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<td>(54) Title: A METHOD OF MODULATING IMMUNE RESPONSE WITH OSTEOPONTIN</td>
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<tr>
<td>(57) Abstract</td>
<td>A method of modulating (augmenting or reducing) an individual’s (e.g., a human’s) immune response and pharmaceutical compositions useful in the method.</td>
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A METHOD OF MODULATING IMMUNE RESPONSE WITH OSTEOPONTIN

RELATED APPLICATION(S)

This application claims the benefit of U.S. Provisional Application 60/049,260 entitled “A Chemoattractant Cytokine Associated with Granulomas” by Richard A. Young, Gerard J. Nau and Patrick Guilfoile, filed June 10, 1997. The teachings of this referenced application are incorporated herein by reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by U.S. Public Health Service grants AI 37869, AI 08812 and AI 01305. The United States Government has rights in the invention.

BACKGROUND OF THE INVENTION

Mycobacterial infections are among the most numerous in the world, with Mycobacterium (M.) tuberculosis believed to have infected one third of the world’s population. (Bloom, B.R. & Murray, C.J. (1992) Science 257, 1055-64). Aggravating the worldwide pandemic have been the emergence of resistant organisms and the concurrent HIV epidemic. These events have renewed interest in understanding the fundamental biology of the interactions between pathogenic mycobacteria and their host.

Exposure to M. tuberculosis can lead to a pulmonary infection characterized by macrophage recruitment to the site of infection, followed in many cases by granuloma

SUMMARY OF THE INVENTION

The present invention relates to a method of modulating (augmenting or reducing) an individual’s immune response by altering (increasing or decreasing) osteopontin activity and, as a result, increasing or decreasing the activity of the individual’s immune system effector functions. Osteopontin activity can be altered by a variety of approaches, including increasing or decreasing osteopontin production or levels in cells, inactivating or degrading osteopontin (e.g., by binding to a molecule which ties up osteopontin and prevents it from acting or by enzymatically or chemically digesting osteopontin) or interfering with a cell component with which osteopontin must interact, directly or indirectly, (e.g., a member of a pathway in which osteopontin is a participant) to function and preventing the interaction.

In one embodiment, the present invention is a method of augmenting an individual’s osteopontin activity and activating or increasing the activity of the individual’s immune system effector functions (e.g., macrophage activity, T cell function, T cell proliferation). This provides a method for treating an individual and protecting (totally or partially) the individual against a variety of conditions, such as infection, cancer and immune suppression, in which an increased immune response and granuloma formation will be beneficial. In this embodiment, osteopontin activity can be increased by a variety of approaches, which can be used singly or in combination, and result in greater quantities of osteopontin being present in the individual, more active forms and/or increased half life of osteopontin. For example, the quantity of osteopontin in an individual can be increased by administering to him or her osteopontin; an active osteopontin fragment; an osteopontin analogue or derivative; DNA encoding osteopontin or an active fragment or an analogue or derivative of osteopontin; or another molecule or agent which mimics osteopontin activity. Alternatively, an endogenous osteopontin gene can be altered, using known genetic engineering techniques, to turn on a silent gene or to enhance expression of a normally-
expressed osteopontin gene (e.g., by introducing a promoter, enhancer and/or other regulatory element into an endogenous osteopontin gene in such a location that the gene is subsequently expressed under control of the introduced element(s)). Osteopontin activity can also be enhanced by reducing inactivation of the protein by cellular mechanisms, such as by inhibiting enzymes which degrade osteopontin, thus prolonging its activity. An individual treated by this method in order to augment his or her immune response can, at the time treatment begins, have a normal immune response (a response within physiologically acceptable parameters) or a compromised (ineffective) immune response. In either case, the individual's immune response (e.g., monocyte and macrophage accumulation, T cell function, T cell proliferation, T cell cytokine production, and granuloma formation) is enhanced (greater than it was prior to carrying out of the present method).

In a second embodiment of the present method of modulating an individual's immune response by altering osteopontin activity and granuloma formation, the individual's immune response is reduced. This is useful, for example, in treating individuals who have an excessive or enhanced immune response, such as in individuals who have an autoimmune condition (e.g., Lupus erythematosus) or are transplant recipients. In this embodiment, an osteopontin inhibitor (e.g., an antibody or other molecule which binds osteopontin or a protein or other molecule with which osteopontin must interact to increase immune response) is administered to the individual in sufficient quantity to have the desired effect. This is particularly beneficial in preventing an anti-inflammatory response or reducing the extent to which it occurs, such as in preventing or treating sepsis, coronary artery disease and autoimmune conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of a screening strategy employing lambda phage cDNA library analysis.

Figure 2 is a graphic representation of results of assessment of mycobacteria burden of infected tissue from wildtype and OPN mutant (spp1m1) mice. CFU determination from liver samples of animals infected at time = 0. Symbols represent means ± SEM; three or four animals per group (p<0.05 at 4 weeks post infection).

Figures 3A-3C are bar graphs showing comparisons of granuloma burden (Figure 3A), granuloma number (Figure 3B), and average granuloma size (Figure 3C) of wildtype versus supp1m1 animals. Measurements were performed in 10 mutant and 9
wildtype animals from 4 separate experiments. Livers from uninfected control animals of each genotype showed granuloma burdens of <0.05%. Values are ± SEM. p<0.05 for each comparison.

Figures 4A-4D show results of measurement of cytokine secretion from splenocytes stimulated *in vitro* 4 weeks after infection (Figures 4A, C, D) or one week after infection (Figure 4B). Figures 4A and 4B, IFN-γ. Figure 4C, IL-4. Figure 4D, IL-10. Each symbol represents an individual animal, closed symbols are spp1^{tm}l animals and open symbols are wildtype animals. Those symbols on the X axis represent cytokine levels below the smallest Y axis value or were undetectable in the ELISA.

Figure 5 is a graphic representation of NO production from PEC stimulated *in vitro*. Thioglycollate-elicited peritoneal exudate cells (PEC) were stimulated *in vitro* with IFN-γ (100 ng/ml) for 6 (Exp. 1) or 16 (Exp. 2) hours followed by addition of TNF-α (10ng/ml (Exp. 1) or 100 ng/ml (Exp. 2)) or LPS (5 μg/ml) for 48 hours. Values are means ± SEM of quadruplicate cultures. * = none detected. There was no detectable NO produced by cells stimulated with either TNF-α or LPS alone.

