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(54) Title: A METHOD OF TRANSFECTION AND COMPOSITIONS THEREFOR

(57) Abstract: The present invention relates to the targeted delivery of molecules to cells expressing toll-like receptors (TLRs). Aspects of the invention provide compounds comprising a positively charged group linked to a TLR ligand. These compounds are useful for *in vitro* and *in vivo* methods of transfection of TLR-expressing cells. Other aspects of the invention relate to the use of such compounds for repression of gene expression and DNA vaccination approaches.

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## A METHOD OF TRANSFECTION AND COMPOSITIONS THEREFOR

### FIELD OF THE INVENTION

5 The present invention relates generally to the field of cellular biology. More particularly, the present invention is directed to a method of transfecting cells, in particular dendritic cells, and to a method of eliciting an immune response. To this end, the present invention provides compounds comprising at least one positively charged group, capable of binding nucleic acids, linked to a moiety capable of interacting with one or more members of the  
10 toll-like receptor (TLR) family.

### BACKGROUND TO THE INVENTION

As used herein the term "derived from" shall be taken to indicate that a specified integer  
15 may be obtained from a particular source albeit not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but  
20 not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also  
25 includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific examples described  
30 herein. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

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All the references cited in this application are specifically incorporated by reference herein.

The present invention is performed without undue experimentation using, unless otherwise  
5 indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, peptide synthesis in solution, solid phase peptide synthesis, and immunology. Such procedures are described, for example, in the following texts that are incorporated by reference:

- 10 1. Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, New York, Second Edition (1989), whole of Vols I, II, and III;
2. *DNA Cloning: A Practical Approach*, Vols. I and II (D. N. Glover, ed., 1985), IRL Press, Oxford, whole of text;
- 15 3. *Oligonucleotide Synthesis: A Practical Approach* (M. J. Gait, ed., 1984) IRL Press, Oxford, whole of text, and particularly the papers therein by Gait, pp1-22; Atkinson et al., pp35-81; Sproat et al., pp 83-115; and Wu et al., pp 135-151;

Reference to any prior art in this specification is not, and should not be taken as, an  
20 acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia.

Delivery of molecules such as lipids, proteins, peptides, DNA, polysaccharides and/or  
combinations thereof (eg. lipopolysaccharides, lipoproteins), into cells is useful for a  
25 multitude of research and clinical purposes. For example, in order for researchers to study intracellular processes such as gene regulation and expression, DNA-protein interactions or protein-protein interactions, and so on, it is often essential to introduce molecules into cells, and desirable to do so with as high efficiency as possible. Currently researchers deliver molecules into cells, i.e. transfect cells, by a variety of means with variable  
30 efficiency. The efficiency of transfection of cells is dependent on a number of factors

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including cell type, rate and stage of cell division, and the individual properties of both the molecules to be transfected and the transfection reagent(s).

DNA vaccines are thought to elicit an immune response via uptake of DNA by antigen-presenting cells such as dendritic cells (DCs), which subsequently express the antigen  
5 encoded by the internalised DNA and present the antigen to the immune system as peptides in the context of MHC molecules. In small animal models, administration of DNA has been successful in inducing protective immune responses, but only low efficacies have been reported in human clinical trials, often requiring high doses of DNA to induce  
10 immune responses (Kutzler, M.A. & Weiner, D.B. 2004 *J Clin Invest*, 114(9), 1241-1244). Adenoviruses and retroviruses have been used as vectors for gene delivery, however concerns exist in relation to the safety of these vectors for human use (Buckley, R.H. 2002 *Lancet*, 360(9341), 1185-1186). To date, transfection of DCs with relatively safe, non-viral vectors has proven difficult.

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DNA is a net negatively charged molecule. More specifically, the phosphate groups within the backbone of DNA are negatively charged. Therefore, cationic molecules, which have a net positive charge, can adsorb DNA via electrostatic interaction, and are potential carriers for DNA. Such cationic molecules include microparticles (Minigo, G. et al. 2007 *Vaccine*,  
20 25(7), 1316-1327; Mollenkopf, H.J. et al. 2004 *Vaccine*, 22(21-22), 2690-2695), peptides (Gratton, J.P. et al. 2003 *Nat Med*, 9(3), 357-362; Riedl, P. et al. 2006 *Methods Mol Med*, 127, 159-169), or liposomes (Jiao, X. et al. 2003 *Hepatology*, 37(2), 452-460; Ewert, K. et al. 2002 *J Med Chem*, 45(23), 5023-5029).

25 Simply carrying DNA to antigen-presenting cells, however, is not sufficient enough to result in transfection, and in order to drive an antigen specific response, there must also be uptake of the DNA. Antigen-presenting cells of the immune system express toll-like receptors (TLRs) on their cell surface, which bind to a variety of ligands, largely derived from microorganisms. For example, TLR-2 is known to bind bacterial lipoproteins, TLR-4  
30 is known to bind bacterial lipopolysaccharides, TLR-6, in association with TLR-1, is known to bind diacylated bacterial lipids, and TLR-9 binds to CpG DNA. Dendritic cell

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subsets have been shown to express no fewer than nine such TLRs. Engagement of one or more TLRs on the surface of DCs induces cell signalling pathways, which can lead to the maturation and activation of DCs, which is required for the induction of protective immunity.

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The lipid moiety, dipalmitoyl-*S*-glyceryl cysteine (Pam<sub>2</sub>Cys), is a synthetic analogue of a bacterial lipoprotein known as MALP-2, derived from the cytoplasmic membrane of *Mycoplasma fermentans*. Pam<sub>2</sub>Cys is a ligand for both TLR-2 and TLR-6 (Okusawa, T. *et al.*, *Infect Immun* 2004, 72(3), 1657-1665). Vaccines comprising Pam<sub>2</sub>Cys coupled to peptide epitopes can induce strong humoral and cellular responses. Engagement of TLR-2 by Pam<sub>2</sub>Cys coupled to peptide epitopes results in DC maturation, activation of transcription factors such as NF- $\kappa$ B, secretion of pro-inflammatory cytokines and eventual migration of DCs to the draining lymph nodes to activate epitope-specific naïve T cells (Jackson, D.C. *et al.* 2004 *Proc Natl Acad Sci U S A*, 101(43), 15440-15445; Zeng, W. *et al.* 2002 *J Immunol*, 169(9), 4905-4912; Chua, B.Y. *et al.* 2007 *Vaccine*, 25(1), 92-101).

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## SUMMARY OF THE INVENTION

In work leading up to the present invention, the inventors sought to produce an efficient means of transfecting cells with nucleic acids. It was also sought to produce a DNA vaccine candidate targeting antigen-presenting cells, in particular dendritic cells, to elicit both humoral and cellular immune responses in a subject.

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In a first aspect the present invention provides a compound comprising a positively charged group linked to at least one TLR ligand.

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In a second aspect the present invention provides a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group.

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- 5 -

In a third aspect the present invention provides a method of transfection comprising contacting a cell expressing a TLR with a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction  
5 between the nucleic acid and the positively charged group.

In a fourth aspect the present invention provides a method of raising an immune response against an antigen, comprising administering to a subject a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one TLR  
10 ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group, and wherein the nucleic acid encodes the antigen or an epitope thereof.

In a fifth aspect the present invention provides a method of raising an immune response against an antigen, comprising administering to a subject cells transfected with a complex comprising nucleic acid and a compound comprising a positively charged group linked to  
15 at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group, and wherein the nucleic acid encodes the antigen or an epitope thereof.

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In a sixth aspect the present invention also provides a method of repressing expression of a gene in a cell expressing a TLR, comprising administering to a subject a complex comprising nucleic acid and a compound comprising a positively charged group linked to  
25 at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group, wherein the nucleic acid is selected from the group consisting of: siRNA; shRNA; DNA encoding siRNA; and DNA encoding shRNA; and is targeted against the gene.

Further provided are uses of a compound comprising a positively charged group linked to  
30 at least one TLR ligand in: the manufacture of a vaccine for the induction of an immune response in a subject; the manufacture of a medicament for the treatment of a subject with

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a genetic abnormality or deficiency; the manufacture of a medicament for the treatment of a subject suffering from aberrant or otherwise unwanted expression of a gene; and in the manufacture of a reagent for the transfection of cells.

## 5 BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** is a diagrammatic representation of the various synthesized fluorescened peptide constructs. Abbreviations; R, arginine; K, lysine; L, linear; FL, fluorescein or carboxylfluorescein.

