorylation in the presence or absence of CSF-1. BMM, pretreated overnight with medium or CSF-1 were stimulated with AO-1 (3 μM) for the time interval indicated. Cell extracts were prepared and phosphorylated p38 levels were assessed by western blotting. Blots were stripped and reprobed for total p38 as a loading control. Similar results were obtained in 2 independent experiments.

FIGURE 6. Effect of CSF-1 pretreatment on CpG DNA- and LPS-induced MAPK p38 and ERK-1 and -2 phosphorylation. A. BMM, pretreated overnight with medium control or CSF-1, were stimulated with AO-1 over the concentration range indicated for 30 min. Cell lysates were prepared and levels of phosphorylated p38, total p38, phosphorylated ERK-1/2 and total ERK-1/2 were assessed by western blotting. B. BMM, pretreated overnight with medium control or CSF-1, were stimulated with a range of LPS concentrations for 30 min. Extracts were analysed as described in A. Similar results were obtained in 2 independent experiments.

FIGURE 7. Effect of CSF-1 on expression of TLRs in BMM. A. BMM were treated overnight with medium or CSF-1. 16 h later, total cellular RNA was isolated, cDNAs prepared and levels of TLR9 mRNA relative to HPRT were estimated by quantitative PCR (n=3 ± SD). Results are representative of 2 experiments. B. cDNAs were prepared as described as in A. and levels of TLR 1, 2, 4, 5 and 6 relative to HPRT were determined. Data from 2 experiments were pooled and results (n=6 ± SEM) are expressed as fold repression in response to CSF-1.

FIGURE 8. Regulation of TLR9 expression in BMM. A. BMM were treated for 18 h with medium, CSF-1 (5×10^4 U/ml), IL-3 (10^3 U/ml) or PMA (100 ng/ml). Total RNA

was prepared and levels of TLR9 and HPRT mRNA were assessed by PCR/southern hybridisation. B. BMM were starved of CSF-1 overnight and then treated with CSF-1 (5 x 10^4 U/ml) for the indicated times. Expression levels of TLR9 and HPRT were assessed as in A.

FIGURE 9. Effect of CSF-1 on TLR9 expression in TEPM. TEPM were cultured for 20 h with CSF-1 (5 x 10^4 U/ml) or medium alone. Levels of TLR9, TLR4 and HPRT mRNAs were estimated by PCR/southern hybridisation in total RNA from these cells. Similar results were obtained in 2 independent experiments.

FIGURE 10. Suppressive effect of CSF-1 on macrophage responses to CpG ODN and PS-CpG ODN. Primary one marrow-derived macrophages were pretreated overnight with CSF-1 (50,000 U/mL) and then were stimulated the following day with CpG ODN (AO-1) or with PS-CpG ODN (AOS-1). After 8 hr, culture supernatants were harvested and levels of IL-6 in supernatants were estimated by ELISA. Determinations were in triplicate and the mean +/- SD is displayed for each data point.

FIGURE 11. Dominant effect of CSF-1 over other cytokines that potentiate CpG DNA responses. Primary bone marrow-derived macrophages were pretreated overnight with a range of different cytokine combinations and were then stimulated the following day with CpG ODN (AO-1) or with LPS. After 24 hr, culture supernatants were harvested and levels of IL-6 in supernatants were estimated by ELISA. Determinations were in triplicate and the mean +/- SD is displayed for each data point.

FIGURE 12. RAW264 ELAM cells (a RAW264 cell line that stably expresses green fluorescent protein driven by the NF-kappaB-responsive E-selectin promoter) were pretreated for 30 min with medium (No Ab) or with different doses of an anti-CSF-1 receptor antibody. Cells were then treated overnight with medium, CSF-1 (10 000 U/mL) or CSF-1 high (50 000 U/mL). The following day cells were stimulated with a CpG-containing phosphorithioate-modified oligonucleotide (AOS; 0.3 μM) or medium. GFP expression was determined by flow cytometry. Average of duplicates and range are displayed.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have reasoned that the previously reported immunomodulatory effects of CSF-1 might be due to regulation of LPS recognition via

the CD14-TLR4-MD2 complex, or at subsequent levels (such as through MyD88, TRAF6, IRAK) that appear to be shared with other microbial agonists such as CpG DNA. To distinguish these alternatives, the present inventors compared the effect of CSF-1 on the response to LPS and CpG DNA by primary murine bone marrow-derived macrophages (BMM). In light of these experiments, the present invention is predicated, at least in part, on the discovery that CSF-1 enhances macrophage responses to LPS but suppresses expression of TLR9 and responses to CpG DNA. These findings provide a mechanism for the differential cytotoxic effect of LPS and CpG DNA and have led the present inventors to propose novel immunotherapeutic strategies. A particular immunotherapeutic application is prophylactic or therapeutic treatment of bacterially-induced septic shock.

For the purposes of this invention, by "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

By "*protein*" is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids, D- or L- amino acids or chemically-derivatized amino acids as are well understood in the art.

A "*peptide*" is a protein having no more than fifty (50) amino acids.

A "*polypeptide*" is a protein having fifty (50) or more amino acids.

As used herein, an "*immunogen*" is any substance or molecule that is capable of being administered to an animal, or to cells isolated from an animal, to produce an immune response. Non-limiting examples of immunogens are attenuated bacteria or bacterial products such as bacterial nucleic acids, proteins, polysaccharides, lipopolysaccharides, oligosaccharides proteoglycans, lipoproteins and glycoproteins; attenuated virus and viral products inclusive of virus-like particles and viral protein subunits in recombinant or native form; recombinant or synthetic polypeptides and peptides, DNA constructs encoding polypeptides and peptides, purified synthetic or

native carbohydrate-containing molecules or any antigenic molecule or substance obtainable or derivable from a foreign pathogens.

The term "*nucleic acid*" as used herein designates single-or double-stranded mRNA, RNA, RNAi, cRNA and DNA inclusive of cDNA and genomic DNA.

As used herein, "*DNA*" is a dexoxyribonucleic acid in single-or double-stranded form, inclusive of single- or double-stranded oligonucleotides and plasmids such as used in gene therapy or DNA vaccination. DNA normally comprises the purine bases adenine (A) and guanine (G), and the pyrimidine bases cytosine (C) and thymidine (T). However, the skilled person will also appreciate that DNA may include modified bases such as methylcytosine, inosine, methylinosine, methyladenosine, thiouridine and methylcytosine, for example.

As used herein, "*immunostimulatory DNA*" is DNA which elicits or potentiates an immune response by cells of the vertebrate immune system, preferably the mammalian immune system, and more preferably the human immune system. Suitably, responsive cells of the immune system include macrophages, NK cells, dendritic cells, B cells and T cells.

A preferred immunostimulatory DNA is phosphorothioate-modified CpG oligonucleotides (PS-CpG ODN)

As used herein "*oligonucleotide*", abbreviated herein to "*ODN*", is single-stranded or double-stranded nucleic acid, preferably DNA, comprising between six (6) and one hundred (100) contiguous nucleotides, or preferably fifteen (15) to thirty (30) nucleotides. A "*polynucleotide*" is a nucleic acid having more than one hundred (100) contiguous nucleotides.

An oligonucleotide may have phosphodiester linkages between constituent bases, abbreviated herein to "*PO-ODN*", or phosphorothioate linkages, abbreviated herein to "*PS-ODN*". Phosphorothiaote linkages are created by replacing a non-bridging oxygen atom of the internucleotide phosphate group with a sulfur, to thereby create a thioester linkage. It will also be appreciated that oligonucleotides may be modified by addition of peptide linkages, enzymes, biotin, radiolabel, fluorochromes, lipids and carbohydrates.

For use *in vivo*, nucleic acids are preferably relatively resistant to degradation (e.g., are stabilized). A "*stabilized nucleic acid*" shall mean a nucleic acid that is

relatively resistant to *in vivo* degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Unmethylated CpG oligonucleotides that are tens to hundreds of kbs long are relatively resistant to *in vivo* degradation. For shorter CpG oligonucleotides, secondary structure can stabilize and increase their effect. For example, if the 3' end of an oligonucleotide has self-complementarity to an upstream region, so that it can fold back and form a stem loop type of structure, then the oligonucleotide becomes stabilized and therefore exhibits more activity. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. Preferred stabilized oligonucleotides of the instant invention have a modified backbone. It has been demonstrated that modification of the oligonucleotide backbone provides enhanced activity of the CpG oligonucleotides when administered *in vivo*. These stabilized structures are preferred because the CpG molecules of the invention have at least a partial modified backbone. CpG constructs, including at least two phosphorothioate linkages at the 5' end of the oligonucleotide and multiple phosphorothioate linkages at the 3' end, preferably 5', provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases. Other modified oligonucleotides include phosphodiester modified oligonucleotides, combinations of phosphodiester and phosphorothioate oligonucleotide, methylphosphonate, methylphosphorothioate, phosphorodithioate, and combinations thereof. Each of these combinations and their particular effects on immune cells is discussed in more detail in PCT Published Patent Application Nos. PCT/US95/01570 and PCT/US97/19791 claiming priority from U.S. Serial Nos. 08/386,063 and 08/960,774, filed on February 7, 1995 and October 30, 1997 respectively, the entire contents of which are hereby incorporated by reference. It is believed that these modified oligonucleotides may show more stimulatory activity due to enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered intracellular localization.