Figures 6A and 6B are graphic representations showing an exaggerated inflammatory response of spp1^{tm}l mice compared to wildtype animals after exposure to thioglycollate or BCG. Figure 6A Thioglycollate was injected i.p. and PEC were harvested 3 days later. Cells were counted on a hemocytometer with trypan blue exclusion. Results are means ± SEM of 5 animals per group. p < 0.05 for difference. Significant differences in cell counts were observed in 5 other experiments where PEC pooled from OPN mutant mice outnumbered those from wildtype mice by 1.6 to 6 fold, 5 to 7 days after administration of thioglycollate. Figure 6B PEC accumulation 3 days after i.p. injection

**DETAILED DESCRIPTION OF THE INVENTION**

Osteopontin expression has been shown to be induced in a mammal in cells of the immune system, such as macrophages and lymphocytes, and cells of affected tissues (cells of pathologic tissues) in granulomatous conditions. In addition, osteopontin has been shown to contribute to host protection against a granulomatous condition and to facilitate clearance of a pathogen from host tissues. Osteopontin is closely associated with cancers and other inflammatory processes. The present invention relates to methods and compositions for modulating (augmenting or reducing) the immune response of a mammal, such as a human, by altering osteopontin activity in the
mammal. The method and compositions are useful to modulate an individual's immune response in any condition or disease in which an enhanced immune response or a reduced immune response is desired, such as in any condition in which increased macrophage activity and/or T cell activation is desired. For example, the present invention is useful in enhancing an individual's immune response to pathogens or infectious agents, including intracellular pathogens and extracellular pathogens (e.g., a bacterium, mycobacterium, virus, protozoan, parasite), cancer or tumor cells, foreign tissue (e.g.; organ or tissue transplant) or self (e.g., in autoimmune conditions, such as rheumatoid arthritis, Lupus erythematosus). Osteopontin can be used to protect against infection (preventing its occurrence or reducing its severity) and against progression of an established infection. Osteopontin can also be used to enhance the efficacy of a DNA or protein vaccine (e.g., by sequential or simultaneous administration of osteopontin protein and the vaccine). In those instances in which an individual's immune response is to be augmented (e.g., in infections, cancers), osteopontin activity is increased, as described herein. In those in which the immune response is to be decreased (e.g., inflammatory response to foreign tissue, autoimmune conditions), osteopontin activity is decreased, also as described herein.

As described herein, Applicants have shown that the osteopontin gene is induced by infection and that osteopontin contributes to host protection against infection and facilitates clearance of the pathogen from host tissues. The osteopontin gene has been shown to be induced by mycobacterial infection; *M. tuberculosis* infection of primary human alveolar macrophage has been shown to cause a substantial increase in osteopontin gene expression. As described in detail below, osteopontin protein was identified by immunochemistry in macrophages, lymphocytes and the extracellular matrix of pathologic tissue sections of patients with tuberculosis. Further, results using a mouse strain with a genetic deletion of the osteopontin gene support the role of osteopontin in augmenting the immune response and accelerating clearance of mycobacteria after infection. When infected with *M. bovis* BCG, mice lacking a functional osteopontin gene had more severe infections and larger numbers of granulomas, compared to wildtype animals. As also described, increased osteopontin expression was found to be associated with silicosis, which is also a granulomatous disease.

Osteopontin, also known as early T lymphocyte activation protein or *Eta-1*, is a cytokine and macrophage chemoattractant. As described herein, it has been shown to be
associated with granulomatous diseases. The association of osteopontin with tuberculosis and granuloma formation, and its function as a cytokine and macrophage chemoattractant support osteopontin’s role in granuloma formation. Further, the role of osteopontin in granuloma formation provides a means of protecting individuals against infections (such as tuberculosis), silicosis and other conditions in which augmentation of the immune response, such as by increasing osteopontin production or availability and activation of immune system effector functions is beneficial. Alternatively, the work described herein provides an approach to moderating an excessive or enhanced immune response by reducing (partially or totally) osteopontin activity. In one embodiment, this is effected through the use of agents that bind or otherwise disable or tie up osteopontin and/or osteopontin receptors. In another embodiment, osteopontin activation is inhibited (and, thus, the immune response or the inflammatory process is lessened) by means of administering agents which block secondary messengers of osteopontin activation. As a result, the adverse effects of a heightened immune response or inflammatory process are also lessened or prevented.

Osteopontin protein and its use have broad applicability in the area of immune modulation. For example, augmentation of the immune response (to enhance or boost an already effective immune response or an ineffective (compromised) immune response) is possible. Osteopontin protein can be administered to an individual (as osteopontin protein, active osteopontin fragment or protein derivative or analogue or by means of a nucleic acid construct which encodes and expresses one of the aforementioned or cells containing such a construct, which results in osteopontin production in the individual) to enhance immune response. As used herein, the term osteopontin encompasses active fragments or portions of osteopontin, osteopontin analogues and osteopontin derivatives. Alternatively, an osteopontin mimic can be administered. As a result, the individual’s immune system effector functions are activated. This is useful, for example, for macrophage killing of such pathogens as bacteria and mycobacteria, e.g., *M. tuberculosis*, chlamydia, mycoplasma, Listeria, Salmonella), or other intracellular pathogens; extracellular or multicellular pathogens (e.g., infectious agents or parasites); augmentation of anti-tumor/cancer responses; and activation of inflammatory reaction against infections in transplant patients, without induction of a widespread activation of the immune system, thus avoiding graft rejection. In addition, T lymphocyte proliferation can be augmented, such as to reconstitute in T depletion syndromes (HIV, idiopathic CD4 cell depletion or after
ablative chemotherapy). In addition, osteopontin protein, active osteopontin fragment, osteopontin derivative or analogue or DNA encoding and expressing one of the aforementioned can be administered to an individual (e.g., a human) to enhance the efficacy of a vaccine (e.g., DNA vaccine or protein/peptide vaccine). Osteopontin (or a fragment/analogue/derivative) can be administered prior to or at the time of administration of the vaccine (e.g., by injection into the dermis of an individual). In either case, this is done to prime or stimulate the individual’s immune system (e.g., to activate macrophages and render them more effective in engulfing and digesting a pathogen).

In one embodiment, pure or essentially pure osteopontin is administered. It can be produced by recombinant protein synthesis. Expression constructs useful for producing recombinant osteopontin include, but are not limited to, constructs which utilize the well described systems of baculovirus and of yeast. Both of these systems are capable of producing large quantities of recombinant human protein that can be easily purified for use in vivo.