10

**Figure 2** is a photographic representation of an agarose gel analysis of R<sub>4</sub> and DNA interactions. Varying amounts (10, 1, 0.5 or 0.1 μg) of R<sub>4</sub> were incubated with 1 μg of either (A) pEGFP-N1 plasmid or (B) pCI-HA plasmid for 30 mins at 37°C. Samples were then ran on a 1% agarose gel containing SYBR-Safe DNA gel stain so that DNA could be visualised under UV light.

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**Figure 3** shows absorbance readings at 260nm of solutions containing R<sub>4</sub> and DNA. Increasing amounts of R<sub>4</sub> were incubated with 10 μg of pCI-HA plasmid for 30 mins at 37°C in a total volume of 100 μl of water. (A) Samples were centrifuged at 9300g to pellet any insoluble complexes and absorbance readings of the collected supernatants were measured at 260nm (light grey bars). Absorbance readings of wash supernatants after pelleted samples were resuspended with 100 μl of water were also measured (dark grey bars). (B) Supernatants and wash supernatants were also run on a 1% agarose gel containing SYBR-Safe DNA gel stain to detect the presence of DNA in these samples.

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**Figure 4** is a graphical representation of the transfection of D1 cells with pEGFP-N1 plasmid and upregulation of MHC Class II expression on D1 cells. D1 cells ( $4 \times 10^5$ ), derived from BALB/c splenocytes, were cultured at 37°C in an atmosphere of 5% CO<sub>2</sub> with 1 μg of pEGFP-N1 plasmid pre-incubated with 3 μg of R<sub>4</sub> or R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) for 30 mins at 37°C, or with a 3:1 ratio of FUGENE to plasmid DNA for 45 minutes at room temperature. After 2 days, cells were harvested and green fluorescence was determined by

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flow cytometry (A). Cells were also stained with fluorochrome conjugated anti-murine MHC Class II (IA/E) antibody before analysis by flow cytometry (B). Cells expressing high levels of MHC Class II are considered to be mature (shaded parts of the histogram) whereas those that express low levels are deemed immature (unshaded). The percentage of cells in this category is also indicated within each panel as an average and standard deviation obtained by analysing triplicate samples.

**Figure 5** is a graphical representation of transfection of D1 cells using different ratio mixtures of transfection reagent to DNA. D1 cells ( $4 \times 10^5$ ) derived from BALB/c splenocytes were cultured for 48 hours, at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , with  $1 \mu\text{g}$  of pCI-HA plasmid pre-incubated with different ratios of  $\text{R}_4$  or  $\text{R}_4(\text{S}_2\text{Pam}_2\text{Cys})$  for 30 mins at  $37^\circ\text{C}$ , or with different ratios of FUGENE for 45 minutes at room temperature. Cells were then harvested and stained with an anti-HA antibody (clone E2.6) that was detected with a FITC-conjugated goat anti-murine Ig antibody before analysis by flow cytometry.

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**Figure 6** is a graphical representation of transfection of human, monocyte-derived dendritic cells (MoDCs) with plasmids, pEGFP-N1 and pCI-HA. Monocyte-derived dendritic cells ( $2 \times 10^5$ ) were cultured at  $37^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  with  $1 \mu\text{g}$  of pEGFP-N1 plasmid pre-incubated with  $3 \mu\text{g}$  of  $\text{R}_4$  or  $\text{R}_4(\text{S}_2\text{Pam}_2\text{Cys})$  for 30 mins at  $37^\circ\text{C}$ , or with a 3:1 ratio of FUGENE to plasmid DNA for 45 minutes at room temperature. After 2 days, cells were harvested and green fluorescence protein in cells transfected with the pEGFP-N1 plasmid was determined by flow cytometry (A). For cells transfected with the pCI-HA plasmid, a fluorochrome conjugated anti-HA antibody was used to detect the surface expression of HA protein (B).

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**Figure 7** is a graphical representation of transfection levels of green fluorescent protein in two different cell lines using different ratio mixtures of transfection reagent to DNA. The human lung epithelial cell line A549 (A) or the murine lung alveolar macrophage cell line MH-S (B) were cultured at a concentration of  $2 \times 10^5$  cells/ml in the absence or presence of plasmid encoding for green fluorescent protein complexed with different dilutions of  $\text{R}_4$ ,

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R<sub>4</sub>Pam<sub>2</sub>Cys or FUGENE for 48 hours at 37°C and 5% CO<sub>2</sub>. Transfection efficiency was determined by measuring the fluorescence of cells on a flow cytometer.

5 **Figure 8** is a graphical representation of HA-specific antibody levels in unimmunised mice, and mice immunised with either complexes of R<sub>4</sub>Pam<sub>2</sub>Cys and DNA, or influenza virus. BALB/c mice (6-8 weeks old) were inoculated sub-cutaneously at the base of the tail on day 0 and 28 with 50µg of DNA plasmid comprising DNA encoding influenza hemagglutinin, either alone or complexed with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) at molar ratios of 1:3 or 1:5. As a positive control, mice were also immunised intra-nasally with split PR8 virus  
10 (3µg/mouse). Unimmunised mice served as negative controls. Sera were obtained from mice 14 days after the last inoculation and ELISA was performed to detect the presence of antibodies specific for influenza PR8 virus.

15 **Figure 9** is a graphical representation of the numbers of antigen-specific, IFN-γ producing CD8+ T cells induced in the spleen (A) and inguinal lymph nodes (B) of mice immunised with complexes of R<sub>4</sub>Pam<sub>2</sub>Cys and DNA. BALB/c mice (6-8 weeks old) were inoculated sub-cutaneously at the base of the tail with 20µg of DNA plasmid encoding for influenza nucleoprotein either alone or complexed with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) at NH<sub>3</sub><sup>+</sup>: PO<sub>4</sub><sup>-</sup> molar ratios of 1:1, 1:2 or 1:5. Complexation was achieved by slowly adding 10µl aliquots of a solution  
20 containing R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) to a solution of DNA every 2 minutes for a total period of 1 hour. All inoculants were dissolved in 0.7M NaCl. Spleens and inguinal lymph nodes were removed from mice 7 or 10 days after immunizations and intracellular cytokine staining was performed to detect the presence of IFN-γ secreting CD8+ T cells specific for nucleoprotein<sub>147-155</sub>. Each bar represents the mean and standard error from two mice.

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**DETAILED DESCRIPTION OF THE INVENTION**

Surprisingly, the inventors have found that the compounds of the present invention form a stable complex with DNA. DNA binds to the cationic portion of the compound and this  
5 complex is targeted, via the TLR ligand portion of the compound, to cells expressing receptors recognising the ligand, e.g. antigen-presenting cells such as DCs.

One advantage provided by the compounds of the present invention is that they are capable of targeting TLR-expressing cells. As the result of binding to TLRs, compounds of the  
10 present invention are internalised and also induce TLR-mediated signalling pathways. In particular embodiments, binding of TLR-2 by the Pam<sub>2</sub>Cys portion of the compounds of the present invention, causes DC maturation, resulting in migration of DCs to lymph nodes and efficient antigen presentation to T lymphocytes.

15 In a first aspect the present invention provides a compound comprising a positively charged group linked to at least one TLR ligand. In some embodiments the compounds of the present invention comprise a positively charged group covalently linked to at least one TLR ligand.

20 In a second aspect the present invention provides a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group.

25 In a third aspect the present invention provides a method of transfection comprising contacting a cell expressing a TLR with a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group.

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In a fourth aspect the present invention provides a method of raising an immune response

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against an antigen, comprising administering to a subject a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group, and wherein the  
5 nucleic acid encodes the antigen or an epitope thereof.

In a fifth aspect the present invention provides a method of raising an immune response against an antigen, comprising administering to a subject cells transfected with a complex comprising nucleic acid and a compound comprising a positively charged group linked to  
10 at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group, and wherein the nucleic acid encodes the antigen or an epitope thereof.

In a sixth aspect the present invention also provides a method of repressing expression of a  
15 gene in a cell expressing a TLR, comprising administering to a subject a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group, wherein the nucleic acid is selected from the group consisting of: siRNA; shRNA; DNA encoding  
20 siRNA; and DNA encoding shRNA; and is targeted against the gene.

In additional aspects the present invention also provides uses of a compound comprising a positively charged group linked to at least one TLR ligand in: the manufacture of a vaccine for the induction of an immune response in a subject; the manufacture of a medicament for  
25 the treatment of a subject with a genetic abnormality or deficiency; the manufacture of a medicament for the treatment of a subject suffering from aberrant or otherwise unwanted expression of a gene; and in the manufacture of a reagent for the transfection of cells.