Modified backbones such as phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated

as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann & Peyman,1990, Chem. Rev. **90** 544; Goodchild, 1990, Bioconjugate Chem. **1** 1650).

Both phosphorothioate and phosphodiester oligonucleotides containing CpG motifs are active in immune cells. However, based on the concentration needed to induce CpG specific effects, the nuclease resistant phosphorothioate backbone CpG oligonucleotides are more potent. Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

Oligonucleotides are readily available in synthetic form, and can be "made to order" from a variety of commercial and laboratory sources. Oligonucleotides generated by digestion of larger nucleic acids are also contemplated.

A "*probe*" may be a single or double-stranded oligonucleotide or polynucleotide, suitably labeled for the purpose of detecting complementary sequences in Northern or Southern blotting, for example.

A "*primer*" is usually a single-stranded oligonucleotide, preferably having 15-50 contiguous nucleotides, which for example, is capable of annealing to a complementary nucleic acid "template" and being extended in a template-dependent fashion by the action of a DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or Sequenase™.

Modulators of CSF-1 activity

The present invention provides methods of modulating immune responses, methods of immunization and pharmaceutical compositions that utilize modulators of CSF-1 activity.

As used herein, a "*modulator of CSF-1 activity*" is a molecule or substance that affects a biological activity mediated by CSF-1. Preferably, said modulator of CSF-1 activity affects an immunological activity mediated by CSF-1.

In particular, the present invention contemplates modulation of immune responses involving CSF-1 responsive cells of the monocyte/macrophage lineage.

Preferred immunological activities of CSF-1 affected by said modulators include suppression of immune responses to immunostimulatory DNA and enhancement of immune responses to LPS.

Such modulators of CSF-1 activity may antagonize binding of CSF-1 to the cognate CSF-1 receptor (CSF-1R or c-fms) or mimic binding of CSF-1 to CSF-1R. Other modulators may interfere with intracellular signaling from CSF-1R or prevent CSF-1R receptor dimerization and activation. Conversely, modulators may facilitate CSF-1R receptor dimerization and activation.

Modulators that promote, mimic or enhance CSF-1 activity are generally referred to herein as "*agonists*". Modulators that diminish, attenuate, reduce or eliminate CSF-1 activity are generally referred to herein as s "*antagonists*".

Modulators of CSF-1 activity may be proteins, peptides or small organic molecules, for example.

Preferred modulators of CSF-1 activity are antagonists.

Non-limiting examples of antagonists are neutralizing anti-CSF-1 antibodies, neutralizing anti-CSF-1R antibodies, mutant forms of CSF-1 or interfering mutant forms of CSF-1R such as peptides corresponding to the CSF-1-binding domain of CSF-1R or corresponding to a dimerization domain of CSF-1R.

Persons skilled in the art will be aware that modulators of CSF-1 activity may be created by any of a number of methods.

With regard to mutant CSF-1 or CSF-1R, these can be created by mutagenizing wild-type CSF-1 or CSF-1R, or by mutagenizing an encoding nucleic acid, such as by random mutagenesis or site-directed mutagenesis. Examples of nucleic acid mutagenesis methods are provided in in Chapter 9 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel *et al.*, *supra* which is incorporated herein by reference.

Random mutagenesis methods include chemical modification of proteins by hydroxylamine (Ruan *et al.*, 1997, Gene **188** 35), incorporation of dNTP analogs into nucleic acids (Zaccolo *et al.*, 1996, J. Mol. Biol. **255** 589) and PCR-based random mutagenesis such as described in Stemmer, 1994, Proc. Natl. Acad. Sci. USA **91** 10747 or Shafikhani *et al.*, 1997, Biotechniques **23** 304, each of which references is incorporated herein. It is also noted that PCR-based random mutagenesis kits are commercially available, such as the Diversify™ kit (Clontech).

Modulators of CSF-1 activity may also be identified by way of screening libraries of candidate molecules such as synthetic chemical libraries, including combinatorial libraries, by methods such as described in Nestler & Liu, 1998, Comb. Chem. High Throughput Screen. **1** 113 and Kirkpatrick *et al.*, 1999, Comb. Chem. High Throughput Screen **2** 211.

It is also contemplated that libraries of naturally-occurring candidate molecules may be screened by methodology such as reviewed in Kolb, 1998, Prog. Drug. Res. **51** 185.

More rational approaches to designing modulators of CSF-1 activity may employ computer assisted screening of structural databases, computer-assisted modelling, or more traditional biophysical techniques which detect molecular binding interactions, as are well known in the art.

Computer-assisted structural database searching is becoming increasingly utilized as a procedure for engineering agonists and antagonist molecules. Examples of database searching methods may be found in International Publication WO 94/18232 (directed to producing HIV antigen mimetics), United States Patent No. 5,752,019 and International Publication WO 97/41526 (directed to identifying EPO mimetics), each of which is incorporated herein by reference.

Generally, other applicable methods include any of a variety of biophysical techniques which identify molecular interactions. Methods applicable to potentially useful techniques such as competitive radioligand binding assays, analytical ultracentrifugation, microcalorimetry, surface plasmon resonance and optical biosensor-based methods are provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN

SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) which is incorporated herein by reference.

In one particular embodiment, the invention provides a method of identifying a CSF-1 antagonist including the steps of:

(i) administering a candidate molecule to an animal; and

(ii) measuring CSF-1 activity; wherein

(a) an enhanced immune response to immunostimulatory DNA; and/or

(b) a reduced immune response to bacterial lipopolysaccharide (LPS);

indicate that CSF-1 activity is reduced in said animal and that said candidate molecule is a CSF-1 antagonist.

CSF-1 activity may be measured *in vivo* or *in vitro* using cells derived from said animal. A non-limiting example of cells derived from said animal is BMM macrophages.

Preferably, said animal is a mammal, including but not limited to mice, rats, rabbits, goats, hamsters and any other mammals used as laboratory models.

Preferably, said mammal is a mouse.

In another particular embodiment, the invention provides a method of identifying a CSF-1 antagonist including the steps of:

(i) administering a candidate molecule to one or more animal cells; and

(ii) measuring CSF-1 activity; wherein

(a) an enhanced immune response to immunostimulatory DNA; and/or

(b) a reduced immune response to bacterial lipopolysaccharide (LPS);

indicate that CSF-1 activity is reduced in said animal cells and that said candidate molecule is a CSF-1 antagonist.

Preferably, said animal cells are monocyte or macrophage cells as hereinbefore described.

As will be described in a particular embodiment hereinafter, said animal cells are RAW264 cells that have been transformed with an expression construct comprising a green fluorescent protein (*gfp*) nucleic acid operably linked to an NF-kB responsive E-

selectin promoter. These cells express green fluorescent protein (GFP) in response to CSF-1, wherein reduced CSF-1 activity (in response to a CSF-1 antagonist such as the monoclonal antibody AFS98) is measured as reduced GFP expression.

Pharmaceutical compositions and vaccines

The invention also provides pharmaceutical compositions that comprise a modulator of CSF-1 activity. Preferably, the pharmaceutical composition comprises a pharmaceutically-acceptable carrier, diluent or excipient.

Such compositions may further comprise an immunogen as hereinbefore defined, so that the presence of said CSF-1 modulator may modify the immune response to said immunogen.

In other embodiments, said pharmaceutical composition may include, for example, immunostimulatory DNA as described in more detail hereinafter. Further examples of immunostimulatory oligonucleotides and methods for identifying same are provided in International Publication WO 00/31540.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference.

Any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the

like may be employed. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, vaccines and DNA vaccines.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Pharmaceutical compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, sachets or tablets each containing a pre-determined amount of one or more therapeutic agents of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the agents of the invention with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmaceutically-effective. The dose administered to a patient, in the context of the present invention, should be sufficient to effect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

In the case of immunotherapeutic compositions, such as "*vaccines*", it is preferred that the pharmaceutical composition includes an adjuvant. As will be understood in the

art, an "*adjuvant*" means a composition comprised of one or more substances that enhances the immunogenicity and efficacy of a vaccine composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other oils of animal origin); block copolymers; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; liposomes; ISCOM® and ISCOMATRIX® adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as muramyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran or with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (e.g. U.S. Pat. No. 5,047,238); vaccinia or animal poxvirus proteins; sub-viral particle adjuvants such as cholera toxin, or mixtures thereof.

So that the invention can be readily understood and put into practical effect, the skilled person is directed to the following non-limiting examples.

EXAMPLES

Materials and Methods

Cell culture and Reagents.