As a result of administering osteopontin (or an active fragment, an analogue or a derivative thereof, DNA encoding one of the aforementioned or cells comprising such DNA, which is expressed by the cells) the immune response of an individual (e.g., a mammal, such as a human) is augmented by increasing osteopontin activity in the mammal and the individual’s immune system effector functions are activated or enhanced (e.g., macrophage activity is increased; T cell activity is increased; helper T cells are turned on, with the result they produce more cytokines; T cell proliferation occurs; or a combination of any of these effects occurs). In one embodiment, a method of augmenting a mammal’s immune response is carried out by administering osteopontin (or a fragment/analogue/derivative, encoding DNA or cells comprising encoding DNA) to increase osteopontin activity, whereby macrophage activity is increased. Conversely, in those instances where the present method is carried out to reduce an individual’s immune response, osteopontin activity is decreased and at least one of the immune system effector functions is reduced in activity (e.g., macrophage activity, T cell activity, helper T cell activity and production of cytokines, T cell proliferation).

Pharmaceutical compositions useful in the present method are also the subject of this invention. Pharmaceutical compositions useful to augment an individual’s immune response comprise (a) a first component selected from the group consisting of:
osteopontin protein, an active osteopontin fragment, an osteopontin derivative or analogue or an osteopontin mimic or DNA encoding one of the aforementioned and (b) a second component, which is a physiologically appropriate carrier or solvent (e.g., water; buffer, physiologic saline). Such compositions can be administered alone to enhance an individual’s immune response or in conjunction with (before or simultaneous with) a DNA or protein vaccine. Pharmaceutical compositions useful to reduce an individual’s immune response comprise (a) a first component selected from the group consisting of: (a) an antibody or other moiety which binds osteopontin and prevents it from functioning or reduces osteopontin function; an osteopontin mimic which (i) interacts with a cellular component with which osteopontin must interact to function and (ii) prevents osteopontin from interacting with the cellular component; DNA encoding such a mimic; an enzyme which degrades or digests osteopontin; DNA encoding such an enzyme; or a mimic of a cellular component with which osteopontin must interact to function, wherein the mimic (i) prevents osteopontin from interacting with the cellular component and (b) a second component which is a physiologically appropriate carrier of solvent (e.g., water, buffer, physiologic saline).

Tuberculosis is a pandemic infection that involves much of the world’s population and is caused by M. tuberculosis. The World Health Organization has raised concern over the epidemic potential of this organism because of increasing antimicrobial resistance. Recently, an isolate of M. tuberculosis with an extraordinary growth rate was isolated with increased rates of transmission. These findings have generated interest in understanding the unique interactions of host cells and mycobacteria: M. tuberculosis can elude host immune responses and persist in a latent state for years. The granuloma is a hallmark of this host-pathogen interaction.


Therefore, a study of macrophage gene expression changes after infection by mycobacteria was undertaken and is described herein. An evaluation of the host genome of a murine macrophage cell line by differential screening of a cDNA library
repeatedly revealed one isolate, osteopontin (OPN), as described in Example 1. Osteopontin gene expression in human pulmonary macrophages increased after infection with virulent MTB and osteopontin protein expression was widespread in human TB pathology (Examples 2 and 3).


In spite of the close association between OPN and tuberculosis infection, until the work described herein, it was unclear if OPN has a salutary effect on the host response to tuberculosis or if its expression is only coincidental with granulomatous inflammation. OPN knockout mice (Liaw, L. et al. (1998) J. Clin Invest 101 (7): 1468-1478) were used to investigate whether OPN has a role in protection against infection by mycobacteria. Results described herein showed that animals lacking OPN had more severe infections due to delay in eliminating the infecting mycobacteria (Example 4). The findings support a new role for OPN as an accessory molecule to augment the clearance of inflammatory stimuli.

In a specific embodiment of the present invention, individuals are protected (partially or totally) against infection by M. tuberculosis or against progression of infection with tuberculosis. For example, individuals infected with M. tuberculosis can be treated to lessen or eliminate the effects of the mycobacterium or to prevent
development of debilitating or life-threatening effects of the infection. Individuals are treated with osteopontin or osteopontin-encoding DNA/DNA construct in such a manner that the immune response is enhanced and granulomas are formed. For example, sufficient osteopontin or osteopontin-encoding DNA/DNA construct is administered to stimulate accumulation of monocytes and macrophages and, as a result, to initiate granuloma formation (perhaps by promoting chemotaxis, adhesion and anchoring of the cells). As a result, the organism is destroyed or disabled and unable to adversely affect an infected individual or produces effects to a lesser extent than would otherwise result.

Alternatively, the immune response and particularly the adverse effects of an overly exuberant inflammatory process can be reduced.

The monoclonal antibody HA1A and IL-1 receptor antagonists have been used in previous attempts at reducing lethal inflammatory responses. However, these agents are often administered too late to be clinically effective - i.e., the damaging cytokines have already been released. High levels of osteopontin protein expression in human tissue pathology indicate that osteopontin is likely to be a molecule important later in inflammatory responses. Therefore, osteopontin is a better target for anti-inflammatory activity. For example, blocking osteopontin function can be useful in preventing or treating sepsis, coronary artery disease, autoimmune diseases, such as rheumatoid arthritis, transplant rejection, rejection of prosthetic porcine heart valves and graft versus host disease.

Osteopontin is also useful diagnostically. Osteopontin protein accumulates under conditions of chronic inflammation. Thus, agents that bind to osteopontin can be used to localize areas of chronic inflammation for procedures such as diagnostic fine needle aspiration and surgical drainage. Strategies include those already employed by nuclear medicine radiologists such as radiolabeled monoclonal antibodies; in this case the diagnostic monoclonal antibodies specifically bind to osteopontin. Alternatively, recombinant osteopontin that is directly coupled to a radioactive label can be used to localize sites of inflammation.

Osteopontin is also closely associated with several carcinomas. Thus, reagents that detect osteopontin accumulation, such as antibodies that bind to osteopontin, can be used to assess the full extent of local tumor extension and of distant metastases.

It should be noted that proteins that modulate the immune response frequently have structurally and functionally related family members. It is likely that osteopontin
represents one of a group of related cytokines. As used herein, the term osteopontin refers to and includes all members of such a group.