The present invention provides compounds comprising a positively charged group linked  
30 to a TLR ligand, i.e. a moiety that binds to a cell surface TLR. Binding of the TLR ligand to the TLR results in uptake of the compound by a cell expressing the TLR, and/or

signalling via the TLR-mediated signalling pathway.

The term "TLR" as used herein means refers to one or more toll-like receptors, which can be defined as a class of membrane-bound receptors that bind to structurally conserved molecules derived from microbes. Thirteen TLRs, TLR-1 to TLR-13, have thus far been  
5 identified, and it is estimated that most mammalian species have between ten and fifteen types of Toll-like receptors. (Du, X. et al 2000. Eur. Cytokine Netw. 11:362-371; Chuang, T.H., and Ulevitch, R.J. 2000. Eur. Cytokine Netw. 11:372-378; Tabeta, K. et al 2004 Proc. Natl. Acad. Sci. USA 101:3516-3521). TLRs are a type of pattern recognition  
10 receptor (PRR) and their ligands are known collectively as pathogen-associated molecular patterns (PAMPs).

A "TLR ligand" as used herein means a molecule that selectively or preferentially binds to a TLR. Examples of TLR ligands include conserved features in pathogens and include:  
15 bacterial cell-surface lipopolysaccharides (LPS), lipoproteins, lipopeptides and lipoarabinomannan; proteins such as flagellin from bacterial flagella; double-stranded RNA of viruses or the unmethylated CpG motifs of bacterial and viral DNA; and certain other RNA and DNA. Endogenous ligands of TLRs have also been identified, including fibrinogen, heat shock proteins (HSPs), and DNA.

20

Table 1 lists TLRs, presently known to be expressed on dendritic cells, together with their corresponding ligands.

*Table 1. Toll-like receptors expressed by DC*

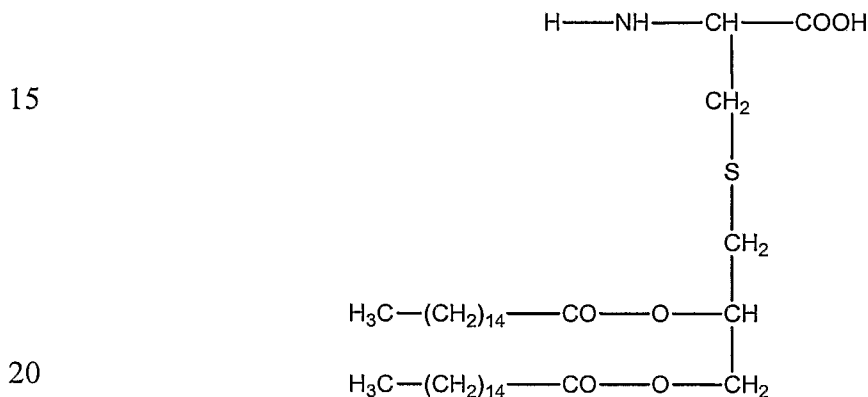
Family	Receptor	Ligand	References
Toll-like receptors	TLR-1	triacylated bacterial lipids (TLR-1 & 2)	(Takeuchi 2002)
	TLR-2	peptidoglycan, yeast zymosan, bacterial lipoproteins	(Schwandner 1999, Ozinsky 2000, Takeuchi 2000, Schjetne 2003)
	TLR-3	double stranded RNA	(Alexopoulou 2001)
	TLR-4	LPS, hsp60 & hsp70	(Poltorak 1998, Chow 1999, Asea 2002, Bulut 2002, Husebye 2006)
	TLR-5	flagellin	(Hayashi 2001)
	TLR-6	diacylated bacterial lipids (TLR-2 & 6)	(Morr 2002, Okusawa 2004)
	TLR-7	imidazoquinoline compounds	(Hemmi 2002)
	TLR-8	single stranded RNA	(Heil 2004)
	TLR-9	CpG DNA	(Hemmi 2000, Takeshita 2001)
	TLR-10	yet to be discovered	(Flacher 2006)
	TLR-11	yet to be discovered	(Pepper 2008)

Abbreviations: CpG, cytosine phosphate guanine; hsp, heat shock protein; LPS, lipopolysaccharide; RNA, ribonucleic acid

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An exemplary compound of the present invention is a cationic peptide linked to the lipopeptide "Pam<sub>2</sub>Cys". One of skill in the art would understand that the term "lipopeptide" means any composition of matter comprising one or more lipid moieties and one or more amino acid sequences that are conjugated. "Pam<sub>2</sub>Cys" (also known as dipalmitoyl-S-glyceryl-cysteine or S-[2, 3 bis(palmitoyloxy) propyl] cysteine has been synthesised (Metzger, J. W. et al. 1995. J Pept Sci 1: 184) and corresponds to the lipid moiety of MALP-2, a macrophage-activating lipopeptide isolated from *Mycoplasma fermentans* (Sacht, G. et al. 1998. Eur J Immunol 28 :4207; Muhiradt, P. F. et al. 1998. Infect Immun 66: 4804; Muhiradt, P. F. et al. 1997. J Exp Med 185: 1951). Pam<sub>2</sub>Cys is known to be a ligand of TLR-2.

Pam<sub>2</sub>Cys has the structure of Formula(I):



Other lipid moieties which may be used to target cell surface TLRs include palmitoyl, myristoyl, stearoyl, lauroyl, octanoyl, or decanoyl. Preferred groups include Pam<sub>2</sub>Cys, Pam<sub>3</sub>Cys, Ste<sub>2</sub>Cys, Lau<sub>2</sub>Cys, and Oct<sub>2</sub>Cys.

Positively charged groups of the present invention include but are not limited to the cationic compounds listed in Table 2 and the polycationic compounds described in US 6,689,478 and US 4,035,558.

*Table 2. Cationic compounds*

Cationic Compounds	References
Penetratin	(Christiaens 2004)
HIV Tat 48-60	(Fawell 1994)
HIV Rev 34-50	(Futaki 2001)
Transportan	(Pooga 1998)
Oligoarginine peptides (linear and branched)	(Buschle 1997, Mitchell 2000)
Oligolysine peptides (linear and branched)	
Pyrrochoricin	(Otvos 2004)
Alpha-helical amphipathic model peptide	(Oehlke 1998)
Polylysine	(Wagner 1990)
Protamine (e.g. salmon protamine)	(Wagner 1990)
FL17 $([(\text{Me}_2\text{NCH}_2\text{CHOHCH}_2)_n]^{n+}\text{Cl}_n)$	(Billingham 1997)
Magnafloc 1697 $([(\text{CH}_2\text{CHCH}_2\text{N}(\text{Me})_2\text{CH}_2\text{CHCH}_2)_n]^{n+}\text{Cl}_n)$	(Billingham 1997)

5 Since nucleic acids, due to the phosphate groups within the backbone of nucleic acids, are net negatively charged molecules, they are bound by the positively charged groups of the compounds of the present invention, via electrostatic interaction, to form a stable complex.

In one embodiment, the positively charge group is a linear or branched peptide comprising arginine or lysine residues.

10

Reference to a “nucleic acid” should be understood as a reference to both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), including double-stranded DNA, double-stranded RNA, single-stranded DNA, single-stranded RNA, small interfering RNA molecules (siRNA), triplexes, quadruplexes and any multi-stranded (multiplex) nucleic acid molecule, nucleic acid hybrids such as peptide nucleic acids (PNA), a molecule comprising 15 both deoxyribonucleic acid bases and ribonucleic acid bases, and any nucleotide variant.

- 15 -

In one embodiment, nucleic acid molecules are provided as "plasmids". Reference to "plasmids" should be understood as a reference to a nucleic acid molecule which is transmissible to a host cell and may undergo replication in the host cell. The nucleic acid molecule should not however, be limited to plasmids, but may be any nucleic acid molecule, including viral DNA or RNA.

One of skill in the art would appreciate that transfecting cells with nucleic acids is useful for many research and clinical applications. Reference to "transfection" should be understood as reference to a process by which exogenous molecules, including nucleic acids, are introduced into cells.

Many research applications require transfection of cells. Currently researchers introduce molecules into cells, i.e. transfect cells, by a variety of means with variable efficiency. Several transfection reagents are currently available to the researcher, including FUGENE. However, the efficiency of transfection of cells is dependent on, for example, cell type, rate and stage of cell division, and the individual properties of both the molecules to be transfected and the transfection reagent(s).