RPMI 1640 medium (Life Technologies, Paisley, UK) containing 10% FCS, penicillin/streptomycin and glutamine (complete medium) was used for culture of BMM. BMM were derived from the femurs of adult BALB/c mice (Harlan Olac, Bicester, UK). In some experiments, adult CD1 outbred mice were used for preparation of BMM with similar results. Briefly, femurs were flushed with complete medium and bone marrow cells were plated out in complete medium containing 10^4 U/ml (100 ng/ml) recombinant human CSF-1 (a gift from Chiron Corporation, Emeryville, CA) on 10 cm bacteriological plastic plates (Bibby Sterilin, Staffordshire, UK) for 7 days in a 37°C incubator containing 5% CO_2. Thioglycollate-elicited peritoneal macrophages (TEPM) were obtained by injecting BALB/c mice i.p. with 1 mL of 10% thioglycollate broth

followed by peritoneal lavage with 10 mL PBS 5 days later. LPS from Salmonella minnesota (Sigma, Poole, UK) was used at a final concentration of 100 ng/ml in all cell culture experiments. Phosphodiester oligodeoxynucleotides (Sigma-Genosys, Poole, UK) were used at a final concentration of 3 μM in cell culture. Oligodeoxynucleotides used were activating oligonucleotide-1 (AO-1) (5'-GCTCATGACGTTCCTGATGCTG-3';SEQ ID NO:1) and nonactivating oligonucleotide (NAO-1) (5'-GCTCATGAGCTTCCTGATGCTG-3' SEQ ID NO:2) (20). IL-3 (a gift from Dr. A. Hapel, ANU, Canberra, Australia), stored at a concentration of 10^4 U/ml at -20°C, was used at 10^3 U/ml in cell culture. PMA (Sigma) was stored as a stock solution (10 mg/ml) in DMSO at -70°C and used at a final concentration of 100 ng/ml.

In vitro *treatment of cells and ELISAs.*

For all in vitro experiments, BMM were plated out in 24 well plates at 5 x 10^5 cells per well in 1 ml complete medium with or without CSF-1 (10^4 U/ml) overnight. The following morning cells were stimulated with 100 ng/ml LPS, 3 μM CpG-containing oligonucleotide (AO-1), 3 μM control oligonucleotide (NAO-1) or medium. After 24 h (unless otherwise stated) supernatants were collected and stored at -20°C until ELISAs were performed. ELISAs were carried out using paired antibodies (Pharmingen, San Diego, CA).

Immunoblotting.

BMM (2 x 10^6) were plated on 60 mm Corning dishes in 5 ml medium or 5 ml medium plus CSF-1 (10^5 U/mL) for 18 h. Culture medium was reduced to 2 ml and cells were treated as described in Figure legends. Cell monolayers were lysed with boiling 66 mM Tris-Cl (pH7.4)/2% SDS/1 mM sodium vanadate/1 mM Sodium pyrophosphate/1 mM Sodium Molybdate/10 mM Sodium Fluoride. Equal amounts of total protein in cell extracts were resolved by SDS-PAGE with 10% polyacrylamide seperating gels, transferred to Immobilon-P (Millipore), blocked and probed with the anti-phospho p42/p44 MAPK rabbit polyclonal antibody (1:1000) (New England Biolabs, Beverly, MA), washed and incubated with HRP-linked anti rabbit IgG (1:2000) (New England Biolabs). Blots were washed and detected using ECL Plus reagents (Amersham Pharmacia Biotech) and Hyperfilm-ECL (Amersham Pharmacia Biotech). Membranes were then sequentially stripped with 63mM Tris-Cl (pH 6.7)/2%

SDS/100mM 2-mercapto-ethanol and reprobed with rabbit anti-phospho p38 (New England Biolabs), rabbit anti-p42/p44 MAPK (New England Biolabs) and rabbit anti-p38 (New England Biolabs).

Total RNA isolation and quantitative PCR.

Total RNA was prepared using RNAzol B (Biogenesis, Poole, UK) according to the manufacturers instructions. RNA was treated with DNase 1 (Ambion, Austin, Texas), and reverse transcribed to cDNA using Superscript reverse transcriptase (Life Technologies, Paisley, UK). Negative control samples (no first strand synthesis) were prepared by performing reverse transcription reactions in the absence of reverse transcriptase. cDNA levels of murine IL-12 (p40), IL-12 (p35), TNF-α, hypoxanthine phosphoribosyl transferase (HPRT), TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 were quantitated by real time PCR using an ABI prism 7700 sequence detector according to the manufacturers instructions (Perkin Elmer Applied Biosystems, Foster City, CA). Amplification was achieved using an initial cycle of 50°C 2 mins, 95°C 10 mins followed by 40 cycles of 95°C 15 seconds, 50 °C 1 min. cDNA levels during the linear phase of amplification were normalised against HPRT controls. Determinations were made in triplicate and mean +/- SD was determined. Primers (f=forward, r=reverse) and 5'-6-carboxy-fluorescein labelled/3'-6-carboxy-tetramethyl-rhodamine labelled probes (p) used to detect expression of the corresponding murine genes were:

IL-12 (p40) (f: 5'-GGA ATT TGG TCC ACT GAA ATT TTA AA-3' SEQ ID NO:3; r: 5'-CAC GTG AAC CGT CCG GAG TA-3' SEQ ID NO:4; p: 5'-ACA AGA CTT TCC TGA AGT GTG AAG CAC CAA AT-3' SEQ ID NO:5); IL-12 (p35) (f: 5'-AAG ACA TCA CAC GGG ACC AAA-3' SEQ ID NO:6; r: 5'-CAG GCA ACT CTC GTT CTT GTG TA-3' SEQ ID NO:7; p: 5'-CAG CAC ATT GAA GAC CTG TTT ACC ACT GGA-3', SEQ ID NO:8); TNF-α (Perkin Elmer Applied Biosystems); TLR1 (f: 5'-TGG ATG TGT CCG TCA GCA CTA-3' SEQ ID NO:9; r: 5'-AGA GCA GCC CTG GTC TTC AA-3', SEQ ID NO:10; p: 5'-CAC ACA CTT GAT GTT AGA CAG TTC CAA ACC GAT-3', SEQ ID NO:11); TLR2 (f: 5'-AAG ATG CGC TTC CTG AAT TTG-3', SEQ ID NO:12; r: 5'-TCC AGC GTC TGA GGA ATG C-3', SEQ ID NO:13; p: 5'-CGT TTT TAC CAC CCG GAT CCC TGT ACT G-3', SEQ ID NO:14); TLR4 (f: 5'-AGG AAG TTT CTC TGG ACT AAC AAG TTT AGA-3', SEQ ID NO:15; r: 5'-AAA TTG

TGA GCC ACA TTG AGT TTC-3', SEQ ID NO:16; p: 5'-GCC AAT TTT GTC TCC ACA GCC AC CA-3', SEQ ID NO:17); TLR5 (f: 5'-GCA CGA GGC TTC TGC TTC A-3', SEQ ID NO:18; r: 5'-GCA TCC AGG TGT TTG AGC AA-3', SEQ ID NO:19; p: 5'-CAT TCT GTG CCC ATT CAA AGT CTT TGC TG-3', SEQ ID NO:20); TLR6 (f: 5'-CTC GGA GAC AGC ACT GAA GTC A-3', SEQ ID NO:21; r: 5'-CGA GTA TAG CGC CTC CTT TGA A-3', SEQ ID NO:22; p: 5'-ATG ATA GAG CAC GTC AAAAAC CAA GTG TTC CTC-3', SEQ ID NO:23); TLR9 (f: 5'-AGG CTG TCA ATG GCT CTC AGT T-3', SEQ ID NO:24; r: 5'-TGA ACG ATT TCC AGT GGT ACA AGT-3', SEQ ID NO:25; p: 5'-TGC CGC TGA CTA ATC TGC AGG TGC T-3', SEQ ID NO:26); HPRT (f: 5'-GCA GTA CAG CCC CAA AAT GG-3', SEQ ID NO:27; r: 5'-AAC AAA GTC TGG CCT GTA TCC AA-3', SEQ ID NO:28; p: 5'-TAA GGT TGC AAG CTT GCT GGT GAA AAG GA-3', SEQ ID NO:29).

PCR/Southern Hybridisation.

Total RNA was treated with DNase 1 (Ambion, Austin, Texas), reverse transcribed to cDNA using Superscript reverse transcriptase (Life Technologies, Paisley, UK) and used as a template for semi-quantitative PCR. No reverse transcriptase negative controls were performed for all samples. Primers used were: TLR9 (f: 5'-CTA CAA CAG CCA GCC CTT TA-3', SEQ ID NO:30; r: 5'-GCT GAG GTT GAC CTC TTT CA-3', SEQ ID NO:31), TLR4 (f: 5'-AGA GAA TCT GGT GGC TGT GG-3', SEQ ID NO:32; r: 5'-TCA ACC GAT GGA CGT GTA AA-3', SEQ ID NO:33) and HPRT (f: 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3', SEQ ID NO:34; r: 5'-GAG GGT AGG CTG GCC TAT AGG CT-3', SEQ ID NO:35). PCR cycling conditions were: 94 °C for 30 seconds, 54 °C for 30 seconds, 72 °C for 60 seconds (21 cycles for HPRT, 21 cycles for TLR4, 21 cycles for TLR9 with BMM cDNA or 24 cycles for TLR9 with TEPM cDNA). PCR products were separated on 1.8 % agarose gels, transferred to zetaprobe nylon membrane (Biorad, Hercules, CA) and subjected to southern hybridisation using cDNA probes for TLR9, TLR4 and HPRT. Probes were labelled by random priming (AP biotech, Piscataway, NJ).

Results

Differential effects of CSF-1 on LPS- and CpG DNA-induced cytokine production from BMM.