As described in Example 1, differential screening of cDNA libraries was used to compare mRNAs of infected and uninfected macrophages and gain insight into the host responses to tuberculosis. This was done because it was predicted that the identification of macrophage genes whose expression is altered following phagocytosis of *M. tuberculosis* would help provide an understanding of pathogenesis. A model system that employed a macrophage cell line was used to survey the population of macrophage mRNAs for those altered by mycobacterial infection. (Rastogi, N., Ptar, M.-C. & David, H.L. (1987) *Curr Microbiol* 16, 79-92; Rastogi, N., Blom-Potar, M.C. & David, H.L. (1989) *Acta Leprol 1*, 156-9). Genes identified with this strategy were then studied in human cells exposed to *M. tuberculosis* and in tissues from patients with tuberculosis. One gene identified repeatedly in this screen was particularly interesting because its protein product, osteopontin, is a cytokine and macrophage chemoattractant. (Patarca, R., Saavedra, R.A. & Cantor, H. (1993) *Crit Rev Immuno1 3*, 225-46; Denhardt, D.T. & Guo, X. (1993) *Faseb J 7*, 1475-82.). Results showed that osteopontin gene expression was induced by mycobacterial infection of human macrophages and osteopontin protein was found in human tissue specimens from patients with clinical tuberculosis.

The present invention will be illustrated by the following examples, which are not intended to be limited in any way. The following methods and materials were used in carrying out the examples presented below.

Work presented herein demonstrates that OPN augments host defenses against a mycobacterial infection. Mice lacking a functional osteopontin gene suffered from more severe infections with *M. bovis* BCG. The OPN mutant animals had delayed eradication of live bacilli and exaggerated macrophage infiltrates. The mutants also had a greater hepatic granuloma burden 4 weeks after infection, consistent with persistence of the bacilli and delayed resolution of the inflammation.

This work supports a model in which the osteopontin mutant mice have a defect in the innate inflammatory response. As described herein, results showed that there was an early defect in the eradication of mycobacteria and in the clearance of the inflammatory effects of thioglycollate. There was not, however, a defect in the ability of macrophages from OPN mutant animals to produce NOS2 or pro-inflammatory cytokines after infection. Mice lacking OPN had greater numbers of inflammatory
macrophages in response to thioglycollate or to acute and chronic mycobacterial infection. Finally, antigen-specific T cell-mediated immunity was normal in the OPN mutant mice. Thus, OPN's effects on augmenting the clearance of mycobacteria appear to be independent of many host defenses mechanisms that are known to be relevant to mycobacterial infections.

Thus, it appears that OPN activates macrophages to be more efficient at eliminating inflammatory stimuli. The susceptibility to infection of the OPN mutant animals does not appear as dramatic as, for instance, IFN-γ mutants. It is logical to conclude that OPN has a co-stimulatory role in macrophage activation to enhance the killing of mycobacteria by macrophages.

The data presented here implicate OPN as a relevant CD44 ligand in granulomatous inflammation. CD44 is believed to be one of several receptors for OPN (Weber, G. F. et al. (1996) Science 271 (5248): 509-512). Mice deficient in CD44 have an exaggerated granulomatous response to i.v. Corynebacterium parvum (Schmits, R.J. et al. (1997) Blood 90 (6): 2217-2233)). The increased granuloma burden in OPN mutant mice after BCG infection is consistent with the exaggerated response of the CD44 mutant mice. The phenotypes of the two mutant mice are similar: a 1.5-fold increase in granuloma size and number was observed in the CD44 mutant animals versus wildtype animals treated i.v. with Corynebacterium parvum and a comparable difference was observed with the OPN mutants after BCG infection. The behavior of the two knockout mice after exposure to inflammatory stimuli suggests CD44 and OPN are important modulators of granulomatous inflammation.

Examples 1-4
The following materials and methods were used in the work described in Examples 1-4.

Bacteria. Mycobacterium bovis BCG (ACTT# 35734) was grown from a frozen stock for 3 days (to O.D.~600 ~ 1.2) in Middlebrook 7H9 broth with 0.5% glycerol, 0.05% Tween 80 and ADC enrichment (Difco). BCG diluted 1:10 in RPMI with 1% FCS was added to J774 cells (bacteria:macrophage ratio approximately 10:1). The streptomycin-dependent strain of Escherichia coli, sd-4 (ATCC #11143), was cultured in LB media with streptomycin at 624 μg/ml. Frozen stocks in 20% glycerol were made from stationary phase cultures after two days growth in LB containing 25 μM/ml streptomycin. On the day of infection these bacteria were thawed, were resuspended in
7H9 medium, and were diluted as for BCG (bacteria:macrophage approximately 1:1). These two ratios were required to achieve comparable percentages of infected macrophages; limited growth of the *E. coli* in the absence of streptomycin necessitated a lower ratio. Latex beads (0.8 μm, carboxylate-modified, Sigma#L-1398) were treated with 70% ethanol before use.

Macrophage culture and infection. J774 cells (ATCC # TIB 67) were grown without antibiotics in RPMI 1640 with 10% low-endotoxin FCS (Gibco BRL, Gaithersburg, MD) until infection. These cells were infected by incubation with either bacteria or beads for four hours, were washed three times with Hank’s Balanced Salt Solution and were cultured for an additional 20 hours before RNA harvest. During and after infection, cells were maintained in 1% low-endotoxin FCS, as previously described (Rastogi, N., Ptar, M.-C. & David, H.L. (1987) *Curr Microbiol* 16, 79-92). Alveolar macrophages were obtained from consenting human donors. Bronchoalveolar lavage cells were harvested by bronchoscopy and plated at a density of 500,000 cells/ml. Alveolar macrophages were purified by adherence to plastic after growing 4 days in RPMI 1640 with 10% FCS. Cells were infected as described above except that cultures of *M. tuberculosis* H37Rv and BCG were passaged through an 25 gauge needle prior to addition to macrophages.

Library Construction and Screening. RNA was isolated using the guanidinium isothiocyanate/CsCl method. (Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, Cold Spring Harbor, NY). 5μg of poly A+ RNA isolated by oligo dT chromatography was used to construct a cDNA library from BCG-infected J774. The Superscript cDNA kit (Gibco BRL) was used to generate first strand cDNA using oligo dT. Second-strand cDNA was synthesized using a modification of the protocol of Gubler and Hoffman. (Gubler, U. & Hoffman, B.J. (1983) *Gene* 25, 263-9). The resulting cDNA was ligated into λgt10 using *EcoRI-NotI* linkers. Recombinant phage were selected and amplified using standard techniques. The cDNA library prepared from BCG-infected J774 cells contained 1.1 X 10^6 independent recombinants with an average insert size of greater than 1kb. Of six random phage isolates, all contained inserts.

A non-radioactive detection method, the Genius System (Boehringer Mannheim, Indianapolis, IN) was employed for library screening. Preparation of digoxigenin-
labeled cDNA probes from PolyA+ RNA, hybridization, and detection with Lumi-Phos 530 or NBT/X-phosphate were used as per the manufacturer’s protocol. Plaques were screened by incubating duplicate filters with digoxigenin-labeled cDNA form E. coli or BCG-infected macrophages.