The compounds of the present invention are particularly useful for transfection of cells expressing TLRs. Therefore, cells either naturally expressing TLRs or cells that have been stably transfected to express a TLR, whether it be the entire receptor or a modified receptor lacking the intracellular signalling domain, can be transfected with nucleic acids complexed with the compounds of the present invention.

A "cell" should be understood as any cell into which the nucleic acid molecule is delivered by the compounds of the present invention. The cell may be an *in vitro*, *in vivo* or *ex vivo* cell. The cell may be isolated or form a part of the organs or tissues of a living animal. A cell may also be a microorganism such as bacteria, yeast, fungi, moulds, parasites, algae and so on. A cell can also be a cultured animal cell or cell line, or an artificial cell.

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Reference to "isolated", in terms of the cells of certain embodiments of the present invention, should be understood as a reference to material that is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a cell present in the tissue of a living organism is not isolated, but the same cell, when separated  
5 from some or all of the co-existing cells in the natural system, is isolated.

One aspect of the present invention provides compounds comprising a positively charged group, linked to a TLR ligand, which can form a stable complex with nucleic acids via electrostatic interaction between the positively charged group and the negatively charged  
10 nucleic acid. The compounds of the present invention provide a means of targeting nucleic acid to cells expressing TLR.

In one embodiment, the present invention provides compounds comprising a cationic peptide, linked to a TLR ligand, which can form a stable complex with DNA via  
15 electrostatic interaction between the cationic peptide and the negatively charged DNA. Such compounds provide a means of targeting DNA to cells expressing TLRs, e.g. antigen-presenting cells, and more particularly DCs.

In another embodiment, the invention provides a method of transfection comprising  
20 contacting a cell expressing a TLR with a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one moiety that binds to a TLR, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group.

25 In a further embodiment, the present invention provides a method of raising an immune response against an antigen, comprising administering to a subject a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one moiety that binds to a TLR, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group, and  
30 wherein the nucleic acid encodes the antigen or an epitope thereof.

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In yet another aspect, the present invention provides a method of raising an immune response against an antigen, comprising administering to a subject cells transfected with a complex comprising nucleic acid and a compound comprising a positively charged group linked to at least one moiety that binds to a TLR, wherein the nucleic acid is associated  
5 with the compound by electrostatic interaction between the nucleic acid and the positively charged group, and wherein the nucleic acid encodes the antigen or an epitope thereof.

Contacting DCs with compounds of the present invention can result in the maturation or activation of DCs, as indicated by an increase in cell surface MHC Class II expression.  
10 Therefore, compounds comprising a TLR ligand can be useful not only for targeting DCs but also for their activation via TLRs and downstream signalling molecules such as MyD88.

Consequently, the compounds of the present invention are useful for activating DCs. In order for dendritic cells to induce an immune response, they must first be activated such that they express the necessary adhesion and co-stimulatory molecules to migrate to lymph nodes and activate T lymphocytes. Compounds comprising TLR ligands complexed with DNA are particularly useful for activating DCs, and when the DNA encodes a protein, the transfected DCs present peptide fragments of the translated DNA-encoded protein to  
15 immune cells, thereby invoking a cellular and humoral immune response. In other words, the compounds of particular embodiments of the present invention when complexed to DNA, are useful for DNA vaccination approaches.  
20

Reference to "DNA vaccination" as used herein means reference to administration of DNA to a subject in order to induce an immune response to the protein encoded thereby. The  
25 compounds of the present invention may be administered directly to a subject by any route, including but not limited to: intravenous; intranasal; intramuscular; oral; rectal and so on.

Reference to "immune response" as used herein means a reference to the concerted action of lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble  
30 macromolecules produced by the above cells or the liver (including antibodies, cytokines,

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and complement) that results in selective damage to, destruction of, or elimination from the human body of invading pathogens, cells or tissues infected with pathogens, cancerous cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

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Reference to "T lymphocyte response" as used herein means a reference to the component of the immune response dependent on T lymphocytes (i.e., the proliferation and/or differentiation of T lymphocytes into helper, cytotoxic killer, or suppressor T lymphocytes, the provision of signals by helper T lymphocytes to B lymphocytes that cause or prevent antibody production, the killing of specific target cells by cytotoxic T lymphocytes, and the release of soluble factors such as cytokines that modulate the function of other immune cells).

"Patient", "subject" or "mammal" are used interchangeably and refer to mammals such as human patients and non-human primates, as well as experimental animals such as rabbits, rats, and mice, and other animals. Animals include all vertebrates, e.g., mammals and non-mammals, such as sheep, dogs, cows, chickens, amphibians, and reptiles.

In addition to their use in DNA vaccination, the compounds of the present invention are useful for a number of other clinical applications. For example, treatments for autoimmune disease or cancer may require transfection of autologous cells, such as hemopoietic stem cells (HSCs) or peripheral blood mononuclear cells (PBMCs). Such cells can be harvested from a subject, cultured *in vitro*, optionally differentiated into DCs by methods well known in the art, and/or transfected with nucleic acid, and subsequently infused back into the subject. In some embodiments, the nucleic acid encodes a vaccine antigen and the transfected cells may be intended to invoke an immune response. In other embodiments, the nucleic acid may encode a cytokine or an antigenic tolerogen.

In another aspect, the present invention provides a method of repressing expression of a gene in a cell expressing a TLR, comprising administering to a subject a complex comprising nucleic acid and a compound comprising a positively charged group linked to a

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TLR ligand, wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group, and wherein the nucleic acid is selected from the group consisting of: siRNA; shRNA; DNA encoding siRNA; and DNA encoding shRNA; and wherein the siRNA or shRNA is targeted against  
5 the gene.

"Treating" or "treatment" includes the administration of the compositions, compounds or agents of the present invention to prevent or delay the onset of the symptoms, complications, or biochemical indicia of a disease, alleviating or ameliorating the  
10 symptoms or arresting or inhibiting further development of the disease, condition, or disorder (e.g., an infectious disease, inflammation, or an autoimmune disease). "Treating" further refers to any indicia of success in the treatment or amelioration or prevention of the disease, condition, or disorder (e.g., an infectious disease, inflammation, or an autoimmune disease), including any objective or subjective parameter such as abatement; remission;  
15 diminishing of symptoms or making the disease condition more tolerable to the patient; slowing in the rate of degeneration or decline; or making the final point of degeneration less debilitating. The treatment or amelioration of symptoms can be based on objective or subjective parameters; including the results of an examination by a physician. Accordingly, the term "treating" includes the administration of the compounds or agents of the present  
20 invention to prevent or delay, to alleviate, or to arrest or inhibit development of the symptoms or conditions associated with an infectious disease, inflammation, or an autoimmune disease. The term "therapeutic effect" refers to the reduction, elimination, or prevention of the disease, symptoms of the disease, or side effects of the disease in the subject. "Treating" or "treatment" using the methods of the present invention includes  
25 preventing the onset of symptoms in a subject that can be at increased risk of an infectious disease, inflammation, or an autoimmune disease but does not yet experience or exhibit symptoms, inhibiting the symptoms of an infectious disease, inflammation, or an autoimmune disease (slowing or arresting its development), providing relief from the symptoms or side-effects an infectious disease, inflammation, or an autoimmune disease  
30 (including palliative treatment), and relieving the symptoms of an infectious disease, inflammation, or an autoimmune disease (causing regression). Treatment can be

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prophylactic (to prevent or delay the onset of the disease, or to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease or condition.

- 5 In additional aspects the present invention provides uses of compounds comprising a positively charged group linked to a TLR ligand in: the manufacture of a vaccine for the induction of an immune response in a subject; the manufacture of a medicament for the treatment of a subject with a genetic abnormality or deficiency; the manufacture of a medicament for the treatment of a subject suffering from aberrant or otherwise unwanted  
10 expression of a gene; and in the manufacture of a reagent for the transfection of cells.

The compounds and the methods set forth herein can be used for both medical applications and veterinary applications. Typically, the product is used in humans, but may also be used in other mammals. The terms "patient" and "subject" are intended to denote a mammalian  
15 individual, and are so used throughout the specification and in the claims. The primary applications of the invention involve human patients or subjects, but the invention may be applied to laboratory, farm, zoo, wildlife, pet or sport animals.

It would be appreciated that the person of skill in the art may introduce any combination of  
20 positively charged group and TLR ligand as befits the desired application.