To compare the effects of CSF-1 on macrophage responses to LPS and CpG DNA, the present inventors used primary bone marrow-derived macrophages (BMM) which respond well to both agents (20), and measured production of inflammatory cytokines by ELISA. Figure 1 demonstrates that overnight pretreatment of BMM with CSF-1 enhanced levels of LPS-induced IL-6, IL-12 and TNF-α protein release into the medium by 15, 72 and 6 fold respectively. In direct contrast, CSF-1 pretreatment suppressed CpG DNA-induced IL-6, IL-12 and TNF-α by 10, 8 and 7 fold respectively. The effect of CSF-1 on both the LPS and the CpG DNA response was apparent at the earliest point at which secreted cytokines could be detected by ELISA and was maximal at 24 h (data not shown). Since IFN-γ can prime macrophage responses to both LPS and CpG DNA (21, 22), the present inventors assessed whether IFN-γ could overcome the suppressive effect of CSF-1 on the DNA response. IFN-γ pretreatment enhanced levels of LPS-induced IL-6 and IL-12 in BMM (Fig. 2). This priming effect was not as striking in CSF-1 pretreated macrophages suggesting that CSF-1 and IFN-γ might provide similar priming signals. Levels of CpG DNA-induced IL-6 and IL-12 were also enhanced by IFN-γ priming, but priming with IFN-γ did not overcome the suppressive effect of CSF-1 on the CpG DNA response (Fig. 2).

The present inventors next assessed whether the effect of CSF-1 on LPS and CpG DNA responses was selective to CSF-1 or was a manifestation of its growth stimulating activity. IL-3 and PMA can both trigger BMM proliferation (23, 24) and IL-3 is known to prime macrophage responses to LPS (25, 26). Therefore, IL-3, PMA and CSF-1 were compared for their ability to regulate IL-6 production in response to LPS and CpG DNA. Figure 3 shows that only CSF-1 was able to enhance the LPS response and suppress the CpG DNA response. IL-3 pretreatment primed responses to both LPS and CpG DNA whilst PMA did not affect the LPS response but suppressed the CpG DNA response slightly (1.5 - 2 fold). Hence, the ability of CSF-1 to enhance LPS responses and suppress CpG DNA responses is unlikely to be related to its ability to trigger macrophage proliferation.

Regulation of LPS- and CpG DNA-induced TNF-α and IL-12 mRNA by CSF-1

To determine the level at which CSF-1 differentially regulates the LPS and CpG DNA response, the present inventors assessed mRNA levels of IL-12 p40 and p35 and

TNF-α in response to LPS and CpG DNA with or without CSF-1 priming (Fig. 4). Priming with CSF-1 enhanced LPS-induced IL-12 (p40) mRNA levels at 4 h by approximately 10 fold but did not alter LPS-induced IL-12 (p35) and TNF-αmRNAs. Whereas CSF-1 was selective in priming LPS-induced cytokine mRNAs, levels of IL-12 (p40), IL-12 (p35) and TNF-α after 4 h CpG DNA were all suppressed by priming with CSF-1 (6, 4.5 and 3 fold respectively). This suppressive effect was also apparent at 2 h post CpG DNA treatment (data not shown).

Effect of CSF-1 on LPS-induced and CpG DNA-induced p38 and ERK-1/2 MAPK activation.

p38 MAPK phosphorylation is an early event in triggering both LPS and CpG DNA-induced gene expression (27-29). Phosphorylation of p38 in response to CpG DNA was suppressed by pretreatment with CSF-1 from the earliest time point examined (Fig. 5). To determine whether CSF-1 altered the ligand dose response curve (sensitivity) or the maximal response, the effect of CSF-1 pretreatment on p38 phosphorylation over a range of CpG DNA doses was determined at 30 min post stimulation. Figure 6a demonstrates that 10 fold higher concentrations of CpG DNA were required to induce p38 phosphorylation in CSF-1 pretreated cells. In contrast, LPS-induced p38 phosphorylation was not affected by CSF-1 pretreatment (Fig. 6b), indicating that enhancement of LPS responses by CSF-1 occurred independently of p38 activation. Phosphorylation of ERK-1/2 is also triggered by LPS and CpG DNA in BMM (20) and the present inventors assessed the effect of CSF-1 pretreatment on CpG DNA and LPS triggered ERK-1 and -2 phosphorylation (Fig. 6, a and b). Since CSF-1 itself triggers sustained phosphorylation of ERK-1 and -2 in BMM (30), basal levels of phosphorylated ERK-1 and -2 were much higher in CSF-1 pretreated BMM than in untreated BMM. Nonetheless, CSF-1 pretreatment blocked the ability of CpG DNA to enhance levels of phosphorylated ERK-1 and -2 over the concentration range examined (Fig. 6a) whilst LPS was still able to activate ERK-1/2 even in the presence of CSF-1 (Fig. 6b). Further, the extent of the LPS-mediated ERK-1/2 phosphorylation was not altered by CSF-1 pretreatment despite the elevated basal activation state (Fig. 6b). These data suggest that CSF-1 alters an early stage of CpG DNA recognition, but acts more distally to activate LPS responses.

The effect of CSF-1 on expression of TLR family members.

Mice deficient for TLR9 are unable to respond to CpG-containing phosphorothioate DNA (10). Whether TLR9 is required for uptake of DNA, directly recognises CpG DNA, or lies downstream in the recognition pathway is yet to be determined (31). The diminished CpG responsiveness observed above hints at a reduction in receptor expression or affinity in response to CSF-1. We therefore assessed the effect of CSF-1 on expression of TLR9 mRNA in BMM. Indeed, CSF-1 treatment resulted in a 20 fold reduction in TLR9 mRNA levels in BMM (Fig. 7a). To assess the specificity of this response, we examined the effect of CSF-1 on expression of other TLR family members since these receptors are instrumental in triggering cellular responses to other bacterial products including LPS, peptidoglycan and bacterial lipoproteins. Overnight treatment with CSF-1 did not significantly affect mRNA levels of the LPS receptor, TLR4 (Fig. 7b). Levels of TLR5 mRNA were also unaffected by CSF-1 but mRNA levels of TLR1, TLR2 and TLR6 were all suppressed by CSF-1 treatment (2.4, 4.8 and 4 fold respectively). Hence, CSF-1 has a selective effect on expression of different TLR family members. This is consistent with our findings that CSF-1 had differential effects on the expression of proinflammatory cytokines in response to different microbial stimuli.

Regulation of TLR9 expression in macrophages by CSF-1

The present inventors analysed the effect on TLR9 expression of other agents that cause BMM proliferation. Levels of TLR9 mRNA were assessed in BMM treated overnight with medium, CSF-1, IL-3 or PMA. Figure 8a demonstrates that only CSF-1 was able to dramatically regulate TLR9 expression; IL-3 did not alter levels of TLR9 mRNA and PMA, which downregulated CpG responses approximately 1.5 to 2 fold (Fig. 3), had a similar effect on TLR9 expression. To assess the timecourse of CSF-1 downregulation of TLR9 expression, BMM starved overnight of CSF-1, were treated with CSF-1 over a 20 h timecourse. Maximal suppression of TLR9 expression occurred between 4 - 8 h, and an effect was apparent by 1 h post-CSF-1 (Fig. 8b).

Although the effects of CSF-1 on BMM are clearly dissociated from its growth promoting effects, these cells are unusual in that mature macrophages *in vivo* are generally not actively proliferating. The present inventors therefore analysed the effect

of CSF-1 on expression of TLR9 in TEPM which are post-mitotic. We have found that, whilst freshly isolated TEPM respond well to CpG DNA, they rapidly lose responsiveness to CpG DNA but retain LPS responses when cultured ex vivo on either tissue culture or bacterial plastic (manuscript in preparation). In keeping with this pattern, TLR9 mRNA levels were 20 to 50 fold less in cultured TEPM than CSF-1 starved BMM (data not shown). Nonetheless, this low basal level of TLR9 expression was still regulated by CSF-1. Overnight treatment of TEPM with CSF-1 further downregulated expression of TLR9, but did not alter levels of of TLR4 compared to control cells (Fig. 9).

The inhibitory Effect of CSF-1 is most pronounced with phosphorothioate CpG DNA

CpG DNA that is contained within bacterial DNA, has therapeutic potential as both an adjuvant and an anti-cancer agent due to its ability to promote development of a Th1-type immune response. Short oligonucleotides that contain a CpG motif (CpG ODN) effectively mimic the action of bacterial DNA, but have a short half life *in vivo* as they are rapidly degraded. The backbone of CpG oligonucleotides can be modified by use of phosphorothioate linkages that dramatically enhance oligonucleotide stability *in vivo*. Hence, for therapeutic applications phosphorothioate-modified CpG oligonucleotides (PS-CpG ODN) are the preferred agents. Although PS-CpG ODN have enhanced stability compared to CpG ODN, this modification also alters some of the actions of CpG DNA on responsive cells. For example, in macrophages CpG ODN is an effective activator of the promoter that drives HIV-1 expression (the HIV-1 LTR) whereas PS-CpG DNA is almost inactive in this assay. Hence, the therapeutic potential of CSF-1 antagonists as modifiers of CpG action is dependent upon the ability of CSF-1 to suppress responses to, not only CpG ODN, but also to PS-CpG ODN. Figure 10 demonstrates that CSF-1 is actually much more effective at downregulating responses to PS-CpG DNA than to CpG DNA. Whereas the effect of CSF-1 is to shift the dose response curve to CpG ODN such that the inhibitory effect of CSF-1 can be overcome by maximal ODN doses, CSF-1 has more pronounced effects on responses to PS-CpG ODN. The suppressive effect of CSF-1 on responses to PS-CpG ODN cannot be overcome by maximal ODN doses, and the suppressive effect over an entire dose

response range is much more dramatic with PS-CpG ODN than with CpG ODN (Figure 10).

