(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 1 142 472 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
20.08.2008 Bulletin 2008/34

(21) Application number: **00900372.4**

(22) Date of filing: **13.01.2000**

(51) Int Cl.:
A01K 67/027 *(2006.01)*

(86) International application number:
PCT/JP2000/000132

(87) International publication number:
WO 2000/041561 (20.07.2000 Gazette 2000/29)

(54) **BACTERIAL CELL COMPONENT-UNRESPONSIVE MODEL MOUSE**

MODELLMAUS, DIE NICHT AUF BAKTERIELLE ZELLKOMPONENTEN ANSPRICHT.

MODELE MURIN NE REPONDANT PAS AUX COMPOSANTS CELLULAIRES BACTERIENS

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **14.01.1999 JP 736599**
12.08.1999 JP 22828299
29.10.1999 JP 30923899

(43) Date of publication of application:
10.10.2001 Bulletin 2001/41

(60) Divisional application:
05077734.1 / 1 716 752

(73) Proprietor: **Japan Science and Technology Agency**
Kawaguchi-shi
Saitama (JP)

(72) Inventors:
- **AKIRA, Shizuo**
  Mino-shi, Osaka 562-0031 (JP)
- **TAKEUCHI, Osamu**
  Mino-shi, Osaka 562-0031 (JP)
- **TAKEDA, Kiyoshi**
  Mino-shi, Osaka 562-0031 (JP)

(74) Representative: **Holliday, Louise Caroline**
D Young & Co
120 Holborn
London EC1N 2DY (GB)

(56) References cited:
- **TAKEUCHI O ET AL: "CELLULAR RESPONSES TO BACTERIAL CELL WALL COMPONENTS ARE MEDIATED THROUGH MYD88-DEPENDENT SIGNALING CASCADES" INTERNATIONAL IMMUNOLOGY, OXFORD UNIVERSITY PRESS, GB, vol. 12, no. 1, January 2000 (2000-01), pages 113-117, XP002945405 ISSN: 0953-8178**
- **OSAMU A. ET. AL. IMMUNITY vol. 9, July 1998, pages 143 - 150, XP002927802**
- **AKIRA S. JAPAN IMMUNOLOGY SOCIETY, PROCEEDINGS OF ACADEMIC MEETING vol. 28, 1998, page 3, XP002930213**
- **TAKO KAWAI, ET. AL. JAPAN IMMUNOLOGY SOCIETY, PROCEEDINGS OF ACADEMIC MEETING vol. 28, 1998, page 280, XP002930214**
- **Taro Kawai, et. al. Collection of programs and Extended Abstracts of lectures, 21st Annual Meeting, Japan Molecular Biology Society, (1998), Page 184 XP002930215**
- **TAKEUCHI O. ET. AL. IMMUNITY vol. 11, October 1999, pages 443 - 451, XP002927803**
- **KAWAI T. ET. AL. IMMUNITY vol. 11, July 1999, pages 115 - 122, XP002927804**
- **HARDIMAN G. ET. AL. GENOMICS vol. 45, 1997, pages 332 - 339, XP002927805**
- **YANG R-B. ET. AL. NATURE vol. 395, 17 September 1998, pages 284 - 288, XP002927806**
- **KIRSCHING C. J. ET. AL. J. EXP. MED. vol. 188, no. 11, 07 December 1998, pages 2091 - 2097, XP002927807**
- **GERARD C. ET. AL. NATURE vol. 395, 17 September 1998, pages 217 - 219, XP002927808**
- **MICHALEK S. M. ET. AL. JOURNAL OF INFECTION DISEASES vol. 141, 1989, pages 55 - 63, XP002927809**

Printed by Jouve, 75001 PARIS (FR)

(Cont. next page)

EP 1 142 472 B1

- **HARBOUR D. V. ET. AL. BRAIN, BEHAVIOR, AND IMMUNITY. vol. 1, 1987, pages 123 - 133, XP002927810**
- **HEINE H. ET AL: 'Cutting-edge: Cells that carry a null allele for Toll-like receptor 2 are capable of responding to endotoxin.' J. IMMUNOL. vol. 163, July 1999, pages 1 - 5**

Description

Technical Field

[0001] The present invention relates to uses of model non-human animals in which the TLR-2 gene has been knocked out.

Background Art

[0002] Cytokines are intracellular signal transmitters which play an important role in an immune response, a response upon infection, hematopoiesis, inhibition of virus infection and tumor cells. Among them, a cytokine which transmits signals between lymphocytes is called interleikin (hereinafter, "IL"). Among ILs, IL-1 is a cytokine which mediates various immune responses and inflammatory responses, and is involved in maintenance of homeostasis of living organisms and produced from various cells such as monocytes, macrophages, keratinocytes, vascular endothelial cells and the like when the living organisms get infected or hurt. It has been known that there are two kinds of IL-1, IL-1α and IL-1β, both of which combine to the same receptor. It has been also known that IL-1 exerts its function simultaneously with the activation by an antigen to T cell and by mitogens, makes T cells release IL-2, and enhances the expression of IL-2 receptors to induce T cell proliferation, and that it acts on monocytes and macrophages in order to induce the production of TNF-α, IL-1, IL-6.

[0003] IL-1 has two kinds of IL-1 receptors (hereinafter "IL-1R"), and both of the IL-1Rs, type I and type II, have three immunogloblin-like domains in their extracellular domains. Type I receptors express in T cells and connective tissue, and type II receptors express in splenic B cells, myeloids and the like, and it has been known that type I receptors induce NF-$_\kappa$B in nuclei. It has been also known that there is an IL-1 receptor antagonist (hereinafter "IL-1ra") which shows no bioactivity in spite that it binds to IL-1R with the affinity similar to that of IL-1α and IL-1β, and that it prevents IL-1 from binding to IL-1R competitively.

[0004] IL-18 is known to promote the production of interferon-γ (hereinafter "IFN-γ"), to enhance the activation of natural killer cells, to induce the production of IFN-y from T cells in cooperation of IL-12, and to act an important role in a Thl (IL-2 producting helper T cells) response. Further, it is known that IL-18 has no structural similarity to IL-12 in spite that it has a functional similarity, and has a structural similarity to IL-1. Moreover, it has been also known that IL-18 is produced as an inactive precursor that requires cleavage by IL-1β-converting enzyme (ICE) /caspasel for its maturation, as in the case of IL-1β, and that IL-18 activates IL-1R-associated kinase (IRAK) and NF-$_\kappa$B.

[0005] A plurality of molecules showing homology to IL-1R have been identified so far, and signal pathways mediated by IL-1R family is being studied intensively now. It has been known that MyD88 is a cytoplasmic protein comprised of an IL-1R homologous domain and a Death domain, and functions as an adaptor molecule which activates NF-$_\kappa$B by recruiting IRAK to IL-1R complex after IL-1 stimulation, and that MyD88 gene was originally separated as a myeloid differentiation primary response gene, which rapidly induces M1 myeloleukemic cells to macrophages by IL-6-stimulated differentiation.

[0006] Toxins in bacterial cells being comprised of lipopolysaccharide, which is a major structural component of the outer membrane encompassing peptidoglycan on the surface of Gram-negative bacteria, are called endotoxin, and it has been known that lipopolysaccharide is comprised of lipid called lipid A and various kinds of saccharide which covalently bind to the lipid A. It has been also known that this endotoxin has a bioactivity mainly involved in fever, decrease of leukocytes and platelet, hemorrhagic necrosis of bone marrow cells, hypoglycemia, induction of IFN, activation of B limphocyte (immune response cell derived from marrow), and the like.

[0007] It has been known that a Toll gene is required to control dorsoventral patterning during the embryonic development of Drosophila (Cell 52, 269-279,1988, Annu. Rev. Cell Dev. Biol. 12, 393-416, 1996), and for antifungal immune responses in adult fly (Cell 86, 973-983, 1996). It has been clarified that the Toll is a type I transmembrane receptor with an extracellular domain containing leucine-rich repeat (LRR) and that its cytoplasmic domain shows high homology to that of mammalian interleukin-1 recepter (IL-1R) (Nature 351, 355-356, 1991, Annu. Rev. Cell Dev. Biol. 12, 393-416, 1996, J. Leukoc. Biol. 63, 650-657, 1998). It has been also clarified that another Toll family member, 18-wheeler, participates in the antibacterial host defense but not in the antifungal immune response, and that particular pathogens induce specific antimicrobial immune responses in Drosophila through the selective activation of the Toll pathways (Proc. Natl. Acad. Sci. USA 94, 14614-14619, 1997, EMBO J. 16, 6120-6130, 1997, Curr. Opin. Immunol. 11, 13-18, 1999).

[0008] Recently, mammalian homologs of Toll, designated as Toll-like receptors (TLRs), have been identified, and so far, six families including TLR2 and TLR4 have been reported (Nature 388, 394-397, 1997, Proc. Natl. Acad. Sci. USA 95, 588-593, 1998, Blood 91, 4020-4027, 1998, Gene 231, 59-65, 1999). It has been known that the TLR families, as in the case of the IL-1R, recruit IL-1R-associated kinase (IRAK) through the adaptor protein MyD88 as a signal transmitter and activate TRAF 6, and then activate NF-$_\kappa$B in the downstream (J. Exp. Med. 187, 2097-2101, 1998, Mol. Cell 2, 253-258, 1998, Immunity 11, 115-122, 1999). Further, the role of the TLR families in mammals is also believed to participate in innate immune recognition as pattern recognition receptors (PRRs), which recognize bacterial cell common structures (Cell 91, 295-298, 1997).

[0009] It has been reported that one of such pathogen-associated molecular patterns (PAMPs) to be recognized

by the PRRs is lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria (Cell 91, 295-298, 1997), that said LPS stimulates host cells and makes them produce various proinflammatory cytokines including TNF-α, IL-1, and IL-6 (Adv. Immunol. 28, 293-450, 1979, Annu. Rev. Immunol. 13, 437-457, 1995), and that the LPS captured by LPS-binding protein (LBP) is delivered to CD14 on the cell surface (Science 249, 1431-1433, 1990, Annu. Rev. Immunol. 13, 437-457, 1995). However, since CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein without a transmembrane domain, the existence of a bona fide signaling receptor of LPS has been believed.

[0010] TLR4, which belongs to the TLR family, is a signaling molecule of LPS, which is a bacterial cell component of Gram-negative bacteria, and transfection of the TLR4 leads to a low constitutive activation of NF-$_{\kappa}$B (J. Exp. Med. 188, 2091-2097, 1998, Nature 395, 284-288, 1998). On the other hand, as TLR2 transmits LPS signal when overexpressed in human embryonic kidney 293 cells in vitro, TLR2 has been thought to be a candidate for the LPS receptor. In addition, Godawski's group has reported that human TLR2 could interact with CD14 to form the LPS receptor complex (J. Immunol. 163, 639-643, 1999). Stimulation treatment with LPS leads to oligomerization of receptors and to subsequent recruitment of IRAK to the receptor complex. In contrast, groups of Poltorak and Qureshi have reported that TLR4 is the causative gene of the LPS hyporesponsiveness of C3H/HeJ mice, that is, the Lps gene, according to positional cloning (Science 282, 2085-2088, 1998, J. Exp. Med. 189, 615-625, 1999).