RNA Analysis. 10 μg of total cellular RNA was run per lane of a 1% Glyoxal/DMSO agarose gel and transferred to nylon membranes (Amersham). Hybridization, washing, and probe striping were as directed by the membrane manufacturer. Probe fragments isolated from agarose gels by using DEAE membrane (Schleicher and Schuell) or by using silica adherence (Gene Clean II, SUN Bioscience Inc.) were labeled by random priming (Primelt II, Stratagene). Relative signals were quantitated with a phosphoimager (Fuji Medical Imaging Systems USA, Stamford, CT) and were normalized to the actin signals.

Quantitative PCR Analysis. 1 μg of total RNA was reverse-transcribed using Superscript reagents (Gibco BRL). The PCR reaction utilized 1/10th of the reverse transcribed cDNA in a 100 μl volume, and included 10 μl 10X Taq buffer, 4 μl 5 mM dNTPs, 50 pmole each primer, 1 μl competitive template and 1 μl (5 units) Taq DNA digest of the yeast RPB1 gene to the unique Nde1 site of the human osteopontin polymerase (Perkin-Elmer). The reaction was incubated for 35 cycles of 94°C for 30 sec., 50°C for 30 sec., and 72°C for 1 min. The competitive template was generated by insertion of a 333 bp DNA fragment from a Nde1 digest of the yeast RPB1 gene to the unique Nde1 site of the human osteopontin gene. The primers used were

5'CACCTGTCATACCAGTTAAGACG-3' (SEQ ID NO.:1) AND 5'CATGGCTGAAATTCATGGCTGT-3' (SEQ ID NO.:2). These primers allowed amplification of a 830 bp cDNA fragment and a 1163 bp fragment from the competitive template. No product was seen when these primers were used to amplify this fragment from 1μg of purified genomic DNA Commercially-available of actin primers were used (Stratagene, La Jolla, CA).

Immunohistochemical Staining.

Tissue sections were obtained from the Gaensler Lung Archive at the Boston University Pulmonary Center and the Boston VA Hospital (Boston, MA). These specimens are paraffin embedded tissues processed using standard clinical techniques.
Immunohistochemical staining was done on a Ventanna ES automated stainer (Ventanna, Tuscon, AZ). Sections 5μm thick were baked for one hour at 60°C on to positively charged slides and then deparaffinized with xylene and hydrated in graded alcohol washes to water. Slides were then processed with Ventanna’s protease 1 reagent for four minutes and incubated with primary antibodies for 32 minutes at 42°C. The manufacturer’s protocol was used for the DAB kit (osteopontin) and the fast red kit (CD68). Images were photographed with Kodak Ektachrome or Ektar film and scanned into Adobe Photoshop to create composite figures.

Monoclonal antibodies MPIIIB10, specific for rat and human osteopontin, and QH1, specific for quail vascular endothelial cells, were obtained from the Developmental Studies Hybridoma Bank (Iowa City, Iowa) and used at 1:200 dilution of hybridoma supernatant (400 and 500 ng/ml respectively). Monoclonal antibody KP-1 recognizing human CD68 was obtained from Dako (Carpinteria, CA) (Pulford, K.A., et al. (1989) J Clin Pathol 42, 414-21).

Example 1: Identification of genes associated with infection of macrophages

The effect of bacterial infection on macrophage gene expression was investigated by differential screening of cDNA libraries. The murine macrophage cell line J774 was used in these initial experiments because previous work has established its usefulness for studying interactions between mycobacteria and macrophages. (Rastogi, N., et al. (1987) Curr Microbiol 16, 79-82; Rastogi, N., et al. (1989) Acta Leprtol I, 156-9). Differential screening of libraries has the advantage of detecting smaller changes in relative mRNA levels than subtractive hybridization.

The screening strategy is summarized in Figure 1. The J774 cells were exposed to latex beads, or Escherichia coli, or Mycobacterium bovis BCG, and recombinant cDNA libraries and probes were prepared from the macrophage polyA+ mRNA. The phage library representing mRNAs of macrophages infected with BCG was plated with host bacteria and duplicate filters were placed on the plaques. These filters were then hybridized with cDNA probes prepared from J774 cells infected with either E. coli or BCG and plaques with different signal strengths were isolated. This screening strategy was designed to identify genes whose expression differed when macrophages were exposed to BCG verses E. coli. The library constructed with mRNA from BCG-
infected J774 cells was screened to maximize the chance of identifying genes whose expression was specifically increased by BCG.

Approximately 730,000 recombinant phage were screened by probing duplicate filters with labeled cDNA prepared form J774 cells that had phagocytized BCG or E. coli. Three clones were isolated that produced stronger signals with cDNA from macrophages infected with E. coli. Sequencing of their insert DNAs revealed that one cDNA clone encoded macrophage inflammatory protein 1-α (MIP-1α) (Davatelis, G. et al. (1988) J. Exp. Med. 167: 1939-1944) and two encoded ferritin (Torti, S. V. et al. (1988) J. Biol. Chem. 263: 12638-12644). Ten clones were isolated that produced stronger signals with cDNA from macrophages infected with BCG. All of these encoded osteopontin Craig, A.M. et al. (1989) J. Biol. Chem. 264: 9682-9689.

Example 2: Differential expression of MIP1α, ferritin, and osteopontin mRNAs

To confirm that the three genes represented by the cDNA clones identified by the screening process were indeed differentially expressed, radiolabeled insert DNAs from the clones were used to probe macrophage mRNA immobilized on nylon filters. The mRNAs of all three genes accumulated to higher levels when macrophages phagocytized the two bacterial species verses the latex beads.

Osteopontin mRNA accumulated to similar levels in macrophages exposed to latex beads of E. coli, but accumulated to at least 5-fold higher levels after infection of macrophages with BCG. There were similar increases in osteopontin mRNA levels in macrophages that had phagocytized live and heat-killed BCG. Neither 7H9 media nor supernatant from a BCG culture were capable of eliciting the increase in osteopontin expression caused by BCG organisms. Thus, osteopontin mRNA levels increase substantially in murine macrophages that phagocytize BCG.

Example 3: Osteopontin expression in human lung macrophages

Work was carried out to determine whether increased osteopontin gene expression occurs in primary human cells in response to phagocytosis of virulent M. tuberculosis. Human alveolar macrophages were exposed to latex beads, BCG, and M. tuberculosis strain H37Rv. To determine precisely the number of osteopontin mRNA molecules present per cell before and after infection, a reverse transcriptase-polymerase chain reaction assay was used with varying levels of competing template. The results demonstrate that the levels of osteopontin mRNA were higher in alveolar macrophages
infected with BCG and virulent *M. tuberculosis*. The human alveolar macrophages that had phagocytized latex beads contained approximately 1 molecule of osteopontin mRNA per cell. In contrast, the alveolar macrophages that had phagocytized BCG and *M. tuberculosis* had about 10 molecules in osteopontin mRNA per cell. This magnitude of increase is osteopontin mRNA following exposure to mycobacteria was similar to that observed with the murine macrophage model.