The present invention will now be described further with reference to the following non-limiting examples:

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**Example 1*****Synthesis of cationic peptides***

Branched peptide constructs were synthesized by using lysine residues to provide the  
5 branching points of a scaffold template. Depending on the number of branch points present  
in the scaffold, di or tetra cationic structures were produced (Figure 1). These branched  
constructs were synthesised on PEG-S RAM resin (Rapp Polymere, Tübingen, Germany;  
substitution factor 0.27mmol/g). Fmoc-lysine(Mtt)-OH ( Novabiochem, Läfelfingen,  
Switzerland) was first coupled to the resin in 4-fold excess with equimolar amounts of  
10 O-benzotriazole-N,N,N,N',N'-tetamethyl-uronium-hexafluorophosphate (HBTU;  
Novabiochem, Darmstadt, Germany), 1- hydroxybenzotriazole (HOBt) and a 1.5-fold molar  
excess of diisopropylethylamine (DIPEA; Sigma, Castle Hill, Australia). Acylation was  
carried out for 40 minutes and complete reaction was confirmed by the  
trinitrobenzylsulfonic acid (TNBSA) test (21). Removal of the Fmoc protective group on  
15 the  $\alpha$ -amino group was achieved with 2.5% diazabicyclo[5.4.0]undec-7-ene (Sigma,  
Steinheim, Germany) and Fmoc-lysine (Fmoc)-OH (Auspep, Melbourne, Australia)  
coupled such that following removal of the Fmoc groups, two primary amino groups were  
exposed to act as branching points. Dimethylformamide (DMF; Auspep, Melbourne,  
Australia) was used to wash the resin between each acylation and deprotection step.  
20 Subsequent amino acids were coupled at a 4-fold excess and acylation carried out for 60  
minutes. For the assembly of tetravalent constructs, an additional round of acylation was  
performed for 60 minutes using Fmoc-lysine(Fmoc)-OH to produce four branch points. To  
enable chemoselective ligation of a group containing an amino acid to a tetravalent  
arginine construct (R4), cysteine was inserted at the C-terminus of the construct to yield  
25 R4-Cys. For carboxyfluorescein conjugation onto branched peptide constructs, N-terminal  
Fmoc groups were not removed following the last acylation reaction. Instead, the Mtt  
protective group present on the  $\epsilon$ -amino group of the C-terminal lysine was removed by  
treatment with 1% TFA in dichloromethane (Ajax Finechem, Seven Hills, Australia). 5(6)-  
carboxyfluorescein (Fluka BioChemika, Switzerland) was then coupled to the exposed  $\epsilon$ -  
30 amino group using a 4-fold excess in the presence of equimolar amounts of HOBt, HBTU  
and a 1.5 fold molar excess of DIPEA for 18 hours in the dark. Following acylation, the

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resin was washed with DMF and N-terminal Fmoc groups were removed. All peptides were cleaved from the solid phase support and side chain protecting groups simultaneously. The purity of peptides was assessed by reverse phase analytical chromatography using a Vydac C4 column (4.6mm × 250mm) or a C8 column (4.6mm × 250mm) installed in a Waters 3 HPLC chromatography system. A flow rate of 1ml/min using 0.1% TFA in H<sub>2</sub>O and 0.1% TFA in acetonitrile as the limit solvent was used to develop chromatograms. Where necessary, peptides were purified using a semi-preparative Waters or GBC HPLC system and a semipreparative Vydac C4 column (10mm × 300mm) at a flow rate of 2.5ml/min. Estimation of peptide content was determined by UV spectrophotometry where the absorbance of fluorescened peptides was determined at 496nm and concentration calculated using a molar extinction coefficient of 83,000 M<sup>-1</sup>cm<sup>-1</sup>. Schematics of the synthesised fluorescened branched peptide constructs are shown in Figure 1.

For lipidation of peptides, lipid moieties were assembled by coupling N-fluorenylmethoxycarbonyl-S-(2,3-dihydroxypropyl)-cysteine (Fmoc-Dhc-OH) at a 4-fold excess in the presence of equivalent amounts of HOBt and DICl in 50% DCM in DMF onto the ε-amino group of the C-terminal lysine. Acylation was carried out for 40 minutes and was repeated until successful coupling was confirmed using the trinitrobenzene sulfonic acid test. Lipidation of the two hydroxy groups of the resin-bound Fmoc-Dhc-peptide was carried out overnight using a solution containing an equimolar amount of DMAP, a 10-fold excess of fatty acid and a 12-fold excess of DICl.

## Example 2

### Retardation of DNA migration by R<sub>4</sub>

To investigate the association between the branched peptide R<sub>4</sub> and DNA, a constant amount of DNA plasmid pEGFP-N1 or pCI-HA, which encode for green fluorescent protein and influenza HA protein respectively, were incubated with varying amounts of R<sub>4</sub> and analysed by agarose gel electrophoresis (Figure 2).

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By increasing the amount of R<sub>4</sub> in each sample, DNA migration could be affected. Partial retardation of DNA plasmid migration towards the anode, as visualised by ethidium bromide fluorescence, was evident when 0.1-0.5µg of R<sub>4</sub> was used whereas DNA samples incubated with higher amounts of R<sub>4</sub> moved at a slower rate. Total retardation of DNA migration was achieved when 10µg of R<sub>4</sub> was present as evidenced by the appearance of a band of DNA at the origin. This band, however, appeared quite faint suggesting that there was little DNA in this sample. This may be due to: R<sub>4</sub> inhibiting the binding of ethidium bromide to the DNA; and/or the interaction between R<sub>4</sub> and DNA results in the formation of an insoluble complex that diffuses into the electrophoresis buffer rather than migrating through the agarose gel. These results suggest that the positively charged amino and guanidine groups of R<sub>4</sub> are able to neutralise the negatively charged phosphate groups within the DNA backbone to retard its ability to migrate towards the anode.

### Example 3

#### 15 *Formation of R<sub>4</sub>-DNA complexes*

In order to confirm the binding of R<sub>4</sub> to DNA, the amount of DNA in the supernatant of centrifuged mixtures containing increasing amounts of R<sub>4</sub> and DNA was determined by measuring the absorbance at 260nm (Figure 3A). The ability of R<sub>4</sub> to precipitate DNA out of solution by neutralisation of its charge would result in less DNA in the solution and would therefore indicate an association between the two. It was found that the amount of DNA in the centrifuged supernatant did not vary dramatically when incubated with 0.1µg to 5µg of R<sub>4</sub>. A slight decrease, however, resulted when 7.5µg of R<sub>4</sub> was used and no DNA was detected in solutions containing more than this amount. This result indicates that the amount of R<sub>4</sub> present is proportional to the precipitation of DNA out of solution. Absorbance readings were also taken on the wash supernatant used to resuspend any centrifuged insoluble material. The presence of little DNA in these samples suggests that the R<sub>4</sub>-DNA precipitates are stable and that DNA is unlikely to dissociate from this complex.

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Agarose gel analysis (Figure 3B) demonstrates that DNA bands were evident in supernatant samples containing 0.1-7.5 $\mu$ g of R<sub>4</sub> but not in samples containing 10 or 15 $\mu$ g of R<sub>4</sub>. Retardation of DNA migration is also evident in samples containing 1 $\mu$ g or more of R<sub>4</sub>.

5

#### Example 4

##### *Transfection of DCs with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys)-DNA*

To determine if the R<sub>4</sub> or R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) constructs were capable of inducing expression of transfected DNA, a murine DC line, D1, was transfected with the pEGFP plasmid using R<sub>4</sub> or R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) (Figure 4A). The expression levels of green fluorescent protein (GFP) in these cells were compared to those levels in D1 cells that were transfected using the commercially available transfection reagent FUGENE.

15 No fluorescence was observed in untreated cells, cells treated with DNA only and cells treated with R<sub>4</sub> together with DNA. However, cells transfected with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) and DNA expressed GFP at a level comparable to the level of expression observed in cells transfected with the pEGFP and the FUGENE reagent.

20 The D1 cell cultures were also found to comprise of two distinct cell populations that were either MHC Class II<sup>low</sup> or MHC Class II<sup>high</sup>, corresponding to immature and mature cells respectively (Figure 4B). Approximately 4% of untreated D1 cells were MHC Class II<sup>high</sup>. Treatment with DNA alone or together with R<sub>4</sub> did not appreciably alter the expression of MHC Class II on D1 cells. D1 cells treated with DNA and FUGENE comprised approximately 36% MHC Class II<sup>high</sup> cells, and D1 cells treated with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) and DNA comprised approximately 82% MHC Class II<sup>high</sup> cells.