[0011] Through generation of TLR4-deficient mice it has been discovered that TLR4 is actually involved in LPS signaling (J. Immunol. 162, 3749-3752, 1999). The findings may be attributed to species-specific differences in the primary structure of TLR, in other words, LPS signaling could be mediated by TLR4 in mice and by TLR2 in humans. However, there is a report showing that mouse TLR2 also activated NF-$_{\kappa}$B in response to LPS (J. Immunol. 162, 6971-6975, 1999). In addition, Chow et al. have reported that they obtained the result showing that human TLR4 activated NF-$_{\kappa}$B-mediated gene expression by stimulation to LPS/CD14 in a dose-dependent or a time-dependent manner, which is consistent with the observation of C3H/HeJ mice, whereas they obtained the result conflicting with that of Kirschning's group when human 293 cells were used, and they have speculated that the differences of outcome may be due to differences in the lot of 293 cells as well (J. Biol. Chem. 274, 10689-10692, 1999).

[0012] Recently, it has been reported that TLR2 may not be involved exclusively in responsiveness to LPS derived from Gram-negative bacteria (J. Immunol. 162, 6971-6975, 1999) but may also act as a signaling receptor for peptidoglycan (PGN) and lipoteichoic acid (LTA). from Gram-positive bacteria , which have another common bacterial structural pattern (J. Biol. Chem. 274, 17406-17409, 1999, J. Immunol. 163, 1-5, 1999). Further, it has been also reported that whole Gram-positive bacteria, soluble PGN, and LTA induced the activation of NF-$_{\kappa}$B in 293 cells expressing TLR2, but not induced the activation of NF-$_{\kappa}$B in the cells expressing TLR1 or TLR4 (J. Biol. Chem. 274, 17406-17409, 1999). Still further, it has been also reported that Chinese hamster ovary (CHO) fibroblast cells which express human TLR2 but not TLR4 were activated similarly by heat-killed Staphylococcus aureus and Streptococcus pneumoniae, and PGN derived from Staphylococcus aureus (J. Immunol. 163, 1-5, 1999).

[0013] Mycoplasmas, known as pathogens in animals and humans, are wall-less bacteria, yet they are capable of activating macrophages. A number of reports have identified this macrophage-activating material as lipoproteins/lipopeptides, and one of these lipopeptides, the 2kD macrophage-activating lipopeptide MALP-2 derived from Mycoplasma fermentans, was biochemically fully characterized and has become available by synthesis (J. Exp. Med. 185:1951, 1997). It is known that the lipid moiety has 2 asymmetric C atoms, and that the synthetic MALP-2 comprised of the S, R racemate had a specific activation similar to the natural compound action at picomolar concentrations in vitro. Little is known about the signal pathways or the cell-surface receptors for MALP-2, except that MALP-2 activates NF-$_{\kappa}$B.

[0014] It is reported that lipoproteins/lipopeptides from mycobacterium and Borrelia burgdorferi induced the activation of host cells through TLR2 in vitro (Science 285, 736-739, 1999, Science 285, 732-736, 1999). Nevertheless, the conclusions obtained from overexpression experiments do not necessarily reflect the function of TLR family in vivo. It is also reported that the results of analysis of the responsiveness based on NF-$_{\kappa}$B activation are not related to biological responses mediated by these stimuli (Infect. Immun. 66, 1638-1647, 1998).

[0015] In addition, it is known that the function of a specific gene can be analyzed in individual level by using transgenic mice in which genes are artificially introduced and expressed, and gene-deficient mice generated by gene targeting in which specific genes on genomes are artificially transformed by homologous recombination with embryonic stem cells (hereinafter "ES cells"). In general, gene-deficient mice are called knockout mice.

Summary of the Invention

[0016] Though in vivo responses to bacterial cell components are expected to vary depending on the difference of expression levels of each TLR on the cell surface, the contribution of individual members of the TLR family to signaling by bacterial cell components' stimuli in vivo remains to be elucidated. An object of the present invention is to provide model non-human animals in which the TLR-2 gene has been knocked out to elucidate the action mechanisms of Mycoplasma lipoproteins or screen for suppressors or promoters of responsiveness to Myco-

plasma lipoproteins.

[0017] The inventors of the present invention have conducted intensive study for attaining the object. They generated TLR2 gene-deficient mice as follows: an exon region including a cytoplasmic region of TLR2 gene is replaced with the neomycin-resistant gene respectively by homologous recombination with plasmid vectors in ES cells and HSV-tk gene was induced into each C-terminal side respectively, and ES cell clones doubly resistant of G418 and gancyclovir were screened; the ES cell clones were microinjected into blastocysts of C57BL/6 mice; TLR2 knockout mice whose function of TLR2 is deficient on their chromosomes were born through the germline at the expected Mendelian ratios. Then the inventors have found that those TLR2 knockout mice are transgenic mice which grow healthy and show no obvious abnormalities until 20 weeks of age, and that those TLR2 knockout mice are unresponsive to peptidoglycan, which is a cell wall component of Gram-positive bacteria and to a lipoprotein/lipopeptides such as mycoplasma lipoprotein MALP-2.

[0018] The present invention relates to the use of a non-human animal in which the TLR-2 gene has been knocked out as a model to elucidate the action mechanisms of Mycoplasma lipoproteins (claim 1), the use of a non-human animal in which the TLR-2 gene has been knocked out for the screening of suppressors and promoters of the responsiveness to Mycoplasma lipoproteins (claim 2), a use according to claim 1 or 2, wherein the non-human animal is rodent (claim 3), a use according to claim 2, wherein the rodent is a mouse (claim 4), a use according any of claims 1 to 4, when the Mycoplasma lipoproteins are brought into contact with peritoneal macrophages of the non-human animals (claim 5), a use according to claim 5, in which the macrophages' production of TNF-α and NO_2 is measured (claim 6), and a use as above, in which the Mycoplasma lipoproteins are S-MALP-2 or R-MALP-2.

Fig. 1 is a graph showing gene maps of the TLR2 knockout mice and the wild-type mice of the present invention.

Fig. 2 is a graph showing survival indices of the TLR2 knockout mice and the wild-type mice of the present invention having an injection of LPS derived from Escherichia coli.

Fig. 3 is a graph showing lipid A- or LPS-induced production amount of IL-6, TNF-α or NO_2^- in the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention.

Fig. 4 is a graph showing the results of responsiveness of splenic B cells of the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to LPS derived from Salmonella minnesota Re-595.

Fig. 5 is a graph showing the results of responsiveness of peritoneal macrophages of the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention to cell wall fractions of Gram-positive bacteria.

Fig. 6 is a graph showing PGN- or LTA-induced production amount of IL-6, NO_2^- or TNF-α in the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention.

Fig. 7 is a graph showing the results of LPS- or PGN-stimulated in vitro kinase assay, Western blot analysis and electrophoretic mobility shift assay in the TLR2 knockout mice, the wild-type mice and the TLR4 knockout mice of the present invention.

Fig. 8 is a graph showing the results of responsiveness of peritoneal macrophages of CH3/HeJ mice to lipopeptide MALP-2.

Fig. 9 is a graph showing the results of responsiveness of human monocytes to lipopeptide MALP-2.

Fig. 10 is a graph showing the results of responsiveness of peritoneal macrophages of the TLR2 knockout mice, the wild-type mice, the TLR4 knockout mice and the MyD88 knockout mice of the present invention to lipopeptide MALP-2.

Fig. 11 is a graph showing the results of lipopeptide MALP-2-stimulated in vitro kinase assay, Western blot analysis and electrophoretic mobility shift assay in the TLR2 knockout mice, the wild-type mice, the TLR4 knockout mice and the MyD88 knockout mice of the present invention.

Best Mode for Carrying out the Invention

[0019] The bacterial cell components used in connection with the present invention are Micoplasma lipoproteins. Thereunder are also understood, for the purposes of the present invention (i) carriers carrying said Mycoplasma lipoproteins and (ii) bacterial cells comprising said Mycoplasma lipoproteins.

[0020] In the present invention, "knocking out" of TLR2 means that a part of or a whole of TLR2 gene on a chromosome is deficient and the function to express TLR2, which is expressed in wild-types, is lost. The non-human animal in which the TLR-2 gene has been knocked out may be a rodent such as a rat or mouse.

[0021] The term "a wild-type non-human animal" in connection with the present invention means a non-human animal being the same species of the non-human animal as the TLR-2 gene knockout. For example, in case of mice, it means TLR2-nondeficient type mice of same species among F2 mice generated at the expected Mendelian ratio. When the deficient type and the wild-type mice of these F2 mice, in particular, the wild-type littermates are used for experiments simultaneously, it becomes possible to conduct precise comparative experiments at individual level. With an example of knockout mice which have deficiency in TLR2, a generating method of the non-human animal whose function of TLR2 gene is deficient on its chromosome will now be explained.

[0022] The TLR2 gene can be cloned, for instance, by

amplifying a mouse genomic library by PCR with a probe derived from a mouse EST. By common DNA recombination techniques, parts of the TLR2 gene sequence (for instance, a part or the whole of the region encoding a cytoplasmic domain of the TLR2 gene product) are replaced with a polyA signal and a selectable marker gene like for instance the neomycin resistance gene. A targeting vector is then constructed by introducing into the 5' terminal side genes like the diphtheria toxin A fragment (DT-A) or the herpes simplex virus thymidine kinale (HSV-tk) genes. The resulting vector is linearized and introduced into embryonic stem cells (ES cells) by electroporation or other suitable method, which are then cultured and subjected to selective pressure with G418, Ganciclovir or other suitable antibiotics to select for ES cells having undergone homologous recombination. The preferred way to verify that homologous recombination has occurred is by Southern blot.

[0023] Chimeric mice can be obtained by microinjecting the recombined ES cells into blastocysts of mice, and putting the blastocysts back into the uteri of recipient mice. Under high chimeric ratio, there will be born many more male chimeras than female ones. In such case, heterologous recombinant mice (+/-: F1) are generated by intercrossing the chimeric mice with female wild-type mice, and the homologous recombinant mice [F2; wild-type mice (+/+), TLR2 knockout mice (-/-)] can be obtained by mating the heterologous recombinant male mice and female mice. All of these mice are generated at the expected Mendelian ratio. As the method of confirming whether MyD88 or TLR2 knockout mice of the present invention are born, for example, the method wherein RNA is isolated from peritoneal macrophages of mice obtained by the above-stated method, and is examined by Northern blot analysis or the like, and the method wherein the expression of TLR2 in the mice is examined by Western blot analysis or the like are exemplified.

[0024] The responsiveness of TLR2 knockout mice to cell wall fractions of bacteria can be assessed in several ways, for instance:

- by contacting in vitro macrophages from TLR2 knockout mice with a lipoprotein/lipopolypeptide, which is a bacterial cell component of bacterium selected from Mycoplasma, Spirochaeta, Escherichia and the likes, and then measuring the production of TNF-α or NO_2^- in the cells; or

- by exposing macrophages or splenic B cells of TLR2 knockout mice to cell wall fractions of Gram-positive bacteria or peptidoglycan (a cell wall component of Gram-positive bacteria), and then measuring the induction of TNF, proliferative response of splenocytes, or the expression MHC class II antigens on the surgace of splenic B cells.