**Example 4: Osteopontin in human granulomatous diseases**


Osteopontin was readily identified throughout specimens of tuberculous lung. The architecture of a tuberculosis lesion with necrosis and with a lymphoid aggregate above alveolar air spaces was evident in sections treated with the isotype control antibody and the hematoxylin counterstain. No staining of this specimen was observed with the isotype control. However, when an adjacent serial section was probed with the MPIIIIB10 antibody, a strong osteopontin signal was detected in the inflammatory border surrounding the necrosis, and immediately adjacent to and within the areas of caseating necrosis. At higher power, macrophages were identified in a serial section with anti-CD68 antibody and the fast red reagent. These cells also expressed osteopontin. Lymphocytes identified at low power and at high power in demonstrated intense osteopontin signal singly and in aggregates. Giant cells expressed CD68 but did not universally stain for osteopontin; in general, epithelioid giant cells expressed osteopontin. Bronchiolar epithelium and small blood vessels stained with the MPIIIIB10,

Specimens from normal lung, an acute inflammatory condition (bacterial bronchopneumonia), and two chronic inflammatory conditions (silicosis and granulation tissue of a pilonidal cyst) were studied to assess the variety of situations in which osteopontin could be detected. Normal lung did not show significant staining for osteopontin either in alveolar networks or in airways. The bronchopneumonia specimen had scattered cells of macrophage and lymphocyte morphology that produced some signal with MIPIII-B10. Lymphoid aggregations associated with the pneumonia were also positive for osteopontin. The two chronic inflammatory conditions showed dichotomous results for the presence of osteopontin. Silicosis is a non-infectious granulomatous disease. The silicotic nodule with its characteristic fibrosis showed an abundance of macrophages that stained with CD68. These cells, and presumably some of the extracellular matrix, showed heavy signal for osteopontin on immunohistochemical staining. Granulation tissue is another pathologic process characterized by mononuclear cell infiltration but distinct from granulomatous inflammation (Warren, K. S. (1976) *Acad Sci* 278: 7-18). While the granulation tissue of the pilonidal cyst showed a large aggregation of giant cells and numerous macrophages based on CD68 staining, there was no osteopontin detected. Thus, only tuberculosis and silicosis, the two granulomatous conditions, expressed high levels of osteopontin in the tissue pathology.

Example 5: Reduced clearance of *M. bovis* BCG in OPN-deficient mice

Wild type and mutant mice were infected with BCG, and the bacterial load in liver sections was assessed over 12 weeks using standard tissue staining for acid-fast bacilli. Four independent experiments were performed. When sections of liver and spleen obtained 4 weeks after infection from wildtype mice were stained for acid fast organisms, few bacilli were detected. In contrast, the mycobacteria were easily identified in the livers and the spleens of OPN mutant animals.
The mycobacteria burden of infected tissue was measured by serial dilutions of homogenized liver samples from the wild type and OPN mutant mice (Figure 2). Similar numbers of bacilli were found in wild type and mutant mice immediately after infection. However, there was a significant difference in the mycobacteria burden 4 weeks after infection. In three independent experiments, OPN mutant mice had 10 to 40-fold more bacteria per gram of liver tissue than wildtype mice. The OPN mutant animals were, however, capable of reducing the mycobacteria burden by twelve weeks and the rate of CFU decline appeared similar to that of wildtype animals. These results show that the OPN mutant mice are less able to clear BCG early after infection. A similar 10-fold difference in CFU was also observed in these animals’ spleens at the 12-week time point.

Example 6: Increase in granuloma number and size in OPN mutants

Histologic analysis showed that inflammatory cells accumulated in foci within the livers of both the wildtype and the OPN mutant animals after infection by BCG. These granulomas varied in size between animals and were composed of lymphoid and histiocytoid constituents. These cell constituents were further defined by immunohistochemistry of the tissue sections. Both macrophages and CD3-positive cells, presumably T cells, were readily identified within the granulomas. These findings indicate that OPN is not critical for the formation of focal accumulations of mononuclear cell inflammation, i.e. granulomas, in the mice 4 weeks after infection by BCG.

Although there was no apparent difference between granuloma cellular constituents, the macrophage staining suggested a difference in the total amount of hepatic tissue involved by granulomas. This difference was quantitated by measuring the granuloma burden. Liver sections stained for macrophages with the F4/80 monoclonal antibody and the Vector Red alkaline phosphate substrate were subjected to a stereologic evaluation to measure the granuloma burden. The fluorescent emissions from the Vector Red substrate clearly demonstrated that the OPN mutant mice had more macrophages within the liver and overall a greater burden of granulomas (Figure 3A). The granuloma burden of liver specimens from 19 animals was systematically measured as described in the Materials and Methods. The wildtype animals had an estimated 1.1% of the liver tissue area involved by granulomas (Figure 3A-3C). The more
extensive inflammation observed in OPN mutant mice 4 weeks after infection with BCG correlated with the greater bacterial burden observed in these animals.

Example 7: Nramp1 genotyping of ssp1\textsuperscript{m1} mice

Several studies have documented genetic differences that predispose animals to more severe mycobacterial infections. Analyses of inbred mice have shown that alleles of Nramp1 determine resistance or susceptibility to BCG, Leishmania donovani, or Salmonella typhimurium infection Nramp1 encodes an integral membrane protein with a structure that is similar to transport proteins (See, Skamene, E. (1986) Curr. Top Microbiol Immunol. 124 49-66, Vidal, S.M. (1993) Cell 73 (3): 469-485) Nramp1 has an important impact on murine responses to a mycobacterial infection, so it was necessary to evaluate this genetic element in the animals used in these experiments.

The OPN and Nramp1 genotypes were, determined by PCR. DNA from ssp1\textsuperscript{m1} animals generated a 500 bp product in the OPN PCR whereas DNA from wildtype animals yielded a 600 bp fragment; a heterozygous animal is shown for comparison. The genomic DNA from the same animals was subjected to PCR genotyping for Nramp1 as described by Medina et al. (Medina, E. et al. (1996) Immunology 88 (4): 479-481). Genomic DNA from C57/B6 and 129/J animals yielded products for the susceptible and the resistant allele, respectively. DNA from all the ssp1\textsuperscript{m1} and wildtype animals tested revealed that these animals possess the resistant allele for Nramp1. Thus, the wildtype and the OPN mutant mice do not differ in their alleles for Nramp1.