***The inhibitory effect of CSF-1 cannot be overcome by other cytokines that are likely to be present* in vivo.**

Macrophage responses to CpG DNA are suppressed by CSF-1 *in vitro*, but the ability of CSF-1 to suppress responses to CpG DNA *in vivo* is likely to be influenced by the presence of other cytokines. For example, during many immune responses interferon-gamma (IFN-γ), interleukin-3 (IL-3) and granulocyte macrophage colony stimulating factor (GM-CSF) are present *in vivo*. We have found that all of these cytokines are able to dramatically potentiate the macrophage response to CpG DNA. Hence, the ability of CSF-1 antagonists to enhance CpG responses is dependent upon the supposition that the suppressive effect of CSF-1 is dominant over the effect of other cytokines present *in vivo* that can potentiate CpG DNA responses. The present inventors have already demonstrated that IFN-γ can enhance CpG DNA responses and that CSF-1 dramatically inhibits macrophage responses to CpG DNA even in the presence of IFN-γ. Figure 11 shows that both IL-3 and GM-CSF can also prime macrophage responses to CpG DNA, and that CSF-1 blocks the ability of these cytokines to potentiate CpG DNA responses. Hence, the suppressive action of CSF-1 on CpG DNA responses is dominant over the effects of all cytokines that are known to enhance responses to CpG DNA. Figure 11 also provides a further demonstration of the ability of CSF-1 to selectively target CpG DNA responses. Macrophage responses to LPS were enhanced by IL-3 and GM-CSF, and CSF-1 (which itself enhanced LPS responses) did not suppress the potentiating effects of IL-3 and GM-CSF on the LPS response (in fact, CSF-1 acted additively with IL-3 to enhance the LPS response).

Since CSF-1 dramatically suppressed TLR9 expression and CpG responsiveness in macrophages, antagonists of CSF-1 action might enhance CpG responses *in vivo*. The present inventors determined whether an antagonist of CSF-1 action, AFS98 (a monoclonal antibody against the murine CSF-1 receptor; ref 47) could block the ability of CSF-1 to suppress the macrophage response to CpG DNA. Figure 12 shows that pretreatment of ELAM cells (a RAW264 cell line expressing green fluorescent protein under the control of the NF-κB-responsive E-selectin promoter) with CSF-1 at either

10,000 or 50,000 U/ml suppressed the response to CpG DNA (AOS). Pre-incubation with an anti-CSF-1R antibody prevented CSF-1 from suppressing the CpG DNA response. At the lowest antibody concentration (1/1000 dilution) CSF-1 was able to partially suppress the CpG response. This result confirms that AFS98 inhibits CSF-1 action and completely blocks the ability of CSF-1 to suppress CpG DNA responses.

Examples of CSF-1 antagonists useful according to the present invention are neutralising antibody against the CSF-1 receptor, neutralising antibody against CSF-1, recombinant extracellular domain of the CSF-1 receptor, or new CSF-1 antagonists identified by screening.

This could also be applied to enhance CpG DNA efficacy as an adjuvant and as a protective agent against intracellular pathogens such as *Leishmania* and *Listeria*.

Discussion

LPS and CpG DNA have differing toxicities *in vivo*. Administration of LPS can lead to fever, shock and multi-organ failure resulting in death. There are no reports of toxicity of bacterial DNA alone, although pretreatment of mice with *E. coli* DNA can enhance the toxicity of LPS *in vivo* (32), probably by the induction of IFN-γ. Even phosphorothioate-stabilised CpG oligonucleotides (PS-ODN) have minimal toxicity (4). The relative non-toxicity of CpG DNA is an attractive feature for its use as a vaccine adjuvant and for other therapeutic strategies.

Analysis of macrophage gene expression *in vitro* has suggested that the differing toxicities of LPS and CpG DNA may be due to both qualitative and quantitative differences in cytokine gene induction. For example, CpG DNA was a relatively poor stimulus for IL-1β (22) and LPS but not CpG DNA stimulated nitric oxide production from macrophages without IFN-γ priming (21, 22). However the situation *in vivo* is likely to be more complex due to the presence of many other cytokines which will modify the responses to LPS and CpG DNA. Here we have found that CSF-1, which is present constitutively *in vivo* and is a macrophage growth and survival factor, differentially affects the responses to LPS and CpG DNA. In the presence of CSF-1, the LPS response was elevated and the CpG DNA response suppressed so that LPS became far more effective than CpG DNA at stimulating IL-6, IL-12 and TNF-α production from BMM (62, 27 and 23 fold respectively).

The ability of CSF-1 to selectively enhance the LPS and suppress the CpG DNA response of BMM is unlikely to be related to its activity as a growth factor. IL-3 and PMA did not have selective effects on LPS and CpG DNA responses; IL-3 pretreatment primed BMM for enhanced IL-6 production in response to both LPS and CpG DNA whilst PMA pretreatment did not have significant effects on LPS-induced IL-6 and modestly suppressed CpG DNA-induced IL-6 synthesis. Further, IL-3 and PMA had little effect on TLR9 expression whereas CSF-1 markedly suppressed expression of this molecule. IL-3 was actually more effective at priming CpG DNA responses than LPS responses. Although the ability of IL-3 to synergise with LPS for macrophage activation has been documented (25, 26), its ability to regulate responses to CpG DNA has not been reported. Given that CpG DNA drives strong Th1 responses *in vivo* and that IL-3 is a product of activated T cells, IL-3 may be involved in amplification of responses to CpG DNA *in vivo*, as has been suggested for IFN-γ (21).

Since concentrations of CSF-1 are markedly and rapidly enhanced in serum, spleen, liver, lung and kidney after LPS administration (13) and during infection (12, 33), macrophages recruited to the site of infection during a bacterial challenge would be expected to have an impaired response to CpG DNA. The implications of this are not obvious, since the role of bacterial DNA in an infection is not clear and will remain so until experiments using bacterial infection models in TLR9 gene-targeted mice are performed. Intact bacterial pathogens do not display their DNA, and detection of CpG DNA during a bacterial challenge may imply that the host has successfully destroyed the invading organism. In this case, CSF-1 may be important in dampening down inappropriate inflammatory responses to bacterial DNA. On the other hand, both LPS and CpG DNA rapidly downregulate cell surface expression of the CSF-1 receptor in BMM (20). Hence, macrophages present at the site of infection will have already encountered bacterial cell wall products such as LPS and are unlikely to be CSF-1 responsive. Such cells may therefore be hypersensitive to the effects of CpG DNA. Consistent with this model we have found that LPS and CpG DNA can synergise for IL-6 and IL-12 production from BMM.

TLR9 is an essential component of the DNA response (10), although whether TLR9 directly recognizes CpG DNA itself remains to be determined. The expression of

TLR9 was profoundly reduced by CSF-1, which provides a clear explanation for the suppression of bacterial DNA responses by CSF-1. By extension, suppression of TLR1, 2 and 6 mRNA levels by CSF-1 would be expected to suppress the macrophage response to their functional ligands such as peptidoglycan and bacterial lipoproteins (9, 34-36). CSF-1 is immunosuppressive for antigen-specific and mitogen triggered lymphocyte responses (37, 38). It is conceivable that its ability to downregulate expression of specific TLR family members in macrophages is involved in this phenomenon.

By contrast to the suppressive action of CSF-1, a variety of inflammatory stimuli including LPS selectively upregulated TLR2 but not TLR4 expression (39, 40). The differential regulation of TLR members implies that, as in *Drosophila*, different kinds of pathogens could elicit different outcomes. In keeping with this view, Sing et al. have reported that gram-negative organisms induce γ-interferon, whereas gram-positive bacteria lack this activity (41).

While the ability of CSF-1 to prime murine macrophage responses to LPS has previously been reported for IL-6 and TNF-α production (14, 15), its effect on LPS-induced IL-12 production has not been documented. In the case of IL-6, synergy between CSF-1 and LPS might be partially due to CSF-1-induced GM-CSF production (42). We have not fully investigated the mechanism by which CSF-1 augments LPS responses of BMM. We did find that CSF-1 did not affect levels of LPS-induced TNF-α mRNA despite having a marked effect on TNF-α protein secretion, implying that post-transcriptional mechanisms are responsible for this effect. In support of this, p38 phosphorylation in response to LPS was not altered by CSF-1 pretreatment. In the case of IL-12, LPS-induced p40 but not p35 mRNA was enhanced by CSF-1 priming. Whether the effect of CSF-1 on LPS-induced IL-12 (p40) mRNA is due to enhanced transcription or enhanced mRNA stability has not yet been investigated. One possibility is that CSF-1 and LPS synergise at the level of transcription since CSF-1 triggers sustained phosphorylation and activation of Ets-2 (30) and Ets-2 is necessary for full activation of the IL-12 promoter in response to LPS (43, 44).