[0025] TLR2 knockout mice are unresponsive to a lipoproteins/lipopeptides and to peptidoglycans, are hyporesponsive to Cell wall fractions of Gram-positive bacteria, and are responsive to LPS, LTA and IL-4. Therefore, TLR2 knockout mice can be used as models for elucidating the mechanisms of action of such materials.

[0026] Specifically, in connection with the present invention, a TLR2 knockout non-human animal is used to elucidete the action mechanisms of Mycoplasma lipoproteins.

[0027] The non-human animals in which the TLR2 gene has been knocked-out can be used for the screening of a suppressor or a promoter of responsiveness to Mycoplasma lipoproteins.

[0028] The screening method may comprise the following steps: (i) macrophages, splenocytes obtained from the TLR2 knockout non-human animal and a subject material are brought into contact in advance in vitro; (ii) the macrophages or the splenocytes are cultured in the presence of Mycoplasma lipoproteins; and (iii) the macrophage activity level or the splenocyte activity level of the macrophages or of the splenocytes is measured and assessed. Alternatively the screening method may comprise the following steps: (i) macrophages or splenocytes obtained from the TLR-2 knockout non-human animal and Mycoplasma lipoproteins are brought into contact in advance in vitro; (ii) the macrophages or the splenocytes are cultured in the presence of a subject material; and (iii) the macrophage activity level or the splenocyte activity level of the macrophages or of the splenocytes is measured and assessed.

[0029] Alternatively, the screening method may comprise the following steps: (i) a subject material is administered in advance to the TLR-2 knockout non-human animal; (ii) macrophages or splenocytes obtained from the non-human animal are cultured in the presence of Mycoplasma lipoproteins; and (iii) the macrophage activity level or the splenocyte activity level of the macrophages or of the splenocytes is measured and assessed. Alternatively, the screening method may comprise the following steps: (i) a subject material is administered in advance to the TLR-2 knockout non-human animal; (ii) the non-human animal is made to be infected with Mycoplasma; and (iii) the macrophage activity level or the splenocyte activity level of the macrophages or of the splenocytes obtained from the non-human animal is measured and assessed.

[0030] Alternatively the screening method may comprise the following steps: the TLR-2 knockout non-human animal is made to be infected with bacteria in advance; (ii) macrophages or splenocytes obtained from the non-human animal are cultured in the presence of a subject material; and (iii) the macrophage activity level or the splenocyte activity level of the macrophages or of the splenocytes is measured and assessed. Alternatively the screening method may comprise the following steps: (i) the TLR-2 knockout non-human animal is made to be infected with Mycoplasma in advance; (ii) a subject material is administered to the non-human animal; and (iii)

the macrophage activity level or the splenocyte activity level of the macrophages or of the splenocytes obtained from the non-human animal is measured and assessed.

[0031] Alternatively the screening method may comprise the following steps: (i) a subject material is administered in advance to the TLR-2 knockout non-human animal; (ii) the non-human animal is made to be infected with Mycoplasma, and (iii) the macrophage activity level or the splenocyte activity level in the non-human animal is measured and assessed. Alternatively, the screening method may comprise the following steps: (i) the TLR-2 knockout non-human animal is made to be infected with Mycoplasma in advance; (ii) a subject material is administered to the non-human animal; and (iii) the macrophage activity level or the splenocyte activity level in the non-human animal is measured and assessed.

[0032] As a method of measuring and assessing the macrophage activity level, the method of measuring and assessing the production amount of cytokine and/or nitrous ion in the macrophage is exemplified, and as a method of measuring and assessing the splenocyte activity level, a method of measuring and assessing the expression amount of MHC class II in the splenocyte is exemplified. In the measurement and the assessment of the macrophage activity level or the splenocyte activity level, it is preferable to assess the levels in comparison to the measured value of a wild type non-human animal, in particular, a littermate wild type non-human animal of the TLR-2 knockout non-human animal as control, because there will be no dispersion caused by individual differences. This can be applied to the assessment of bioactivity of various subject materials and elucidation of the action mechanism of Mycoplasma lipoprotein, using a TLR-2 knockout non-human animal.

[0033] Examples of a suppressor or a promoter, which is the object of the screening methods of the present invention, include a suppressor or a promoter of responsiveness to Mycoplasma lipoproteins other than the suppressor or the promoter of bacterial infection, or the agonist or the antagonist to TLR2.

[0034] The screening method of an agonist or an antagonist to TLR2 can be performed by the combined use of TLR-2 and TLR4 knockout mice. In other words, it is possible to conduct the screening of the agonist or the antagonist to TLR2 and/or TLR4 by administering a subject material to each of TLR2 and TLR4 knockout mice, and to wild-type mice if necessary, and by comparing and assessing the activity levels of macrophages or splenocytes derived from the TLR2 knockout mice and the TLR4 knockout mice.

[0035] The screening method may involve administration of a subject material to the TLR-2 knockout non-human animal and then assessment of the the bioactivity of the subject material. The bioactivity of the subject material may, for example, be its endotoxin activity, interleukin-1 activity and/or interleukin-18 activity.

[0036] The present invention will be explained more specifically with examples below, but the technological scope of the present invention is not limited to these examples.

Example 1 (Generation of TLR2 knockout mice)

[0037] TLR2 gene was screened from 129/SvJ mouse gnomic library (Stratagene) using a probe derived from a mouse EST clone (accession number D77677) similar to human TLR2 gene, and subcloned into pBluescript vector (Stratagene), then characterized by restriction enzyme mapping and DNA sequencing. A targeting vector was constructed by replacing a gene fragment at an exon region 1.3 kb containing cytoplasmic domain of TLR2 gene with pMC1-neo (Stratagene) having Poly A signal. The targeting vector was flanked by a 4.8 kb 5' genomic fragment and a 1.0 kb 3' fragment and contained an HSV-tk cassette at the 5' terminal. The targeting vector was linearized with SalI and electroporated into E14.1 embryonic stem cells (ES cells). 120 clones resistant to G418 and gancyclovir were screened for homologous recombination by PCR and 9 clones were confirmed by Southern blot analysis using the probe indicated in Figure 1A.

[0038] Chimeric mice were generated by microinjection of 3 targeted ES clones containing a homologously recombined mutant TLR2 allele into blastocysts of C57BL/6 mice. Male chimeric mice were bred to C57BL/6 females to produce heterozygous mice. The heterozygous mice were intercrossed to obtain homozygotes (Fig 1B). TLR2-deficient mice of the present invention could be generated at the expected Mendelian ratio, and did not show any obvious abnormality until 20 weeks.

[0039] To confirm that the homologous recombination caused inactivation of the TLR2 gene, total RNA (15 μg) was extracted from peritoneal macrophages (5 x 10^6) of wild-type (+/+) and TLR2 knockout (-/-) mice and then electrophoresed, transferred to a nylon membrane, and Northern blot analysis was conducted using cDNA specific to [^{32}P]-labelled TLR2, or cDNA specific to GAPDH (glycelaldehyde-3-phosphate dehydrogenase) as the method previously described (Immunity 9, 143-150, 1998). As a result, TLR2 mRNA was not detected in peritoneal macrophages of TLR2-deficient mice (Fig 1C). In addition, it was shown that the expressions of CD3, B220, CD4, and CD8 in thymocytes and splenocytes of TLR2 knockout mice were not different from those of wild-type mice (data not shown).

Example 2 (Responsiveness of TLR2 knockout mice to endotoxin)

[0040] 1 mg of LPS derived from Escherichia coli (O55:B5) was injected into each of TLR2 knockout mice (n=5), TLR4 knockout mice (n=5) and wild-type mice (n=5) of the present invention, and LPS unresponsiveness was examined by their survival rate. The results are shown in Fig. 2. Fig. 2 confirms that though TLR2 knockout mice (TLR2-/-) and wild-type mice responded to LPS and almost all of them died within 4 days after injection, none

of TLR4 knockout mice (TLR4-/-) died even after 6 days after injection, and that TLR4 knockout mice are unresponsive to endotoxin.

Example 3 (Responsiveness of TLR2 knockout mice to cell components of Gram-negative bacteria)

[0041] Each of TLR2 knockout (TLR2-/-), TLR4 knockout (TLR4-/-) and wild-type (wild-type) mice were intraperitoneally injected with 2 ml of 4% thioglycollate medium (DIFCO). Three days later, peritoneal exudate cells were isolated from the peritoneal cavity of each mouse. These cells were cultured in RPMI1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) for 2 hours at 37°C and washed with ice-cold Hank's buffered salt solution (HBSS; GIBCO) to remove nonadherent cells. Adherent cells were used as peritoneal macrophages for following experiments.

[0042] Each of obtained peritoneal macrophages were cultured for 24 hours with 1.0 ng/ml of synthetic lipid A derived from Escherichia coli (compound· 506; Daiichi Pure Chemicals) or LPS derived from Salmonella minnesota Re-595 (Sigma) in the presence or absence of IFN-γ(30 unit/ml). Synthetic lipid A, which was soluble in endotoxin-free water and containing 0.025% of triethylamine, was used as said synthetic lipid A. After the culture, production amounts of IL-6 (Fig. 3A), TNF-α (Fig. 3B) and NO_2^- (Fig. 3C) in culture supernatants were measured. Production amount of IL-6 was measured by enzyme-linked immunosorbent assay (ELISA; ENDOGEN), and that of TNF-α was measured by ELSIA, according to manufacturer(Genzyme)'s instructions, and that of NO_2^- was measured by the Greiss method using NO_2/NO_3 Assay Kit (Dojindo Laboratories).

[0043] These results indicate that macrophages of wild-type mice and TLR2 knockout mice showed similar responsiveness to LPS and lipid A, and produced IL-6 and TNF-α, and it was confirmed that production of TNF-α would be further increased when IFN-γ was added to LPS or lipid A before the culture. By contrast, macrophages of TLR4 knockout mice produced neither IL-6 nor TNF-α. Further, production of NO_2^- was confirmed by culturing macrophages of wild-type and TLR2 knockout mice with IFN-γ-added lipid A or LPS. The obtained results were same as those aforementioned even in the case the injection amount of lipid A or LPS was arranged to be 1 μg/ml (data not shown).

[0044] Next, each of peritoneal macrophages of wild-type, TLR2 knockout and TLR4 knockout mice were cultured in the presence of LPS derived from Salmonella minnesota Re-595 at various concentrations shown in Fig. 3D, and production of TNF-α was measured. The results indicate that macrophages of wild-type mice and TLR2 knockout mice showed similar tendency to increase in response to LPS in a dose-dependent manner, while macrophages of TLR4 knockout mice produced no TNF-α in response to any concentration of LPS.