Example 8: T cell and APC function and unaltered in OPN mutant mice

OPN is produced by T cells early after activation and it has been proposed to be a factor relevant to developing T cell immunity (Partarca, R. et al. (1989) J. Exp Med 170 (1): 145-161). T lymphocytes that react with specific mycobacterial antigens and the cytokines they produce are critical to the host defense against a mycobacterial infection. The production of INF-\gamma is a particularly important feature of an effective immune response (31, 32). It was possible that OPN mutant mice failed to generate T cells reactive to the relevant mycobacterial antigens or those that did develop were unable to secrete INF-\gamma. To test this possibility, the induction of antigen-specific immunity in ssp1\textsuperscript{m1} versus wildtype animals was analyzed.

Splenocytes from animals infected 4 weeks earlier were stimulated \textit{in vitro} with ConA, an irrelevant antigen (OVA), the relevant mycobacterial antigen (PPD), or
culture medium only. The results in Figure 4A demonstrate that splenocytes from OPN mutant animals were capable of producing INF-γ specifically after stimulation with the relevant antigen, PPD. This INF-γ production was comparable to splenocytes from wildtype animals at the time that the difference between colony forming units was the greatest. There was no difference in the kinetics of induction of T cell immunity as splenocytes of both genotypes produced similar levels of INF-γ one week after infection (Figure 4B). As expected, the T cell response was predominately Th1 insofar as no IL-4 was detected from the splenocyte cultures stimulated with PPD (Figure 4C). Finally, evidence has emerged that IL-10 antagonizes the host response to mycobacteria, (Bermudez, L.E. and J. Champs; (1993) Infect Immunol 61 (7): 3093-3097; Denis, M. and E. Ghadirian (1993) J. Immunol 151 (10): 5425-5430) probably at the level of macrophage stimulation by INF-γ (Murray, P.J. et al. (1997). There was not, however, a significant difference in the IL-10 produced by splenocytes from spp1^{m1} versus wildtype animals (Figure 4D). Therefore, the OPN mutant mice mounted T cell responses comparable to those of the wildtype animals; the kinetics of induction and the type of the cytokines produced were similar between genotypes. These in vitro analyses also show that the OPN mutant mice had functional antigen-presenting cells in their splenocyte populations. These antigen-presenting cells were capable of processing and presenting the PPD to the relevant T cells, leading to cytokine secretion.

Example 9: RNI production is normal in OPN mutant mice

Of the known anti-microbial pathways used by professional phagocytes, the production of reactive nitrogen intermediates (RNI) is crucial to the successful eradication of M. tuberculosis infections. The production of NO by macrophages during an infection depends on the induction of nitric oxide synthase 2 (NOS2). It was possible that the OPN mutant mice were deficient in signaling the production of NO. Therefore, tests were carried out to test for RNA in vitro and for the expression of NOS2 in vivo. PEC preparations that contain inflammatory macrophages respond to INF-γ and LPSs by producing NO, which can be measured by the surrogate marker NO_{2}^{−}(40) (Ding, A.H. et al. (1988) J. Immunol 141 (7) 2407-2412). Figure 5A shows the production of NO by PEC from wildtype and spp1^{m1} mice activated with inflammatory stimuli. Cells from both strains of mice produced NO after stimulation by INF-γ and LPSs or plus TNF-α. There was not a consistent difference observed in the amount of NO in the supernatant between the genotypes.
Because the requirements of NO production in vivo culture system, the expression of NOS2 was assessed in vivo. Immunohistochemical staining of liver sections revealed widespread NOS2 protein in the granulomas of both wildtype and OPN mutant animals 4 weeks after infection. Macrophages infiltrating the livers of both wildtype and mutant animals one week after infection also stained faintly for NOS2. As was observed with the T cell activity (Figure 4A), the NOS2 was present in tissues at the time of the greatest difference in colony forming units between the genotypes.

Example 10: Macrophage recruitment in OPN mutant mice

Macrophages from the spp1tm1 mice cleared viable mycobacteria poorly even though the NO data suggested these cells could respond to INF-γ and TNF-α normally. Because there was inefficient triggering of macrophages to eliminate the BCG, it seemed likely that such an activation defect may be manifested by the inability to clear other inflammatory agents. Therefore, the acute inflammatory reaction to a non-specific irritant, thioglycollate was quantitated. Intraperitoneal injection of thioglycollate was used to elicit inflammatory cells, which are mainly macrophages from spp1tm1 and wildtype mice. In repeated experiments the PEC from spp1tm1 mice out numbered those from wildtype mice (Figure 6A). Similar results were obtained when BCG was used as the inflammatory stimulus; OPN mutant mice had greater inflammatory exudates than the wildtype mice 72 hours after infection (Figure 6B).

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. The teachings of all references cited herein are expressly incorporated herein by reference in their entirety.
CLAIMS

What is claimed is:

1. A method of augmenting the immune response in a mammal, comprising increasing osteopontin activity in the mammal, whereby macrophage activity is increased, thereby enhancing the immune response.

2. The method of Claim 1 wherein osteopontin activity is increased in the mammal by administering to the mammal a composition selected from the group consisting of:

   (a) osteopontin or an active portion thereof;
   (b) an osteopontin derivative;
   (c) an osteopontin analogue;
   (d) DNA encoding osteopontin or an active portion thereof;
   (e) a DNA construct comprising DNA encoding osteopontin or an active portion thereof;
   (f) cells modified to contain DNA encoding osteopontin or an active portion thereof, wherein the DNA is expressed in the cells; and
   (g) an osteopontin mimic.

3. The method of Claim 2 wherein the mammal is a human.

4. A method of providing an immune response in a mammal against Mycobacterium tuberculosis, comprising increasing osteopontin activity in the mammal, whereby macrophage activity is increased, thereby enhancing the immune response in the mammal.

5. The method of Claim 4 wherein osteopontin activity is increased by administering to the mammal a composition selected from the group consisting of:

   (a) osteopontin or an active portion thereof;
   (b) an osteopontin derivative;
(c) an osteopontin analogue;
(d) DNA encoding osteopontin or an active portion thereof;
(e) a DNA construct comprising DNA encoding osteopontin or an active portion thereof;
(f) cells modified to contain DNA encoding osteopontin or an active portion thereof, wherein the DNA is expressed in the cells; and
(g) an osteopontin mimic.