Transfection efficiency using different ratio mixtures of transfection reagent to DNA was also investigated by incubating D1 cells with increasing amounts (0.1, 1 or 3 $\mu$ g) of R<sub>4</sub>, R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) or FUGENE pre-incubated with a constant amount (1 $\mu$ g) of pCI-HA plasmid (Figure 5). Cell surface influenza HA protein expression was determined using a

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HA-specific antibody. In the case of when  $R_4(S_2Pam_2Cys)$  or FUGENE were used, transfection efficiency, as indicated by the percentage of cells expressing surface HA protein, was proportional to the amount of transfection agent used. The exception to this was when  $R_4$  was employed. Here, although HA expression levels were lower than those  
5 induced by DNA mixtures containing  $R_4(S_2Pam_2Cys)$  or FUGENE, transfection appeared to be optimal when there was an equal weight to weight ratio of  $R_4$  to DNA. A higher ratio, however, was found to be detrimental to transfection efficiency.

At all of the ratios examined,  $R_4(S_2Pam_2Cys)$  was found to be superior to FUGENE at  
10 inducing the expression of antigen. The maximum percentage of cells that transfected using  $R_4(S_2Pam_2Cys)$  and DNA was 60% at a 3:1 ratio. In comparison, only around 30% of HA positive cells were detected when an equivalent ratio of FUGENE to DNA was used.

15

#### **Example 5**

##### ***Transfection of human monocyte-derived dendritic cells (MoDCs)***

The ability of  $R_4(S_2Pam_2Cys)$  to enhance transfection of human monocyte-derived dendritic cells (MoDCs) was investigated. In an experiment where MoDCs were  
20 transfected with the pEGFP plasmid (Figure 6A), only  $R_4(S_2Pam_2Cys)$  was efficient at inducing the expression of GFP. Transfection of cells with pEGFP plus either FUGENE or  $R_4$  was relatively inefficient, with the transfection levels in these cells similar to those treated with the plasmid alone. This result was repeated in experiments where MoDCs were transfected with the pCI-HA plasmid together with each of the transfection reagents  
25 (Figure 6B).

#### **Example 6**

##### ***Transfection of non-DC cell lines with $R_4(S_2Pam_2Cys)$ -DNA***

30 To investigate if  $R_4$  or  $R_4(S_2Pam_2Cys)$  constructs complexed with DNA were capable of transfecting non-DC cell lines, the human lung epithelial cell line A549 and the murine

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lung aveolar macrophage cell line MH-S were transfected with the pEGFP plasmid using R<sub>4</sub> or R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) (Figure 7A and 7B). The human lung epithelial cell line A549 (A) or the murine lung aveolar macrophage cell line MH-S (B) were cultured at a concentration of 2×10<sup>5</sup> cells/ml in the absence or presence of plasmid encoding for green fluorescent protein complexed with different dilutions of R<sub>4</sub>, R<sub>4</sub>Pam<sub>2</sub>Cys or FUGENE for 48 hours at 37°C and 5% CO<sub>2</sub>. Transfection efficiency was determined by the measuring the fluorescence of cells on a flow cytometer. Both these cell lines have been reported in other studies to express TLR-2 (Oshikawa 2003, Slevogt 2007). Little to no fluorescence was observed in untreated A549 or MH-S cells and in cells treated with R<sub>4</sub> together with DNA. However, A549 or MH-S cells transfected with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) and DNA expressed GFP at a level comparable, if not higher, to the level of expression observed in cells transfected with the pEGFP plasmid and the FUGENE reagent.

### Example 7

#### Specific antibody production in mice immunised with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys)-DNA

The ability of R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) complexed with DNA to induce antibodies was assessed. BALB/c mice (6-8 weeks old) were inoculated sub-cutaneously at the base of the tail on day 0 and 28 with 50µg of DNA plasmid comprising DNA encoding influenza hemagglutinin, either alone or complexed with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) at molar ratios of 1:3 or 1:5. As a positive control, mice were also immunised intra-nasally with split PR8 virus (3µg/mouse). Unimmunised mice served as negative controls. Sera were obtained from mice 14 days after the last inoculation and an ELISA was performed to detect the presence of antibodies specific for influenza PR8 virus. Serum anti-HA antibodies were measured by ELISA. No HA-specific antibodies were detected in unimmunised or "naïve" mice but substantial levels of antibodies that could bind to influenza virus were detected in mice immunised with two doses of R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) and DNA mixed at molar ratios of 1:3 and 1:5 (Figure 8).

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### Example 8

#### *Induction of influenza nucleoprotein-specific, IFN- $\gamma$ -positive CD8+T cells in mice immunised with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys)-DNA*

5 The ability of R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) complexed with DNA to induce cell-mediated immune responses was assessed. BALB/c mice (6-8 weeks old) were inoculated sub-cutaneously at the base of the tail with 20 $\mu$ g of DNA plasmid encoding for influenza nucleoprotein either alone or complexed with R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) at NH<sub>3</sub><sup>+</sup>: PO<sub>4</sub><sup>-</sup> molar ratios of 1:1, 1:2 or 1:5. Complexation was achieved by slowly adding 10 $\mu$ l aliquots of a solution containing  
10 R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) to a solution of DNA every 2 minutes for a total period of 1 hour. All inoculants were dissolved in 0.7M NaCl. Spleens and inguinal lymph nodes were removed from mice 7 or 10 days after immunizations and intracellular cytokine staining was performed to detect the presence of IFN- $\gamma$  secreting CD8+ T cells specific for nucleoprotein<sub>147-155</sub>. Each bar represents the mean and standard error from two mice.

15

Only very low levels of activated CD8+ T cells were detected in the spleens and inguinal lymph nodes of mice inoculated with DNA alone (Figure 9A and 9B respectively) and non were detected in naïve mice. Administration of complexes containing different ratios of R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) to DNA, however, induced NP-specific IFN- $\gamma$  producing CD8+ T cells  
20 detectable in the spleen 7 days after immunisation (Figure 9A). Particularly high levels of activated CD8+ T cells were detected in both the lymph nodes and spleens of mice inoculated with a 1:1 ratio of R<sub>4</sub>(S<sub>2</sub>Pam<sub>2</sub>Cys) to DNA (Figures 9A and 9B).

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**THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:**

1. A compound comprising a positively charged group linked to at least one TLR ligand.
- 5 2. A compound according to claim 1, wherein the positively charged group is covalently linked to the TLR ligand.
3. A compound according to claim 1 or claim 2, wherein the positively charged group comprises one or more amino acids.
4. A compound according to any one of claims 1 to 3, wherein the positively  
10 charged group is a branched or linear peptide.
5. A compound according to claim 4, wherein the peptide comprises at least one arginine or lysine residue.
6. A compound according to claim 4 or claim 5, wherein the peptide comprises at least four arginine residues or at least four lysine residues.
- 15 7. A compound according to any one of claims 1 to 6, wherein the TLR ligand comprises a lipid or a peptidoglycan or a lipoprotein or a lipopolysaccharide.
8. A compound according to any one of claims 1 to 6, wherein the TLR ligand comprises palmitoyl, myristoyl, stearoyl, lauroyl, octanoyl, or decanoyl.
9. A compound according to any one of claims 1 to 6, wherein the TLR ligand is  
20 selected from the group consisting of: Pam<sub>2</sub>Cys, Pam<sub>3</sub>Cys, Ste<sub>2</sub>Cys, Lau<sub>2</sub>Cys, and Oct<sub>2</sub>Cys.
10. A compound according to any one of claims 1 to 9, wherein the TLR ligand binds either TLR-2 or TLR-6.
11. A compound according to any one of claims 1 to 10 wherein the TLR ligand  
25 binds to TLR-2.

- 34 -

12. A complex comprising nucleic acid and a compound according to any one of claims 1 to 11 wherein the nucleic acid is associated with the compound by electrostatic interaction between the nucleic acid and the positively charged group.
13. A method of transfection comprising contacting a cell expressing at least one  
5 TLR with a complex according to claim 12.
14. A method of raising an immune response against an antigen, comprising administering to a subject a complex according to claim 12, wherein the nucleic acid encodes the antigen or an epitope thereof.
15. A method of raising an immune response against an antigen, comprising  
10 administering to a subject cells transfected with a complex according to claim 12, wherein the nucleic acid encodes the antigen or an epitope thereof.
16. A method of repressing expression of a gene in a cell expressing a TLR, comprising administering to a subject a complex according to claim 12, wherein the nucleic acid is selected from the group consisting of: siRNA; shRNA; DNA encoding  
15 siRNA; and DNA encoding shRNA, and wherein the siRNA or shRNA is targeted against the gene.
17. The use of a compound or complex according to any one of claims 1 to 12 in the manufacture of a vaccine for the induction of an immune response in a subject.
18. The use of a compound or complex according to any one of claims 1 to 12 in  
20 the manufacture of a medicament for the treatment of a subject with a genetic abnormality or deficiency.
19. The use of a compound or complex according to any one of claims 1 to 12 in the manufacture of a medicament for the treatment of a subject suffering from aberrant or otherwise unwanted expression of a gene.
- 25 20. The use of a compound or complex according to any one of claims 1 to 12 in the manufacture of a reagent for the transfection of cells.