Example 4 (Responsiveness to LPS of Salmonella minnesota Re-595)

[0045] Responsiveness of splenocytes of various mice (wild-type, TLR2-/- and TLR4-/-) to LPS of Salmonella minnesota Re-595 were examined. Splenocytes (1×10^5) of each mouse were isolated and then cultured and stimulated in 96-well plates with various concentrations of LPS shown in Fig. 4A. 1 μCi of [^{3}H]-thymidine (DuPont) was added 40 hours after the onset of the culture, and cells were further cultured for 8 hours, then [^{3}H] uptake was measured by a β scintillation counter (Packard) (Fig. 4A). As a result, the cell proliferative response was promoted in response to LPS in a dose-dependent manner in splenocytes of wild-type and TLR2 knockout mice as well. By contrast, whatever the concentration of LPS as a stimulus would be, no LPS-induced cell proliferative response was observed in splenocytes of TLR4-deficient mice.

[0046] In addition, the expression of major histocompatibility complex (MHC) class II (I-A^b) on the surface of B cells in response to Re-595 LPS was examined by flow cytometry. Splenic B cells (1×10^5) of each of wild-type, TLR2 knockout (2-/-) and TLR4 knockout (4-/-) mice were isolated and cultured for 48 hours in 96-well plates with various concentrations (0, 10^1, 10^2, 10^3, 10^4 or 10^5 ng/ml) of LPS or 100 U/ml of IL-4. After the culture, the cells were collected and stained by combining I-A^b molecule on the surface of the cells and FITC-labelled□antibody which is constructed by combining phycoerythrin (PE; PharMingen)-conjugated anti-B220 antibody or biotinylated anti-mouse I-A^b antibody (PharMingen) and fluorescein isocyanate (FITC; PharMingen)-conjugated streptavidin. The stained cells were analyzed on fluorescence-activated cell sorter Calibur (FACS Calibur) using CELLQuest software (Becton Dickinson) (Fig. 4B). As a result, Re-595 LPS enhanced the expression of I-A^b molecule on the surface of splenic B cells of wild-type and TLR2 knockout mice. In contrast, I-A^b molecule expression in splenic B cells of TLR4-deficient mice were not enhanced by Re-595 LPS, even when stimulated with high concentration of LPS (10^5 ng/ml). The above-mentioned results indicate that TLR2 knockout mice show responsiveness to LPS as wild-type mice did. When stimulated with IL-4, each knockout mice show normal expression of I-A^b molecule on the surface of splenic B cells.

Example 5 (Unresponsiveness of macrophages of TLR2 knockout mice to cell wall components derived from Gram-positive bacteria)

[0047] Responsiveness of each peritoneal macrophages of said wild-type (wild-type), TLR2 knockout (TLR2-/-), TLR4 knockout (TLR4-/-) mice and the like to cell wall components derived from Gram-positive bacteria were examined with prepared cell wall specimens of S. aureus, C. diphtheriae and N. coeliaca. The cell specimens were prepared in accordance with the method pre-

viously described (Biken J. 18, 77-92, 1975, Infect. Immun. 38, 817-824, 1982), that is, bacterial cells grown under appropriate cultural conditions were disrupted with either a Braun mechanical cell homogenizer (model MSK; B. Braun Apparatebau) or a Dyno-Mill (type KDL; Willy A, Biochofen Manufactureing Engineers). A crude cell wall fraction obtained by differential centrifugation of a disrupted cell suspension was purified and prepared by removal of components noninherent in cell walls with protease treatment.

[0048] Peritoneal macrophages of each mouse were cultured for 24 hours in the presence of various concentrations (0, 0.1, 1, 10 or 100 μg/ml) of said preparations and stimulated, then concentration of tumor necrosis factor (TNF-α) released from each macrophage was measured by ELISA (Fig. 5). By these results, it has been found that production of TNF-α in response to cell wall components derived from Gram-positive bacteria was more impaired in macrophages of TLR2 knockout mice than in those of wild-type and TLR4 knockout mice.

Example 6 (Responsiveness of TLR2 knockout mice to cell wall components of Gram-positive bacteria)

[0049] Next, it was investigated that which cell wall component of Gram-positive bacteria activated macrophages via TLR2. As it has been reported that both peptidoglycan, which is a cell wall component of Gram-positive bacteria, and lipoteichoic acid (LTA) activate monocytes / macrophages (Infect. Immun. 60, 3664-3672, 1992, Immunity 1, 509-516, 1994, J. Biol. Chem. 271, 23310-23316, 1996, Infect. Immun. 64, 1906-1912, 1996), production amounts of IL-6 and NO_2^- in response to peritoneal macrophages of various kinds of mouse were measured in accordance with the same method as in example 8, with 10 μg/ml of Staphylococcus aureus PGN (Fluka; Fig. 6A) or 10 μg/ml of Staphylococcus aureus LTA (Sigma; Fig. 6C). Further, production of TNF-α in peritoneal macrophages of various kinds of mouse in response to PGN (Fig. 6B) or LTA (Fig. 6D) were measured in accordance with the same method as in example 10.

[0050] The results shown in Fig. 6A indicates that: peritoneal macrophages of wild-type and TLR4 knockout mice produced IL-6 in response to PGN, in contrast, those of TLR2 knockout mice produced no IL-6; NO_2^- was produced when peritoneal macrophages of wild-type and TLR4 knockout mice were cultured with PGN in the presence of IFN-γ, in contrast, no NO_2^- was produced when those of TLR2 knockout mice were used; IL-6 was produced in peritoneal macrophages of wild-type and TLR2 knockout mice in response to LTA, in contrast, no IL-6 was produced in those of TLR4 knockout mice (Fig. 6C); NO_2^- was produced when peritoneal macrophages of wild-type and TLR2 knockout mice were cultured with LTA in the presence of IFN-γ, in contrast, no NO_2^- was produced when those of TLR4 knockout mice were used (Fig. 6C).

[0051] As shown in Fig. 6B, peritoneal macrophages of TLR4 knockout mice, as well as those of wild-type mice, increased production of TNF-α in response to PGN in a dose-dependent manner, in contrast, those of TLR2 knockout mice showed substantial impairment in production of TNF-α, indicating that TLR2 knockout mice were unresponsive to PGN. As shown in Fig. 6D, peritoneal macrophages of TLR2 knockout mice, as well as those of wild-type mice, induced production of TNF-α in response to LTA in a dose-dependent manner, in contrast, no TNF-α was produced in those of TLR4 knockout mice, indicating that TLR4 knockout mice were unresponsive to LTA. These results demonstrate that PGN, which is a cell wall component of Gram-positive bacteria, activates macrophages via TLR2, and that LTA activates macrophages via TLR4.

Example 7 (LPS or PGN-stimulated in vitro kinase assay and Western blot analysis.

[0052] TLR family members are known as intracellular signaling molecules which activate a serine-threonine kisase IRAK via an adapter protein MyD88, and subsequently activate rel-type transcription factor, NF-$_\kappa$B (Mol. Cell 2, 253-258, 1998, J. Exp. Med. 187, 2097-2101, 1998, Immunity 11, 115-122, 1999). Whether LPS and PGN activate the intracellular signaling molecules was examined as follows: peritoneal macrophages (1×10^6) of various kinds of mouse were stimulated with 1 ng/ml of LPS of Salmonella minnesota Re-595 or 1 μg/ml of PGN of Staphylococcus aureus for the period indicated in Fig. 21; these bacterial cell components were lysed in lysis buffer (buffer containing Triton X-100 at a final concentration of 1.0 %, 137 mM of NaCl, 20 mM of Tris-HCl, 5 mM of EDTA, 10 % of glycerol, 1 mM of PMSF, 20 μg/ml of Aprotinin, 20 μg/ml of Leupeptin, 1 mM of Na_3VO_4, and 10 mM of β-glycerophosphate; pH 8.0): the cells were immunoprecipitated with anti-IRAK antibody (Hayashibara Biochemical Laboratories, Inc.); in vitro kinase assay were conducted as previously described (Biochem. Biophys. Res. Commun. 234, 183-196, 1998, Immunity 11, 115-122, 1999); autophosphorylation of IRAK were measured (Auto shown in Fig. 7A and B).

[0053] The lysates were dissolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blotted with anti-IRAK antibody (Transduction Laboratories) and visualized by using the enhanced chemiluminescence system (DuPont) (WB in Fig. 7A and B). These results show that IRAK activation in response to LPS was observed in wild-type (wild-type) and TLR2 knockout (TLR2-/-) mice, but not observed in TLR4 knockout (TLR4-/-) mice. In contrast, IRAK activation in response to PGN was observed only in wild-type and TLR4 knockout mice. Thus indicates that LPS is recognized via TLR4, and that PGN is recognized via TLR2 respectively.

[0054] NF-$_\kappa$B activation in response to LPS or PGN was also investigated. Macrophages of various kinds of

mouse were stimulated with said LPS or PGN, then nuclear extracts from the macrophages were purified and incubated with a probe specific to DNA binding site of NF-$_\kappa$B, and visualized by electrophoretic mobility shift assay as described previously (Immunity 9, 143-150, 1998). The results are shown in Fig. 7C and D. Arrows in Fig. 7C and D indicate the position of a complex comprised of NF-$_\kappa$B and the specific probe, and arrowheads indicate the position of specific probe only. As a result, DNA binding activity of NF-$_\kappa$B in response to LPS was detected in nuclear extracts from macrophages of wild-type and TLR2 knockout mice, but not in those of TLR4 knockout mice. In contrast, NF-$_\kappa$B activation in response to PGN was observed in macrophages of wild-type and TLR4 knockout mice but not in those of TLR2 knockout mice. Thus indicates that TLR4 is essential for LPS-induced NF-$_\kappa$B activation, and that TLR2 is essential for PGN-induced NF-$_\kappa$B activation.

Example 8 (Stereospecific lipopeptide synthesis and HPLC purification of R- and S-MALP-2).

[0055] The stereoisomers of S-(2,3-dihydroxypropyl)-L-cystein were synthesized as described previously (Int. J. Peptide Protein. Res. 38, 545, 1991) using two reagents, (S)-(-)-glycidol and (R)-(+)-glycidol (Sigma-Aldrich), which contain enantiomers purified to 99 % or over respectively, as starting materials. The isomers of N_α-fluorenylmethoxycarbonyl-protected S-[2(S), 3-bis (palmitoyloxy)propyl]-L-cystein and S-[2(R), 3-bis(palmitoyloxy) propyl]-L-cystein were synthesized respectively from these steroisomers and coupled according to the previously described method, and a carrier-bound fluorenylmethoxycarbonyl-protected peptide was obtained. 10 mg of crude MALP-2 was further purified in batch treatment by reversed phase HPLC using SP 250/10 Nucleosil 300-7 C8 column (Macherey & Nagel) and was eluted at 40°C with a linear gradient of water/2-propanol containing 0.1% trifluoroacetic acid. Elution of active material was monitored by the NO release assay. The final product was characterized by mass spectroscopy and amino acid analysis for determination of the exact peptide content. MALP-2 was prepared to be a concentration of 1 mg/ml using a solution of water/2-propanol 1:1 (v/v) and stored at 4°C.

Example 9 (Responsiveness of peritoneal macrophages of CH3/Hej mice to lipoprotein/lipopeptide).