6. The method of Claim 5 wherein the mammal is a human.

7. A method of augmenting the immune response of an individual to a pathogen, comprising administering to the individual a composition selected from the group consisting of:
(a) osteopontin or an active portion thereof;
(b) an osteopontin derivative;
(c) an osteopontin analogue;
(d) DNA encoding osteopontin or an active portion thereof;
(e) a DNA construct comprising DNA encoding osteopontin or an active portion thereof;
(f) cells modified to contain DNA encoding osteopontin or an active portion thereof, wherein the DNA is expressed in the cells; and
(g) an osteopontin mimic.

8. The method of Claim 7 wherein the individual is a human.

9. The method of Claim 8 wherein the intracellular pathogen is selected from the group consisting of: bacteria, mycobacteria, viruses, protozoa and parasites.

10. A method of reducing the immune response in an individual comprising decreasing osteopontin activity in the individual, whereby activity of an immune system effector function is decreased.
11. The method of Claim 10 wherein osteopontin activity is decreased by administering to the individual an inhibitor of osteopontin selected from the group consisting of:
   (a) an antibody which binds osteopontin and prevents it from functioning;
   (b) an osteopontin mimic which (i) interacts with a cellular component with which osteopontin must interact to function and (ii) prevents osteopontin from interacting with the cellular component;
   (c) an enzyme which degrades osteopontin;
   (d) DNA encoding an osteopontin mimic of (b);
   (e) DNA encoding an enzyme of (c); and
   (f) a mimic of a cellular component with which osteopontin must interact to function, wherein the mimic (i) interacts with osteopontin and (ii) prevents osteopontin from interacting with the cellular component.

12. The method of claim 11 wherein the individual is a human.

13. A pharmaceutical composition for use in augmenting an individual’s immune response, comprising:
   (a) a first component selected from the group consisting of:
       (1) osteopontin protein;
       (2) an active osteopontin fragment;
       (3) an osteopontin derivative;
       (4) an osteopontin analogue; and
       (5) DNA encoding one of (1) through (4); and
   (b) a physiologically appropriate carrier.

14. A pharmaceutical composition for use in reducing an individual’s immune response, comprising:
   (a) a first component selected from the group consisting of:
       (1) an antibody or other moiety which binds osteopontin activity;
       (2) an osteopontin mimic which (i) interacts with a cellular component with which osteopontin must interact to function and
          (ii) prevents osteopontin from interacting with the cellular component;
(3) DNA encoding the mimic of (2); and
(4) a mimic of a cellular component with which osteopontin must interact to function, wherein the mimic (i) interacts with osteopontin and (ii) prevents osteopontin from interacting with the cellular component;
(5) an enzyme which degrades osteopontin; and
(6) DNA encoding the enzyme of (b); and
(b) a physiologically appropriate carrier.

15. Use of osteopontin or an active portion thereof; an osteopontin derivative; an osteopontin analogue; DNA encoding osteopontin or an active portion thereof; a DNA construct comprising DNA encoding osteopontin or an active portion thereof; cells modified to contain DNA encoding osteopontin or an active portion thereof, wherein the DNA is expressed in the cells; or an osteopontin mimic in a method of augmenting the immune response in a mammal, wherein the method comprises increasing osteopontin activity in the mammal, whereby immune system effector function are activated in the mammal, thereby enhancing the immune response.

16. The use of Claim 15 wherein the mammal is a human.

17. The use of Claim 15 wherein the immune response augmented is to a pathogen selected from the group consisting of: bacteria, mycobacteria, viruses, protozoa and parasites.

18. Use of: (a) an antibody which binds osteopontin and prevents it from functioning; (b) an osteopontin mimic which (i) interacts with a cellular component with which osteopontin must interact to function and (ii) prevents osteopontin from interacting with the cellular components; (c) an enzyme which degrades osteopontin; (d) DNA encoding an osteopontin mimic of (b); (e) DNA encoding an enzyme of (c); (f) a mimic of a cellular component with which osteopontin must interact to function, wherein the mimic (i) interacts with osteopontin and (ii) prevents osteopontin from interacting with the cellular component, in a method of reducing the immune response in a mammal, wherein
the method comprises decreasing osteopontin activity in the mammal, thereby reducing the immune response.
BCG-Infected Macrophages

Harvest mRNA

Synthesize Lambda Phage cDNA Library

Plate Phage

Make Replica Filters

Hybridize Probes to Replica Filters

Identify Plaques with Different Signal Strengths

BCG-Infected E. coli-Infected Macrophages

Harvest mRNA

Synthesize cDNA Probes

FIGURE 1
FIGURE 2
FIGURE 3A  Granuloma Burden

FIGURE 3B  Granuloma Number

FIGURE 3C  Granuloma Size
FIGURE 5
A. Thioglycollate-elicited

B. BCG-elicited

FIGURE 6A

FIGURE 6B
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/19 A61K39/395 A61K48/00

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)

IPC 6 A61K C07K

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 database and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>ROLLO E E ET AL: &quot;Differential effects of osteopontin on the cytotoxic activity of macrophages from young and old mice.&quot; IMMUNOLOGY, (1996 AUG) 88 (4) 642-7, XP002077096 see the whole document</td>
<td>1-3, 13-18</td>
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<td>EP 0 705 842 A (HOECHST AKTIENGESELLSCHAFT) 10 April 1996 see the whole document</td>
<td>1-18</td>
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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

1. Special categories of cited documents:
   - "A" document defining the general state of the art which is not considered to be of particular relevance
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   - "O" document referring to an oral disclosure, use, exhibition or other means
   - "P" document published prior to the international filing date but later than the priority date claimed
   - "X" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
   - "Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
   - "YP" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
   - "S" document member of the same patent family

Date of the actual completion of the international search: 11 September 1998

Date of mailing of the international search report: 25/09/1998

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040, Tx: 31 651 apo nl, Fax: (+31-70) 340-3016

Authorized officer

Moreau, J
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
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</table>
| P,X      | NAUG J: "Osteopontin in chronic inflammation and host resistance."
          | MEETING ON CYTOKINE AND CHEMOKINE SIGNALING IN LEUKOCYTE DEVELOPMENT AND
          | FUNCTION HELD AT THE THIRTY-SECOND NATIONAL MEETING OF THE SOCIETY FOR
          | 0 (SUPPL.), 17, XP002077040
          | see abstract 62
| P,X      | WO 98 07750 A (CHILDREN'S MEDICAL CENTER CORPORATION) 26 February 1998
          | see the whole document

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INTERNATIONAL SEARCH REPORT

Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.; because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.; because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.; because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invoice payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  

Remark on Protest  ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.
<table>
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