Figure 1

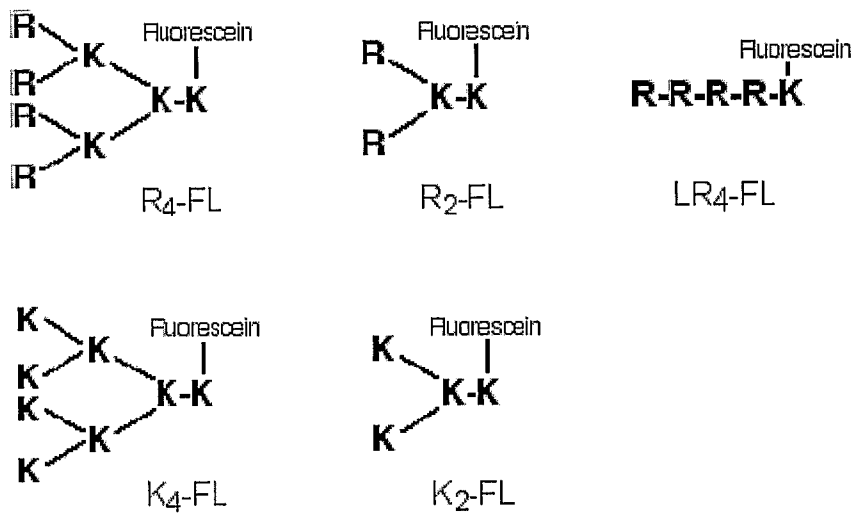


Figure 2

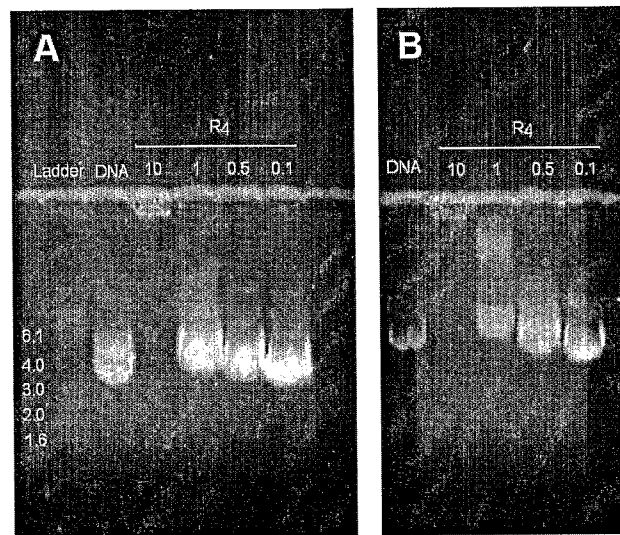
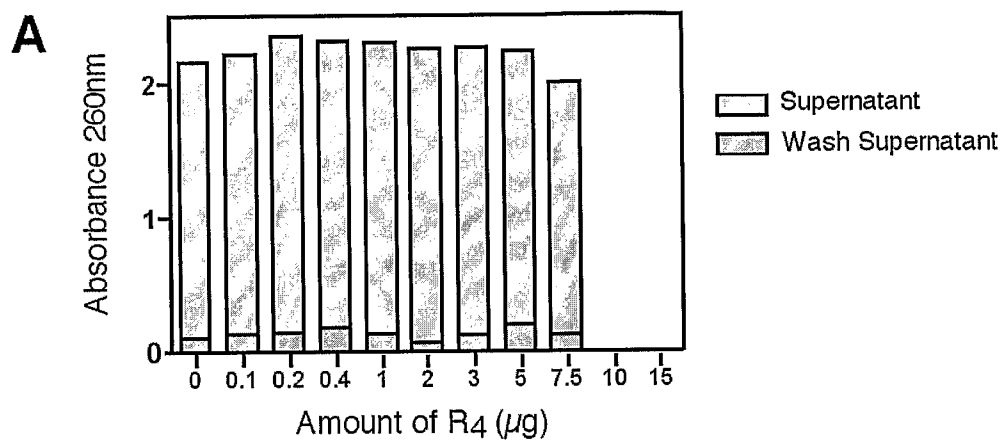