[0056] PEC (peritoneal exudate cells) were isolated from endotoxin-hyporesponsive mice derived from CH3/HeJ, and these PEC (6 x 10^5) were cultured overnight at 37°C in 24-well cell culture plates having 1.25 ml of Dulbecco MEM medium (DMEM) which contained 5 % of FCS and 25 μM of 2-mercaptoethanol. Peritoneal macrophages were prepared by removing nonadherent cells from the cultured material and exchanging the culture liquid for fresh one. The peritoneal macrophages were cultured in the presence of both various concentrations (0.1, 1, 10, 10^2, 10^3, 10^4, 10^5 or 10^6 pg/ml) of R-MALP-2 or S-MALP-2 obtained by the method described in example 8 and recombined interferon-γ (rIFN-γ) at a concentration of 30 unit/ml, and production amounts of NO_2^-, TNF-α and IL-6 in the culture supernatants were measured (Fig. 8). TNF-α was measured by ELISA (Genzyme) at 3 hours after the onset of the culture, IL-6 was measured by ELISA (ENDOGEN) at 21 hours after the onset of the culture, and NO_2^- was measured by Greiss method using NO_2/NO_3 assay kit (Dojindo Laboratories) at 46 hours after the onset of the culture. These results indicate that R-MALP-2 shows higher specific activity to peritoneal macrophages than S-MALP-2.

Example 10 (Responsivenss of human monocytes to a lipoprotein/lipopeptide) .

[0057] Human monocytes from healthy volunteers were washed and used for experiments. With various concentrations (0.1, 1, 10, 10^2, 10^3, 10^4, or 10^5 pg/ml) of R-MALP-2 or S-MALP-2 obtained by the method in example 8, human monocytes (7.5 x 10^5) were stimulated for 20 hours. After stimulation, production amounts of IL-8, MCP-1 and TNF-α were measured by ELISA (Fig. 9). The results indicate that R-MALP-2 shows higher specific activity to human monocytes which have not yet differentiated to macrophages or the like than S-MALP-2 as shown in macrophages derived from the mice in example 9.

Example 11 (Unresponsiveness of TLR2 knockout mice to a lipoprotein/lipopeptide)

[0058] Responsiveness of each peritoneal macrophage of wild-type (wild-type), TLR2 knockout (TLR2-/-), TLR4 knockout (TLR4-/-), and MyD88 knockout (MyD88-/-) mice to a lipoprotein/lipopeptide was examined with MALP-2 derived from mycoplasma. Peritoneal macrophages of each mouse were isolated by the method described in example 9, and each of peritoneal macrophages were cultured for 24 hours with various concentrations (0, 0.1, 1, 10, 10^2, 10^3, or 10^4 pg/ml) of R-MALP-2 or S-MALP-2 obtained in example 8, in the presence (Fig. 10B and D) or absence (Fig. 10A and C) of rINFγ (30 unit/ml). After the culture, production amounts of TNF-α and NO_2^- in the culture supernatants were measured (Fig. 10).

[0059] The results indicate that production of TNF-α and NO_2^- increased in response to R-MALP-2 in a dose-dependent manner in peritoneal macrophages of wild-type and TLR4-deficient mice, whereas neithor TNF-α nor NO_2^- was produced in those of TLR2- and MyD88-deficient mice (Fig. 10A and B). Similar results were obtained with S-MALP-2 as well (Fig. 10C and D). Further, it has been confirmed that peritoneal macrophages of TLR2- and MyD88-deficient mice were unresponsive to R-MALP-2- or S-MALP-2-stimulated IL-6 production (da-

ta not shown). Thus indicates that a lipoprotein/lipopeptide derived from mycoplasma, such as R-MALP-2 or the like, activates macrophages via TLR2 and MyD88.

Example 12 (lipoprotein/lipopeptide-stimulated in vitro kinase assay and Western blot analysis).

[0060] In order to examine whether a lipoprotein/lipopeptide activates intracellular signaling molecules with the results of example 11, peritoneal macrophages of the 4 kinds of mouse (1×10^6) were stimulated with 0.3 ng/ml of R-MALP-2 for 10 minutes, and in vitro kinase assay (Auto in Fig. 11A), Western blot analysis (WB in Fig. 11A), electrophoretic mobility shift assay (Fig. 11B) were conducted with anti-IRAK antibody as in example 12. In addition, in vitro kinase assay (Auto in Fig. 11C) and Western blot analysis (WB in Fig. 11C) with anti-JNK1 antibody were also conducted. As a result, activation of IRAK, NF-$_\kappa$B and JNK in response to MALP could not confirmed in macrophages of TLR2 and MyD88 knockout mice. These results indicate that the lipoprotein/lipopeptide derived from mycoplasma causes vital reaction via TLR2 and MyD88 signaling pathway.

Industrial Applicability

[0061] A TLR2 knockout mouse is unresponsive to peptidoglycan which is a cell wall component of Gram-positive bacteria, a lipoprotein/lipopeptide, and hyporesponsive to cell wall fractions of Gram-positive bacteria. Therefore, by using these knockout mice, it becomes possible to obtain useful information of signaling receptors of selective components such as Mycoplasma lipoproteins. It is also possible to conduct screenings of a promoter or a suppressor of responsiveness to Mycoplasma lipoproteins (such as an agonist or an antagonist to TLR2).

Claims

1. The use of a non-human animal in which the TLR-2 gene has been knocked out as a model to elucidate the action mechanisms of Mycoplasma lipoproteins.
2. The use of a non-human animal in which the TLR-2 gene has been knocked out for the screening of supressors and promoters of the responsiveness to Mycoplasma lipoproteins.
3. A use according to claim 1 or 2, wherein the non-human animal is a rodent.
4. A use according to claim 2, wherein the rodent is a mouse.
5. A use according to any of claims 1 to 4, wherein the Mycoplasma lipoproteins are brought into contact with peritoneal macrophages of the non-human animals.
6. A use according to claim 5, in which the macrophages' production of TNF-α and NO_2^- is measured.
7. A use according to any of the preceding claims, wherein The Mycoplasma lipoprotein is R-MALP-2 or S-MALP-2.

Patentansprüche

1. Verwendung eines nicht-menschlichen Tieres, bei dem das TLR-2-Gen ausgeschaltet wurde, als ein Modell zur Aufklärung der Wirkmechanismen von Mycoplasma-Lipoproteinen.
2. Verwendung eines nicht-menschlichen Tieres, bei dem das TLR-2-Gen ausgeschaltet wurde, für das Screening nach Suppressoren und Promotoren der Responsivität auf Mycoplasma-Lipoproteine.
3. Verwendung nach Anspruch 1 oder 2, wobei das nicht-menschliche Tier ein Nagetier ist.
4. Verwendung nach Anspruch 2, wobei das Nagetier eine Maus ist.
5. Verwendung nach einem der Ansprüche 1 bis 4, wobei die Mycoplasma-Lipoproteine mit peritonealen Makrophagen der nicht-menschlichen Tiere in Kontakt gebracht werden.
6. Verwendung nach Anspruch 5, bei dem die Produktion von TNF-α und NO_2^- durch die Makrophagen gemessen wird.
7. Verwendung nach einem der vorangehenden Ansprüche, wobei das Mycoplasma-Lipoprotein R-MALP-2 oder S-MALP-2 ist.

Revendications

1. Utilisation d'un animal non humain dans lequel le gène TLR-2 a été inactivé comme modèle pour élucider les mécanismes d'action de lipoprotéines de Mycoplasma.
2. Utilisation d'un animal non humain dans lequel le gène TLR-2 a été inactivé pour la sélection de suppresseurs et de promoteurs de la capacité de réponse aux lipoprotéines de Mycoplasma.
3. Utilisation suivant la revendication 1 ou 2, dans laquelle l'animal non humain est un rongeur.

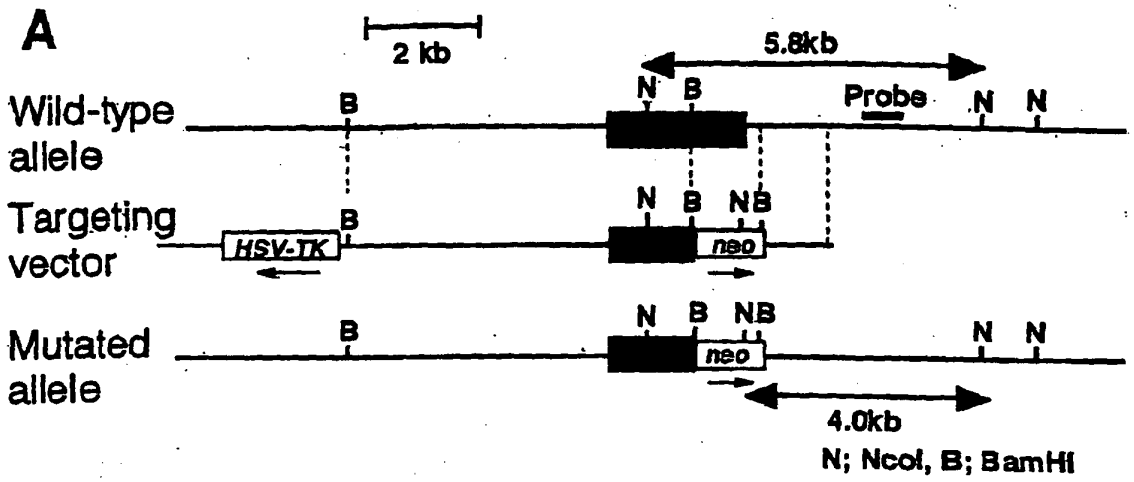
4. Utilisation suivant la revendication 2, dans laquelle le rongeur est une souris.

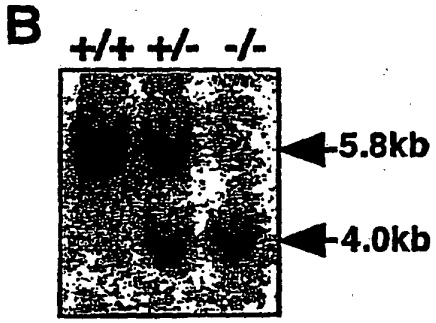
5. Utilisation suivant l'une quelconque des revendications 1 à 4, dans laquelle les lipoprotéines de Mycoplasma sont mises en contact avec des macrophages péritonéaux des animaux non humains.

6. Utilisation suivant la revendication 5, dans laquelle la production par les macrophages de TNF-α et NO_2^- est mesurée.

7. Utilisation suivant l'une quelconque des revendications précédentes, dans laquelle la lipoprotéine de Mycoplasma est R-MALP-2 ou S-MALP-2.