The majority of the results described herein utilised BMM as a primary macrophage model – the present inventors have found that TEPM cultured *ex vivo* retain responsiveness to LPS but rapidly lose responsiveness to CpG DNA. Because of the

rapid decline in CpG DNA responsiveness *ex vivo*, we have been unable to address the effect of CSF-1 pretreatment on CpG responses in TEPM. Nonetheless, the low basal expression of TLR9 in TEPM was further downregulated by overnight treatment with CSF-1 (Fig. 9) implying that this phenomenon is likely to occur with all CSF-1 responsive macrophage populations. Apart from macrophages and B cells, dendritic cells are activated by CpG DNA and are important mediators of CpG DNA responses *in vivo* (45).

In summary, CSF-1 reprograms macrophage responses to different microbial stimuli. This is the first report of a cytokine or growth factor that has differential effects on macrophage responses to LPS and CpG DNA and it highlights the importance of regulated expression of TLRs. Discordant regulation of TLRs may underly different toxicities of TLR agonists *in vivo* and may have relevance for the role of CSF-1 during bacterial infections. Further, CSF-1 and CSF-1 receptor antagonists may enhance the efficacy of CpG DNA in therapeutic strategies and/or increase its toxicity *in vivo*.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All patent and scientific literature, algorithms and computer programs referred to in this specification are incorporated herein be reference in their entirety.

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CLAIMS

1. A method of modulating an immune response in an animal, said method including the step of modulating CSF-1 activity, to thereby modulate the immune response of said animal.

2. The method of Claim 1, wherein CSF-1 activity is reduced.

3. The method of Claim 1, wherein the immune response to immunostimulatory DNA is enhanced.

4. The method of Claim 1, wherein the immune response to bacterial lipopolysaccharide (LPS) is reduced.

5. The method of Claim 4, when used as a prophylactic or therapeutic treatment of bacterially-induced septic shock.

6. The method of Claim 1, wherein the step of modulating CSF-1 activity includes administration of a CSF-1 antagonist to said animal.

7. A method of immunizing an animal, said method including the step of administering a modulator of CSF-1 activity to said animal, in combination with an immunogen, to thereby elicit a modified immune response to said immunogen.

8. The method of Claim 7, wherein CSF-1 activity is reduced.

9. The method of Claim 7, wherein the immune response to immunostimulatory DNA is enhanced.

10. The method of Claim 1, wherein the modulator of CSF-1 activity is a CSF-1 antagonist.

11. A pharmaceutical composition comprising a modulator of CSF-1 activity and a pharmaceutically-acceptable carrier, diluent or excipient.

12. The pharmaceutical composition of Claim 11, wherein the modulator of CSF-1 activity is a CSF-1 antagonist.

13. The pharmaceutical composition of Claim 11, wherein the CSF-1 antagonist is an anti-CSF-1 antibody.

14. The pharmaceutical composition of Claim 11 for prophylactic or therapeutic treatment of bacterially-induced septic shock.

15. The pharmaceutical composition of Claim 11, wherein the modulator of CSF-1 activity is a peptide that inhibits CSF-1 receptor dimerization.

16. The pharmaceutical composition of Claim 11, further comprising an immunogen.

17. The pharmaceutical composition of Claim 16, wherein the immunogen is immunostimulatory DNA.

18. The pharmaceutical composition of Claim 17, wherein the immunostimulatory DNA is in the form of one or more immunostimulatory oligonucleotides.

19. The pharmaceutical composition of Claim 18, wherein the one or more immunostimulatory oligonucleotides comprise phosphorothioate CpG DNA.

20. A method of identifying a CSF-1 antagonist including the steps of:

(i) administering a candidate molecule to an animal; and

(ii) measuring CSF-1 activity; wherein

(a) an enhanced immune response to immunostimulatory DNA; and/or

(b) a reduced immune response to bacterial lipopolysaccharide (LPS);

indicate that CSF-1 activity is reduced in said animal and that said candidate molecule is a CSF-1 antagonist.

21. A method of identifying a CSF-1 antagonist including the steps of:

(i) administering a candidate molecule to one or more animal cells; and

(ii) measuring CSF-1 activity; wherein

(a) an enhanced immune response to immunostimulatory DNA; and/or

(b) a reduced immune response to bacterial lipopolysaccharide (LPS);

indicate that CSF-1 activity is reduced in said animal cells and that said candidate molecule is a CSF-1 antagonist.

22. The method of Claim 21, wherein said animal cells are monocyte or macrophage cells.

23. The method of Claim 21, wherein said animal cells are RAW264 cells.

24. The method of Claim 23, wherein said animal cells are RAW264 cells that express recombinant green fluorescent protein (GFP) in response to CSF-1, wherein reduced CSF-1 activity is measured as reduced GFP expression.

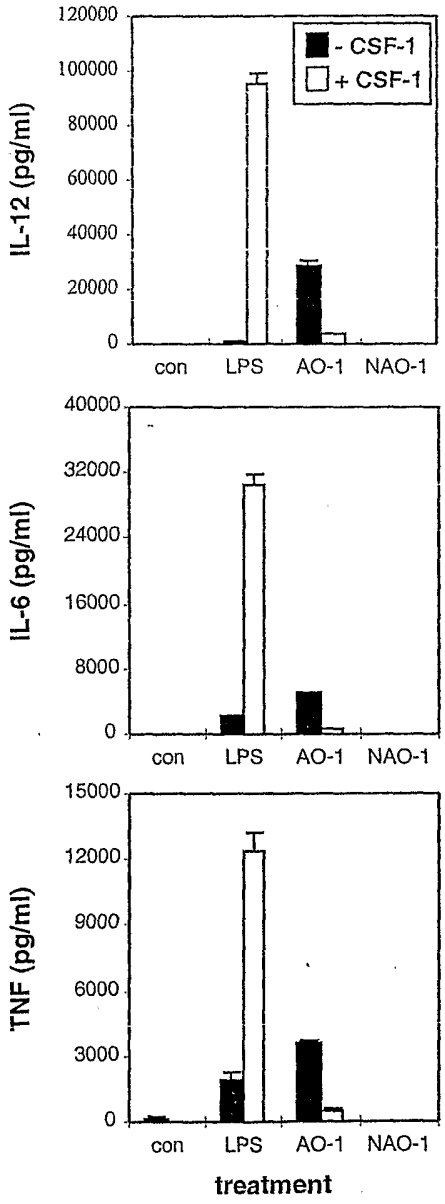
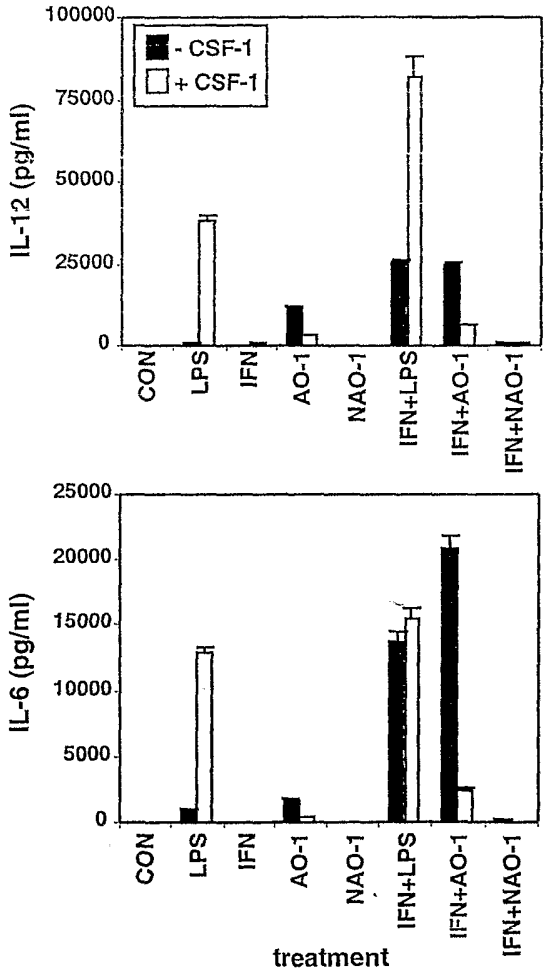

120000
100000
80000
60000
40000
20000
0
IL-12 (pg/ml)
- CSF-1
+ CSF-1
con
LPS
AO-1
NAO-1
40000
32000
24000
16000
8000
0
IL-6 (pg/ml)
con
LPS
AO-1
NAO-1
15000
12000
9000
6000
3000
0
TNF (pg/ml)
con
LPS
AO-1
NAO-1
treatment

FIG. 1


100000
75000
50000
25000
0
- CSF-1
+ CSF-1
IL-12 (pg/ml)
CON
LPS
IFN
AO-1
NAO-1
IFN+LPS
IFN+AO-1
IFN+NAO-1
25000
20000
15000
10000
5000
0
IL-6 (pg/ml)
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LPS
IFN
AO-1
NAO-1
IFN+LPS
IFN+AO-1
IFN+NAO-1
treatment