FIG. 1


A
2 kb
5.8kb
Wild-type allele
Targeting vector
Mutated allele
HSV-TK
neo
Probe
N; NcoI, B; BamHI
4.0kb


B
+/+ +/- -/-
5.8kb
4.0kb

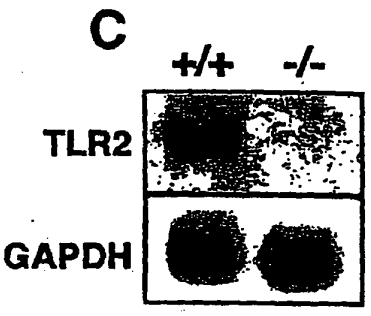

C
+/+ -/-
TLR2
GAPDH

FIG. 2

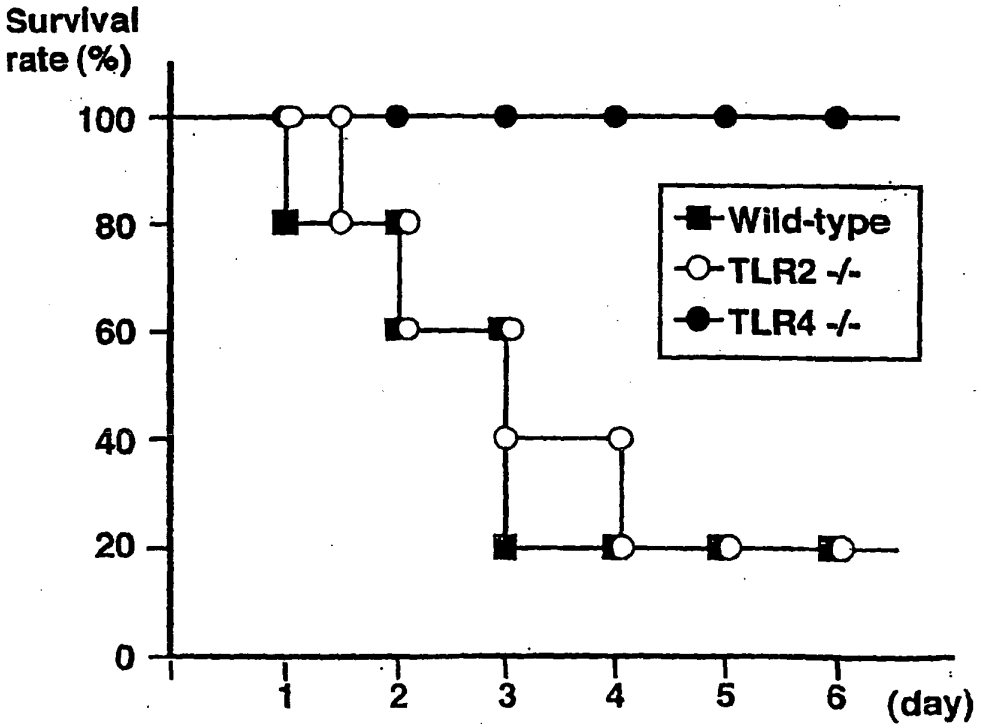

Survival rate (%)
100
80
60
40
20
0
1
2
3
4
5
6
(day)
Wild-type
TLR2 -/-
TLR4 -/-

FIG. 3

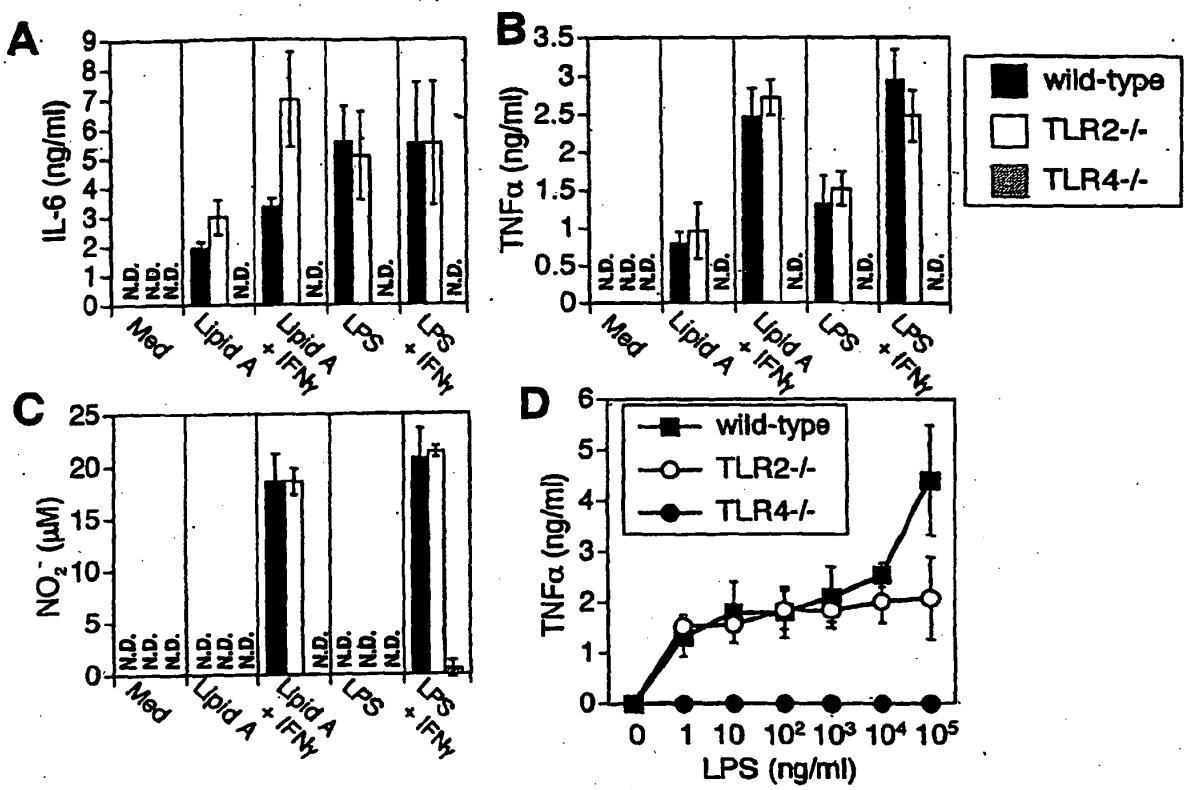

A
IL-6 (ng/ml)
N.D.
Med
Lipid A
Lipid A + IFNγ
LPS
LPS + IFNγ
B
TNFα (ng/ml)
wild-type
TLR2-/-
TLR4-/-
C
NO2- (μM)
D
TNFα (ng/ml)
wild-type
TLR2-/-
TLR4-/-
0 1 10 10² 10³ 10⁴ 10⁵
LPS (ng/ml)

FIG. 4

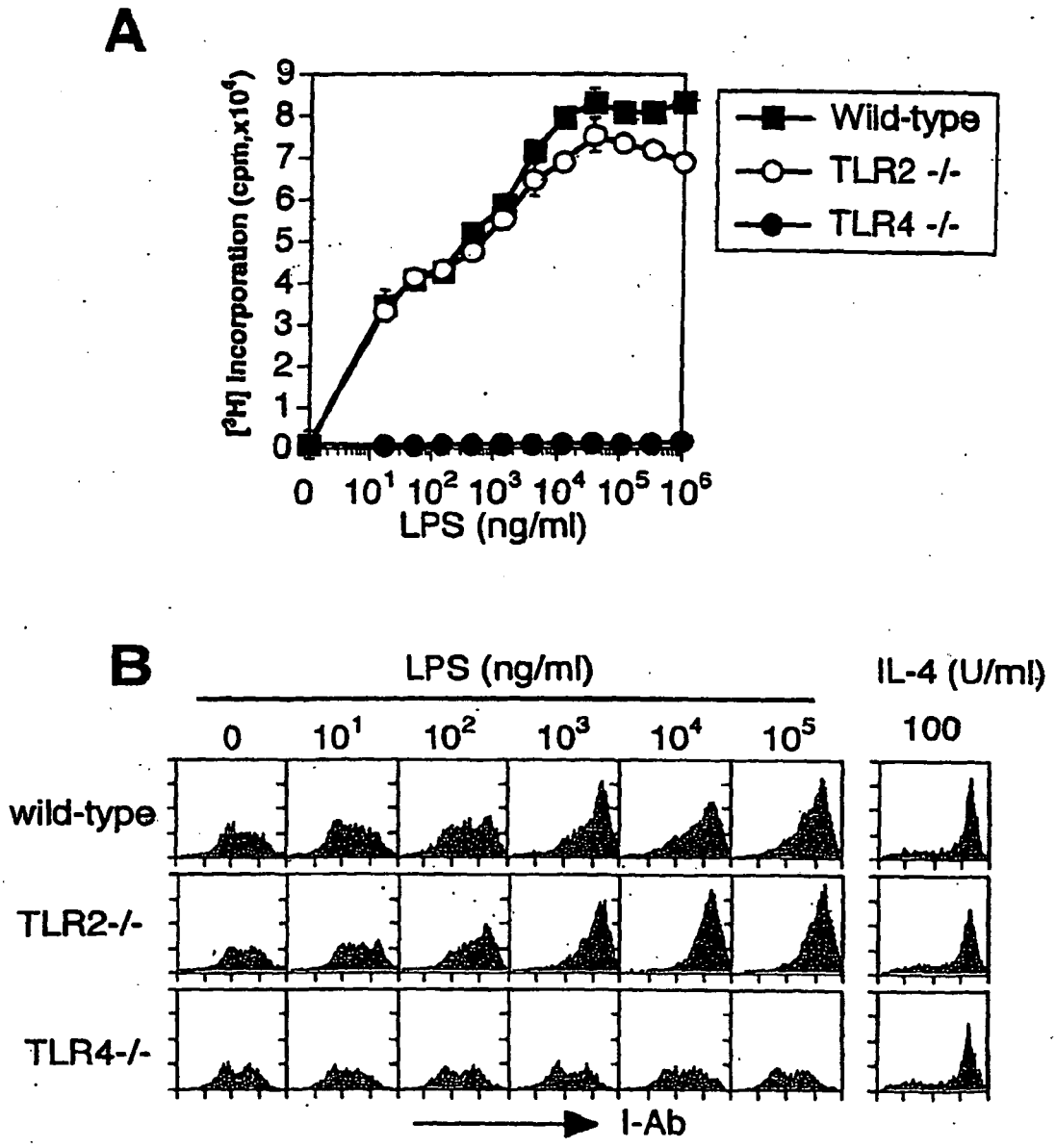

A
[3H] Incorporation (cpm,x10^4)
0
1
2
3
4
5
6
7
8
9
0 10^1 10^2 10^3 10^4 10^5 10^6
LPS (ng/ml)
Wild-type
TLR2 -/-
TLR4 -/-
B
LPS (ng/ml)
IL-4 (U/ml)
0 10^1 10^2 10^3 10^4 10^5
100
wild-type
TLR2-/-
TLR4-/-
I-Ab

FIG. 5

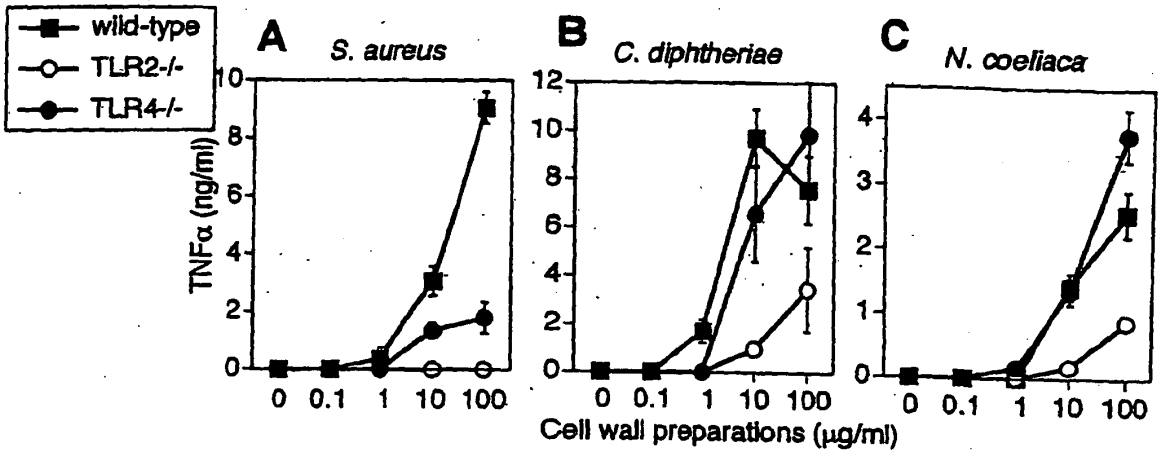

wild-type
TLR2-/-
TLR4-/-
A
S. aureus
B
C. diphtheriae
C
N. coeliaca
TNFα (ng/ml)
0 0.1 1 10 100
Cell wall preparations (μg/ml)

FIG. 6

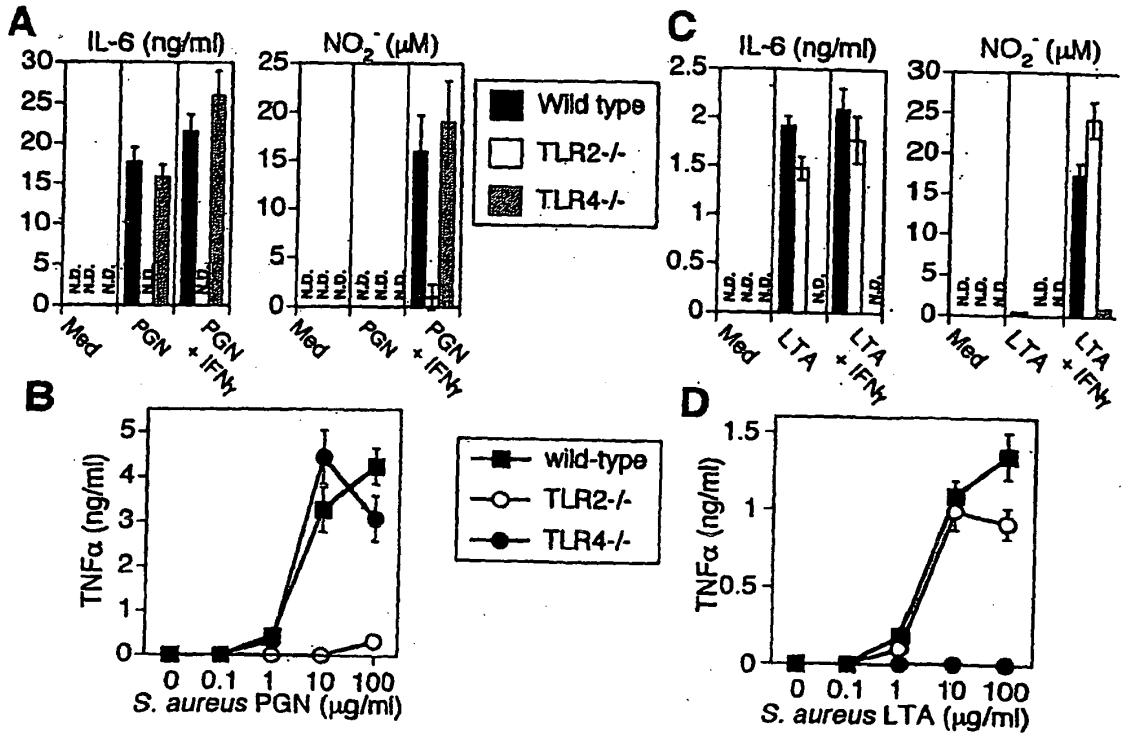

A
IL-6 (ng/ml)
NO2- (μM)
Med
PGN
PGN + IFNγ
Wild type
TLR2-/-
TLR4-/-
C
IL-6 (ng/ml)
NO2- (μM)
Med
LTA
LTA + IFNγ
B
wild-type
TLR2-/-
TLR4-/-
TNFα (ng/ml)
S. aureus PGN (μg/ml)
D
TNFα (ng/ml)
S. aureus LTA (μg/ml)

FIG. 7

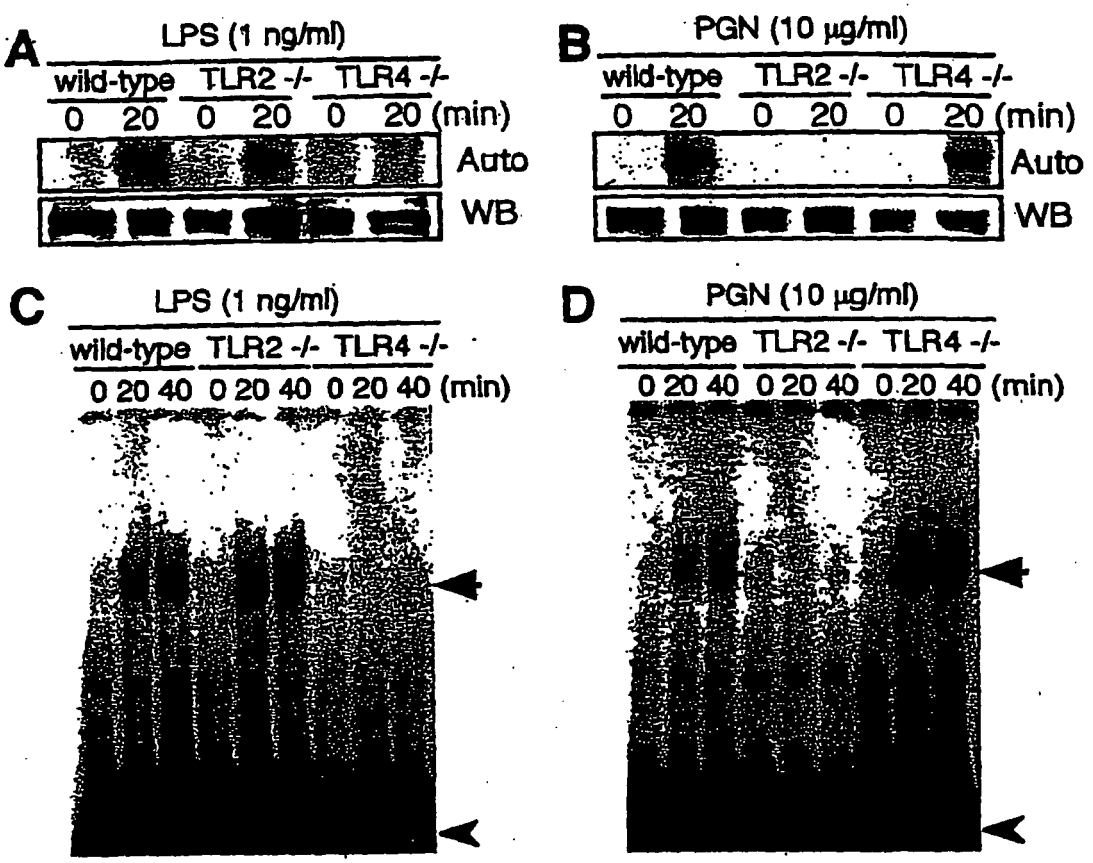

A
LPS (1 ng/ml)
wild-type
TLR2 -/-
TLR4 -/-
0 20 0 20 0 20 (min)
Auto
WB
B
PGN (10 μg/ml)
wild-type
TLR2 -/-
TLR4 -/-
0 20 0 20 0 20 (min)
Auto
WB
C
LPS (1 ng/ml)
wild-type
TLR2 -/-
TLR4 -/-
0 20 40 0 20 40 0 20 40 (min)
D
PGN (10 μg/ml)
wild-type
TLR2 -/-
TLR4 -/-
0 20 40 0 20 40 0 20 40 (min)

FIG. 8

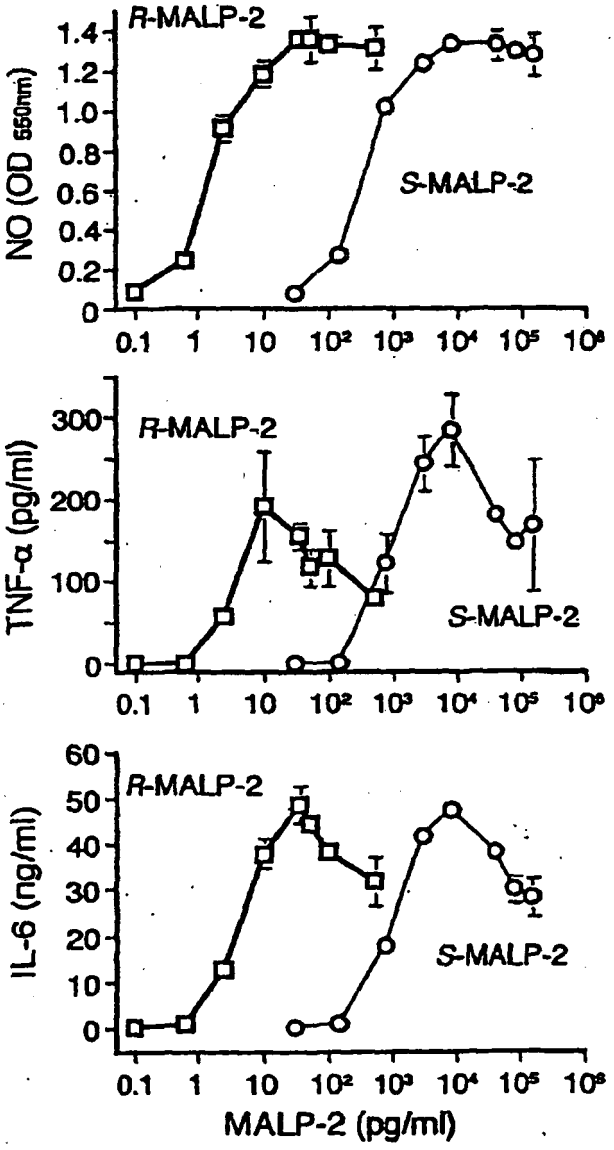

R-MALP-2
S-MALP-2
NO (OD 550nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
TNF-α (pg/ml)
300
200
100
IL-6 (ng/ml)
60
50
40
30
20
10
0.1
1
10
10²
10³
10⁴
10⁵
10⁶
MALP-2 (pg/ml)