FIG. 2

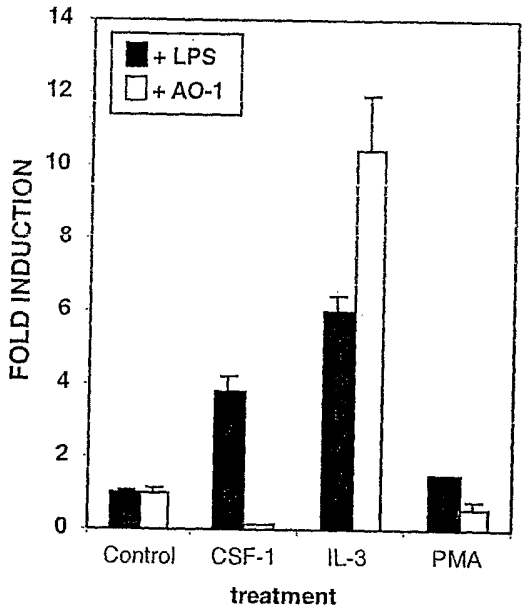

14
12
10
8
6
4
2
0
FOLD INDUCTION
+ LPS
+ AO-1
Control
CSF-1
IL-3
PMA
treatment

FIG. 3

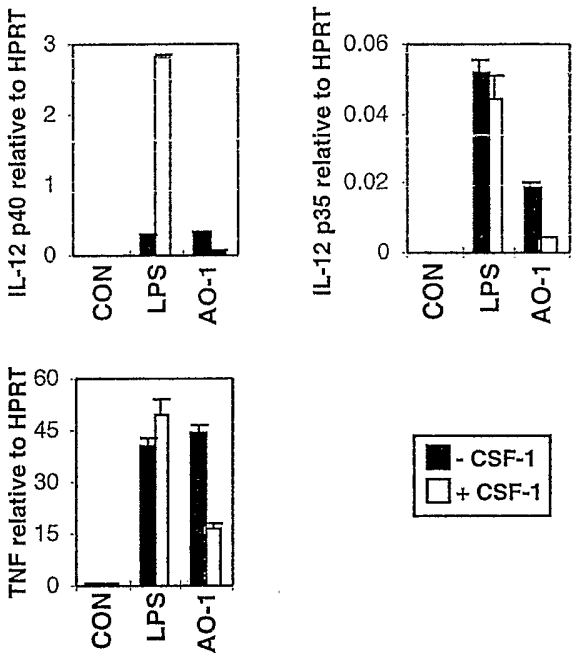

IL-12 p40 relative to HPRT
3
2
1
0
CON
LPS
AO-1
IL-12 p35 relative to HPRT
0.06
0.04
0.02
0
CON
LPS
AO-1
TNF relative to HPRT
60
45
30
15
0
CON
LPS
AO-1
- CSF-1
+ CSF-1

FIG. 4

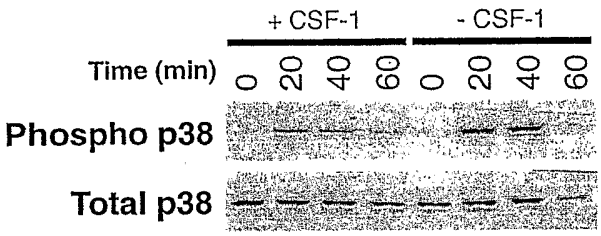
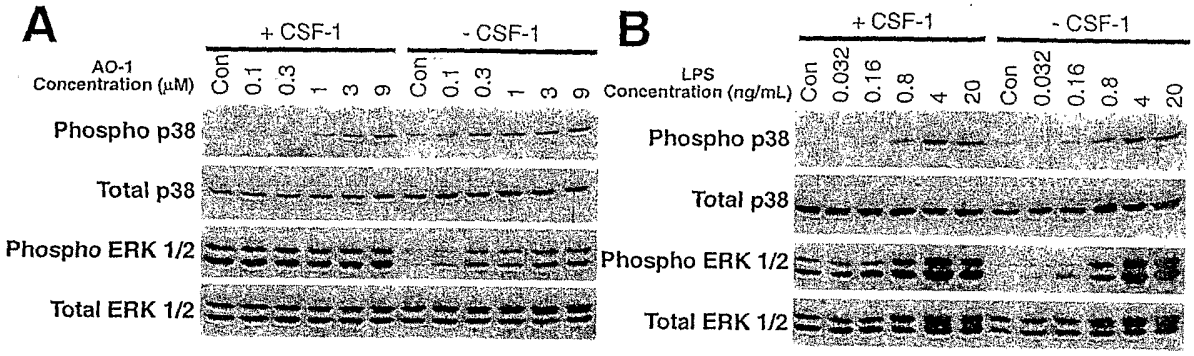


FIG. 5


A
+ CSF-1
- CSF-1
AO-1
Concentration (μM)
Con
0.1
0.3
1
3
9
Phospho p38
Total p38
Phospho ERK 1/2
Total ERK 1/2
B
+ CSF-1
- CSF-1
LPS
Concentration (ng/mL)
Con
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0.16
0.8
4
20
Phospho p38
Total p38
Phospho ERK 1/2
Total ERK 1/2

FIG. 6

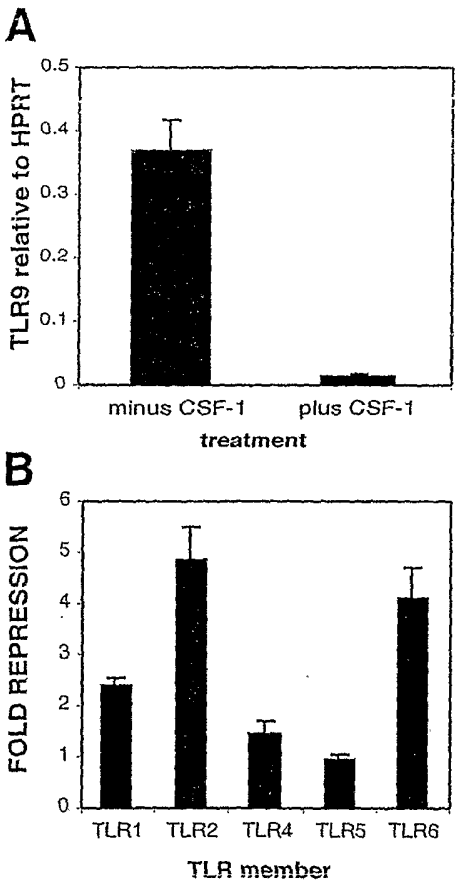
A
TLR9 relative to HPRT
0.5
0.4
0.3
0.2
0.1
0
minus CSF-1
plus CSF-1
treatment
B
FOLD REPRESSION
6
5
4
3
2
1
0
TLR1
TLR2
TLR4
TLR5
TLR6
TLR member

FIG. 7

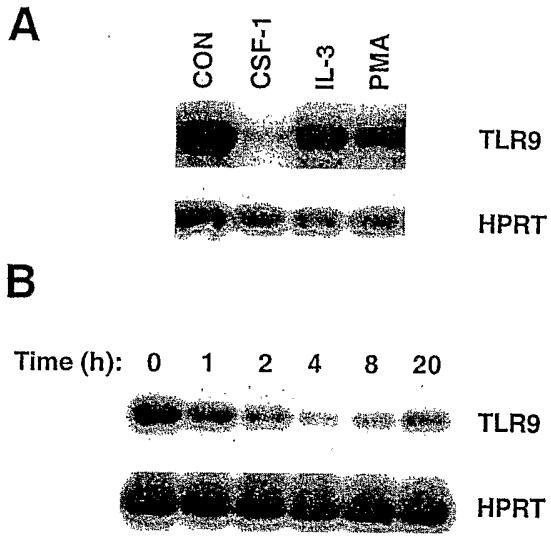

FIG. 8

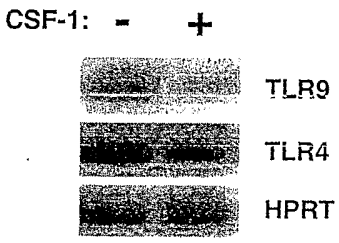

FIG. 9

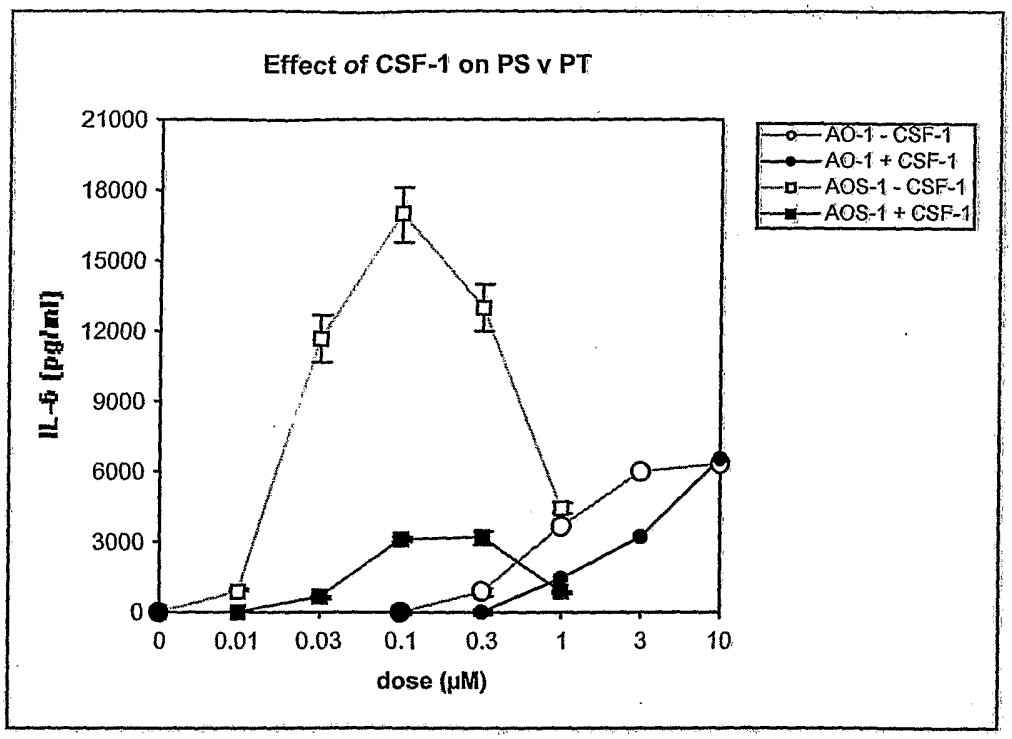
Effect of CSF-1 on PS v PT
IL-6 (pg/ml)
21000
18000
15000
12000
9000
6000
3000
0
0
0.01
0.03
0.1
0.3
1
3
10
dose (µM)
AO-1 - CSF-1
AO-1 + CSF-1
AOS-1 - CSF-1
AOS-1 + CSF-1

FIG. 10

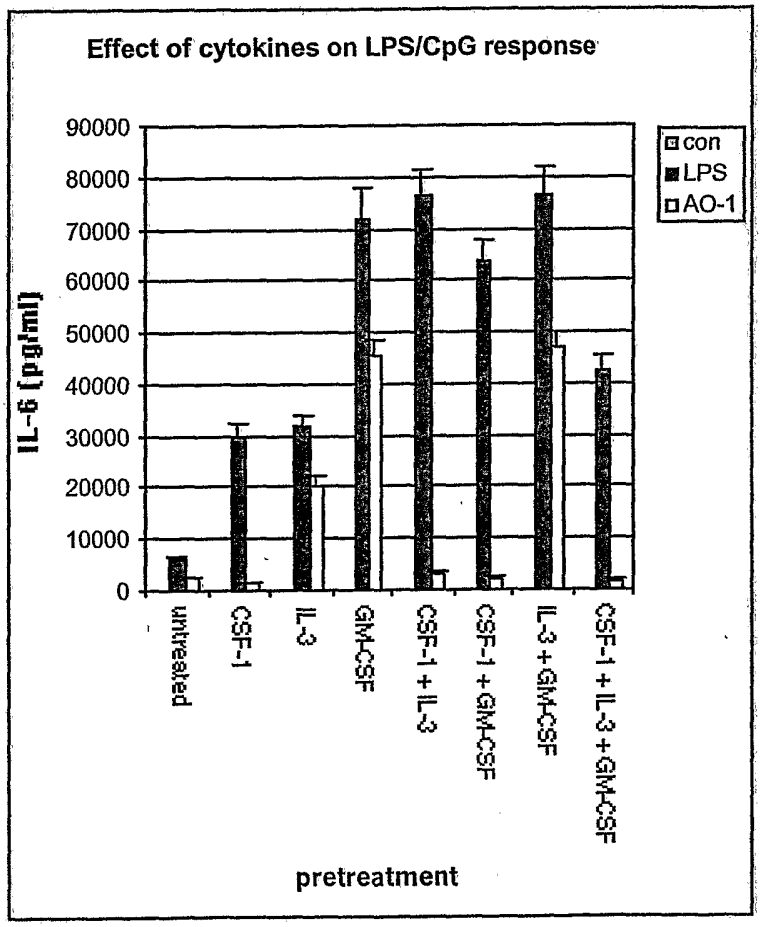
Effect of cytokines on LPS/CpG response
IL-6 [pg/ml]
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
con
LPS
AO-1
untreated
CSF-1
IL-3
GM-CSF
CSF-1 + IL-3
CSF-1 + GM-CSF
IL-3 + GM-CSF
CSF-1 + IL-3 + GM-CSF
pretreatment

FIG. 11

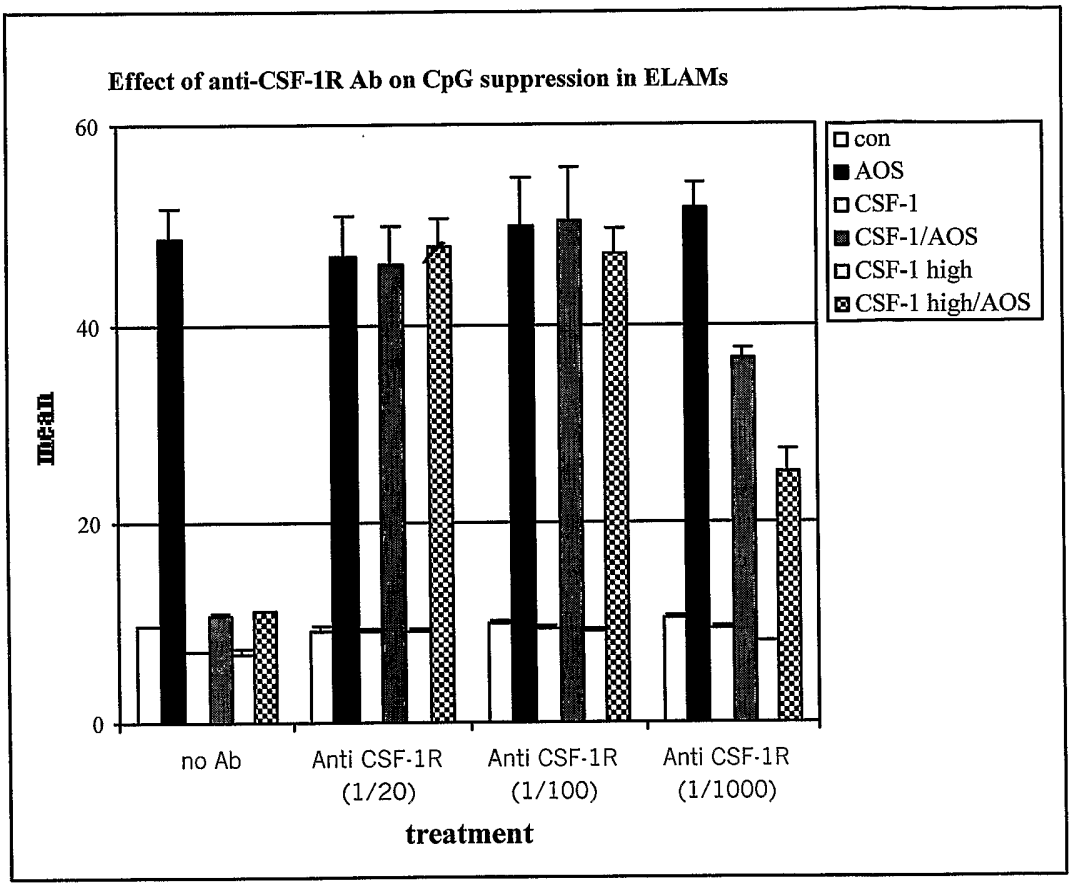
Effect of anti-CSF-1R Ab on CpG suppression in ELAMs
60
40
20
0
mean
con
AOS
CSF-1
CSF-1/AOS
CSF-1 high
CSF-1 high/AOS
no Ab
Anti CSF-1R (1/20)
Anti CSF-1R (1/100)
Anti CSF-1R (1/1000)
treatment

FIG. 12

SEQUENCE LISTING

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22

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24

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25

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01348

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 38/18 A61P 37/02, 37/04, 37/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Medline/WPAT: cpg or septic shock or c fms or CSF 1r and CSF-1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Sester, D et al (1999), J. Immun. Vol 163 pages 6541-6550, "Bacterial/CpG DNA Down-Modulates Colony Stimulating Factor-1 Receptor Surface Expression on Murine Bone Marrow-Derived Macrophages with Concomitant Growth Arrest and Factor-Independent Survival" See Abstract,	1-3, 7-10-12,15-22
X	Evans, R et al (1992), J. Leukocyte Biol. Vol 51 pages 93-96, "Synergistic interaction of bacterial lipopolysaccharide and the monocyte-macrophage colony stimulating factor: potential quantitative and qualitative changes in macrophage-produced cytokine bioactivity"-See Abstract, Fig. 4	1-7, 11-14, 20-22,

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 2002	02 DEC 2002
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **JENNIFER FERNANCE** Telephone No : (02) 6283 2416

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01348

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gao, J et al (June 2001), J. Immun. Vol 166 pages 6855-6860, " Bacterial DNA and Lipopolysaccharide Induce Synergistic Production of TNF-α Through a Post Transcriptional Mechanism" -See Abstract, pages 6857 and Discussion.	1-11, 14, 20-23
X	Krieg, A et al (2000), Curr. Topics in Micro. and Immuno. Vol 247 pages 1-21, "Mechanism of Action of CpG DNA" -See pages 3, 6-15.	7-12, 15-19
X	Chapoval, A et al (1998), J. Leukocyte Biol. Vol. 63 pages 245-252, "CSF-1(M-CSF) differentially sensitizes mononuclear phagocyte subpopulations to endotoxin in vivo: a potential pathway that regulates the severity of gram-negative infections" -See whole document	1-6, 11-14, 20-22
A	Kopp E et al, (1999), Curr. Opinion in Immun. Vol. 11 pages 13-18 -See whole document	1-19