FIG. 9

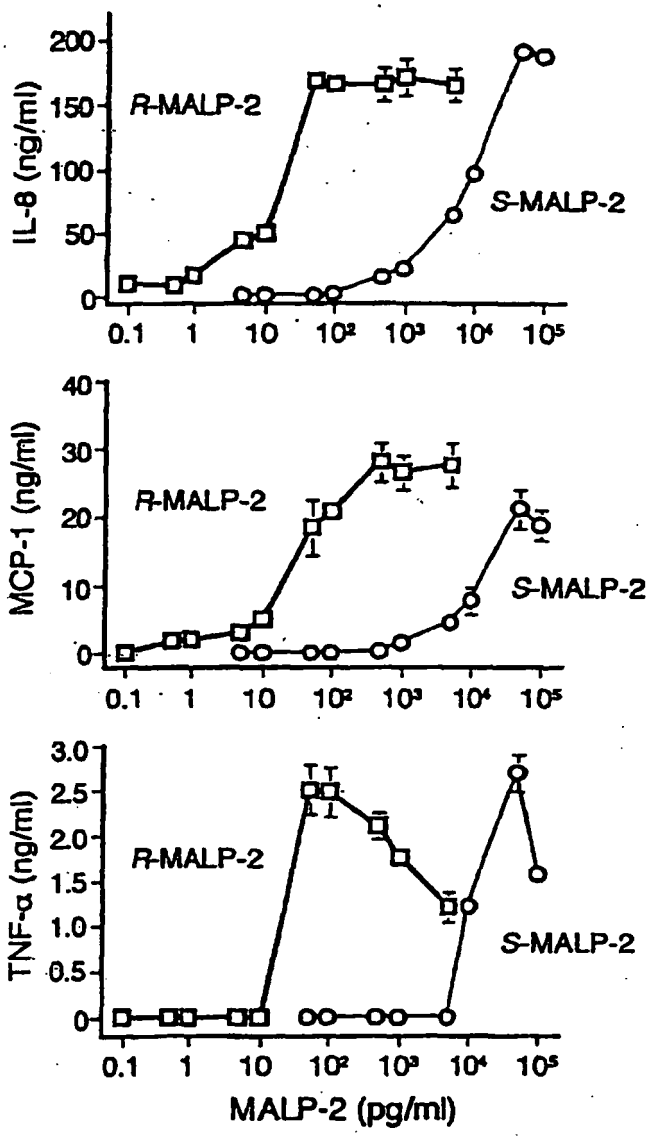

IL-8 (ng/ml)
200
150
100
50
0
R-MALP-2
S-MALP-2
0.1
1
10
10²
10³
10⁴
10⁵
MCP-1 (ng/ml)
40
30
20
10
0
R-MALP-2
S-MALP-2
TNF-α (ng/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0
R-MALP-2
S-MALP-2
MALP-2 (pg/ml)

FIG. 10

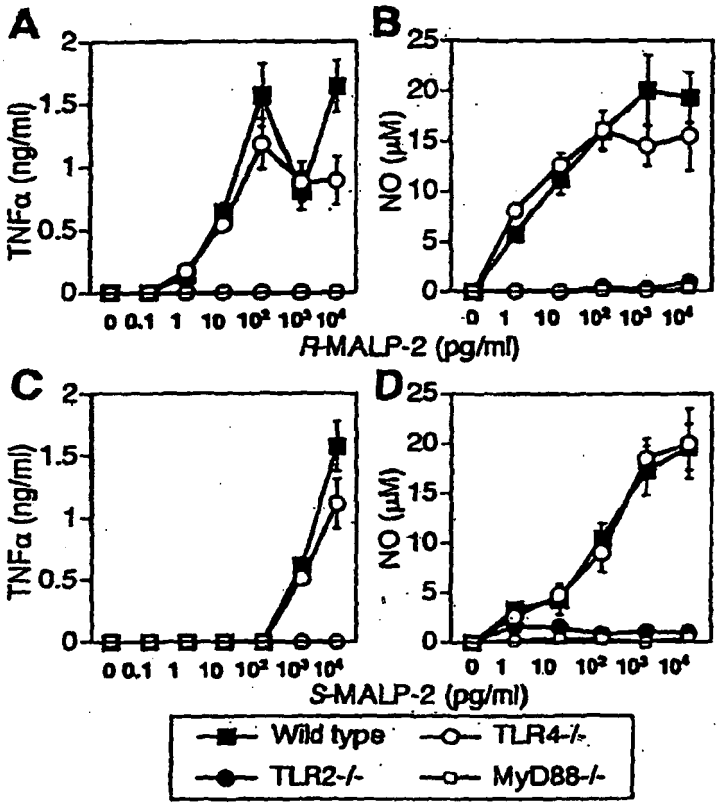
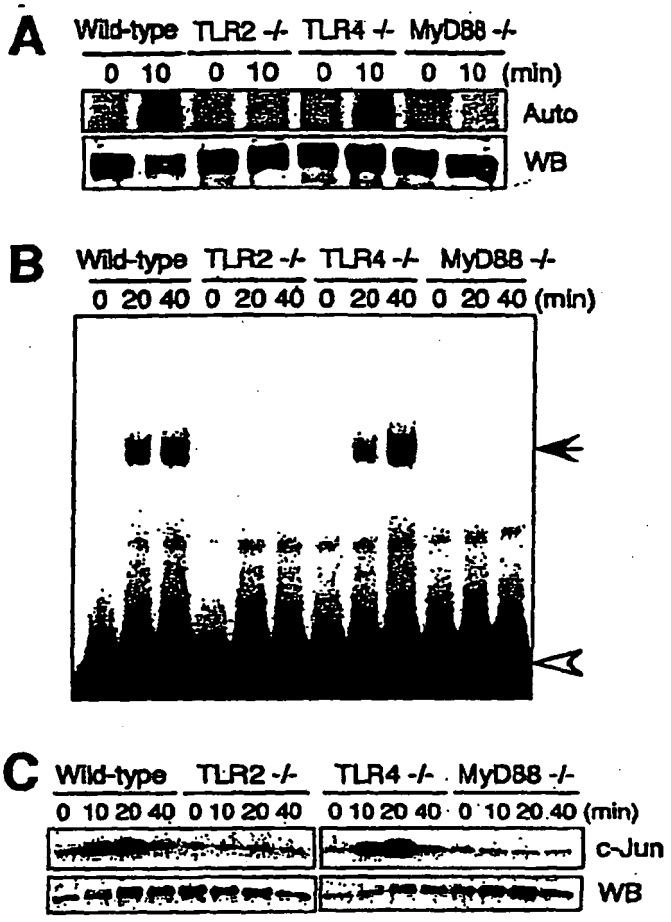

A
TNFα (ng/ml)
2
1.5
1
0.5
0
0 0.1 1 10 10² 10³ 10⁴
B
NO (μM)
25
20
15
10
5
0
0 1 10 10² 10³ 10⁴
R-MALP-2 (pg/ml)
C
TNFα (ng/ml)
2
1.5
1
0.5
0
0 0.1 1 10 10² 10³ 10⁴
D
NO (μM)
25
20
15
10
5
0
0 1 10 10² 10³ 10⁴
S-MALP-2 (pg/ml)
Wild type
TLR4-/-
TLR2-/-
MyD88-/-

FIG. 11


A
Wild-type
TLR2 -/-
TLR4 -/-
MyD88 -/-
0 10 0 10 0 10 0 10 (min)
Auto
WB
B
Wild-type
TLR2 -/-
TLR4 -/-
MyD88 -/-
0 20 40 0 20 40 0 20 40 0 20 40 (min)
C
Wild-type
TLR2 -/-
TLR4 -/-
MyD88 -/-
0 10 20 40 0 10 20 40 0 10 20 40 0 10 20 40 (min)
c-Jun
WB

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Non-patent literature cited in the description

- *Cell,* 1988, vol. 52, 269-279 **[0007]**
- *Annu. Rev. Cell Dev. Biol.,* 1996, vol. 12, 393-416 **[0007] [0007]**
- *Cell,* 1996, vol. 86, 973-983 **[0007]**
- *Nature,* 1991, vol. 351, 355-356 **[0007]**
- *J. Leukoc. Biol.,* 1998, vol. 63, 650-657 **[0007]**
- *Proc. Natl. Acad. Sci. USA,* 1997, vol. 94, 14614-14619 **[0007]**
- *EMBO J.,* 1997, vol. 16, 6120-6130 **[0007]**
- *Curr. Opin. Immunol.,* 1999, vol. 11, 13-18 **[0007]**
- *Nature,* 1997, vol. 388, 394-397 **[0008]**
- *Proc. Natl. Acad. Sci. USA,* 1998, vol. 95, 588-593 **[0008]**
- *Blood,* 1998, vol. 91, 4020-4027 **[0008]**
- *Gene,* 1999, vol. 231, 59-65 **[0008]**
- *J. Exp. Med.,* 1998, vol. 187, 2097-2101 **[0008] [0052]**
- *Mol. Cell,* 1998, vol. 2, 253-258 **[0008] [0052]**
- *Immunity,* 1999, vol. 11, 115-122 **[0008] [0052] [0052]**
- *Cell,* 1997, vol. 91, 295-298 **[0008] [0009]**
- *Adv. Immunol.,* 1979, vol. 28, 293-450 **[0009]**
- *Annu. Rev. Immunol.,* 1995, vol. 13, 437-457 **[0009] [0009]**
- *Science,* 1990, vol. 249, 1431-1433 **[0009]**
- *J. Exp. Med.,* 1998, vol. 188, 2091-2097 **[0010]**
- *Nature,* 1998, vol. 395, 284-288 **[0010]**
- *J. Immunol.,* 1999, vol. 163, 639-643 **[0010]**
- *Science,* 1998, vol. 282, 2085-2088 **[0010]**
- *J. Exp. Med.,* 1999, vol. 189, 615-625 **[0010]**
- *J. Immunol.,* 1999, vol. 162, 3749-3752 **[0011]**
- *J. Immunol.,* 1999, vol. 162, 6971-6975 **[0011] [0012]**
- *J. Biol. Chem.,* 1999, vol. 274, 10689-10692 **[0011]**
- *J. Biol. Chem.,* 1999, vol. 274, 17406-17409 **[0012] [0012]**
- *J. Immunol.,* 1999, vol. 163, 1-5 **[0012] [0012]**
- *J. Exp. Med.,* 1997, vol. 185, 1951 **[0013]**
- *Science,* 1999, vol. 285, 736-739 **[0014]**
- *Science,* 1999, vol. 285, 732-736 **[0014]**
- *Infect. Immun.,* 1998, vol. 66, 1638-1647 **[0014]**
- *Immunity,* 1998, vol. 9, 143-150 **[0039] [0054]**
- *Biken J.,* 1975, vol. 18, 77-92 **[0047]**
- *Infect. Immun.,* 1982, vol. 38, 817-824 **[0047]**
- *Infect. Immun.,* 1992, vol. 60, 3664-3672 **[0049]**
- *Immunity,* 1994, vol. 1, 509-516 **[0049]**
- *J. Biol. Chem.,* 1996, vol. 271, 23310-23316 **[0049]**
- *Infect. Immun.,* 1996, vol. 64, 1906-1912 **[0049]**
- *Biochem. Biophys. Res. Commun.,* 1998, vol. 234, 183-196 **[0052]**
- *Int. J. Peptide Protein. Res.,* 1991, vol. 38, 545 **[0055]**