Figure 3



**B**

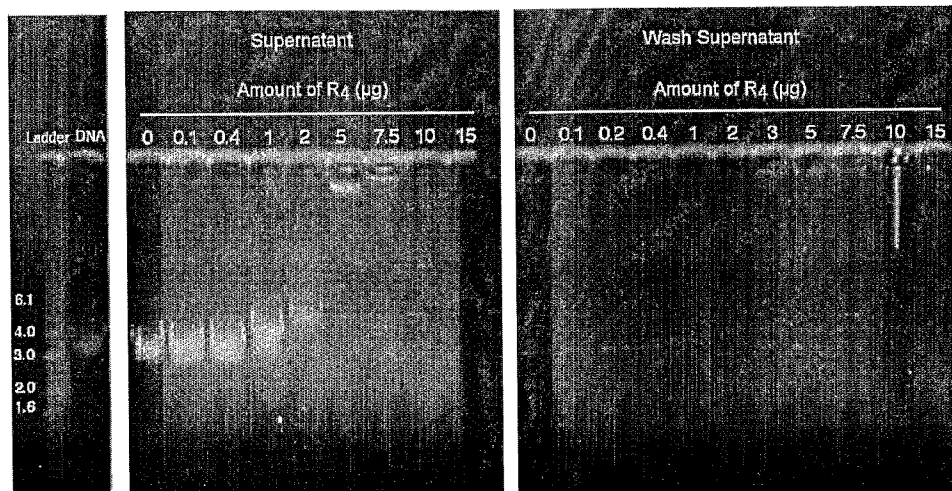


Figure 4

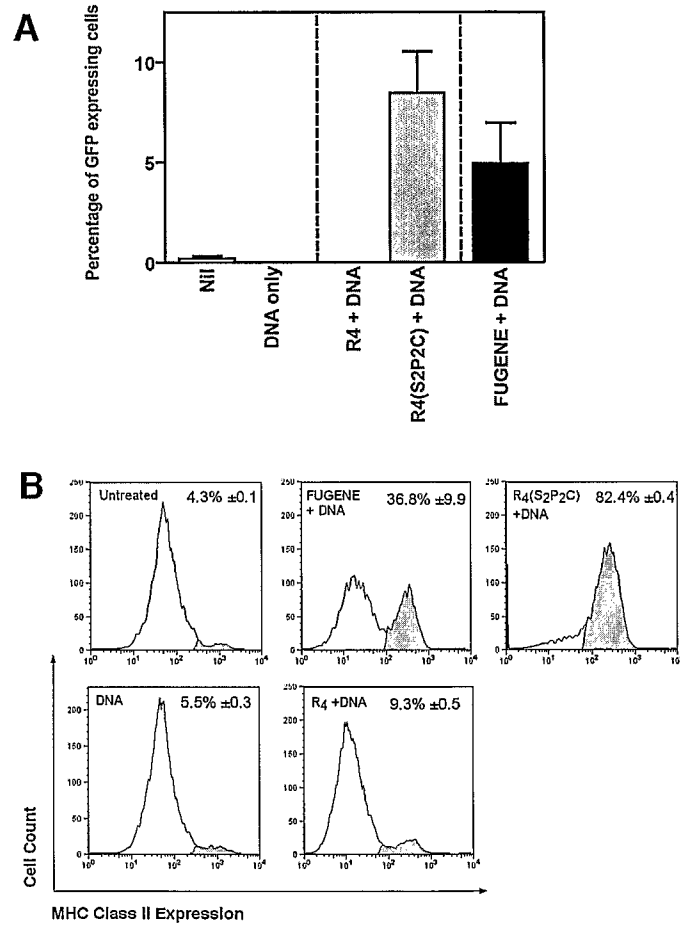


Figure 5

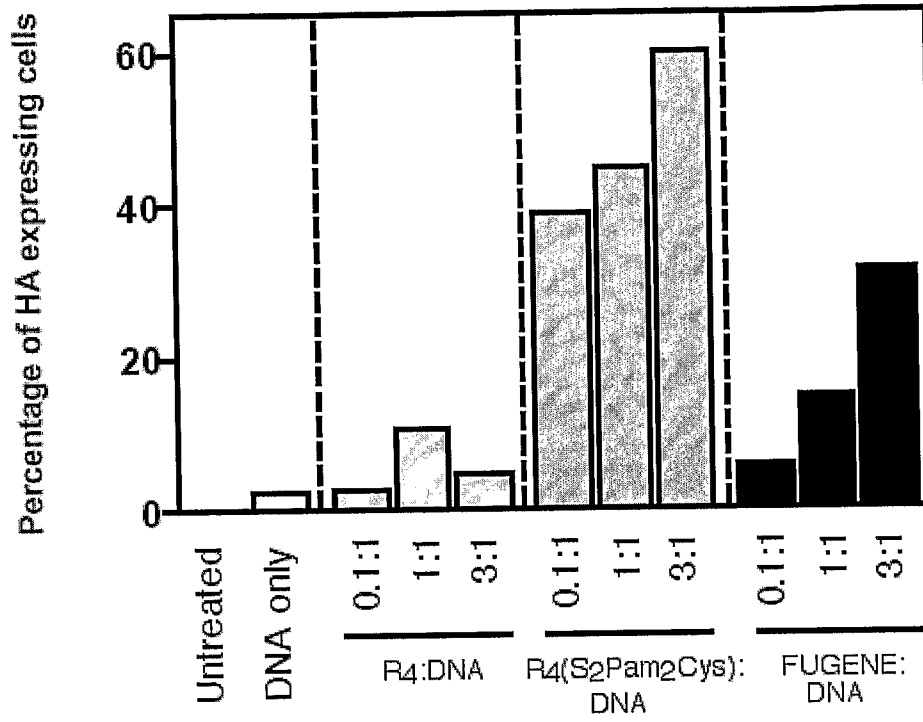


Figure 6

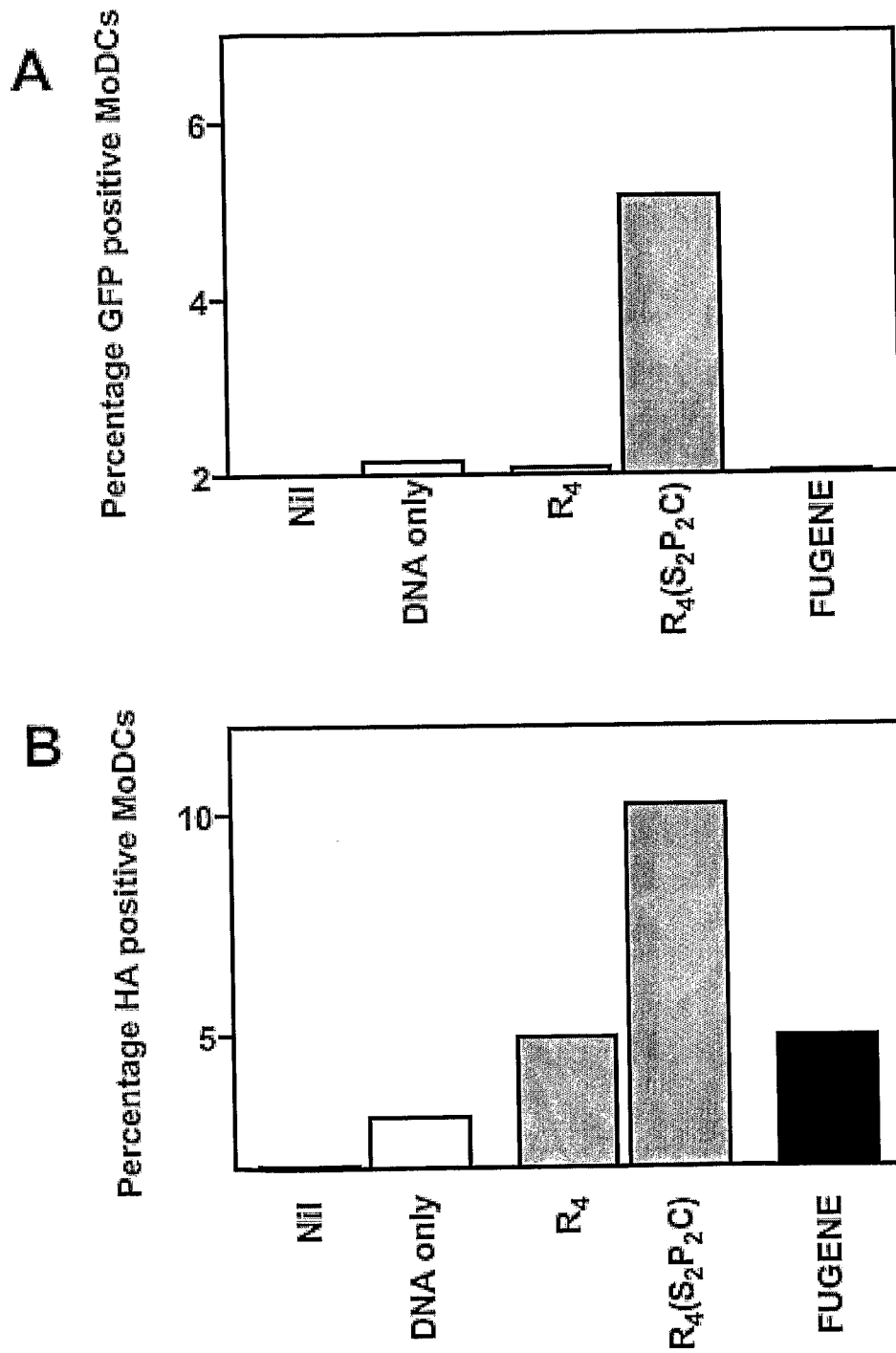


Figure 7

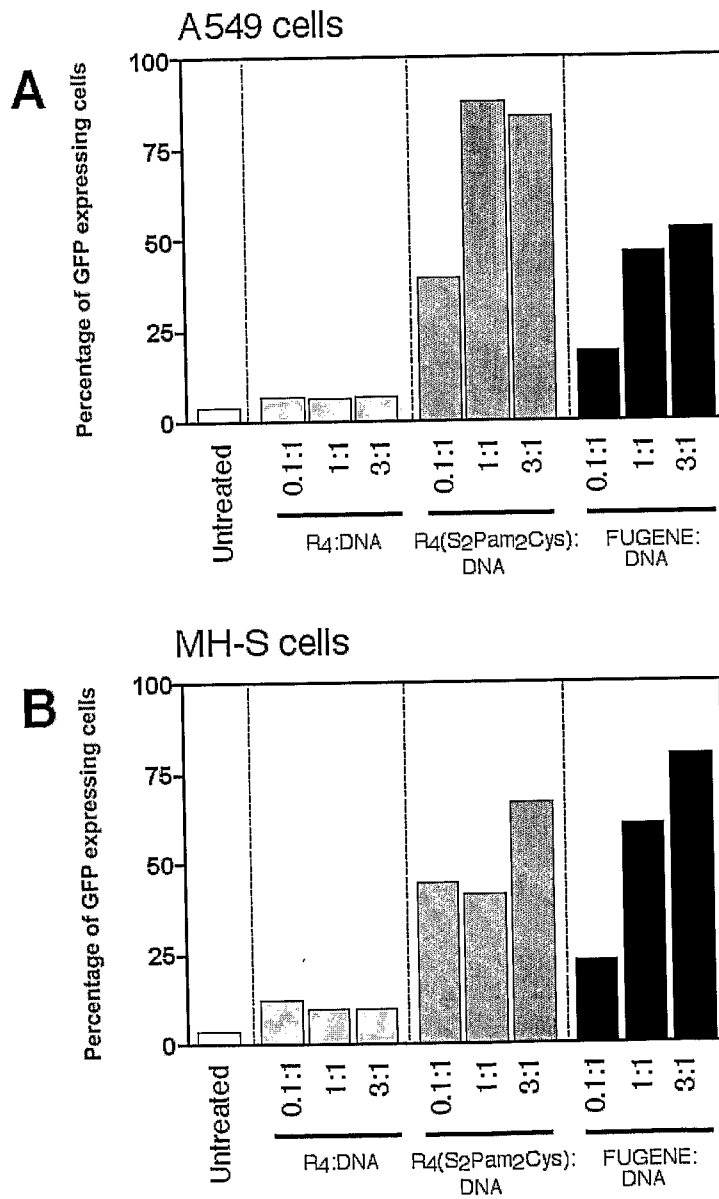


Figure 8

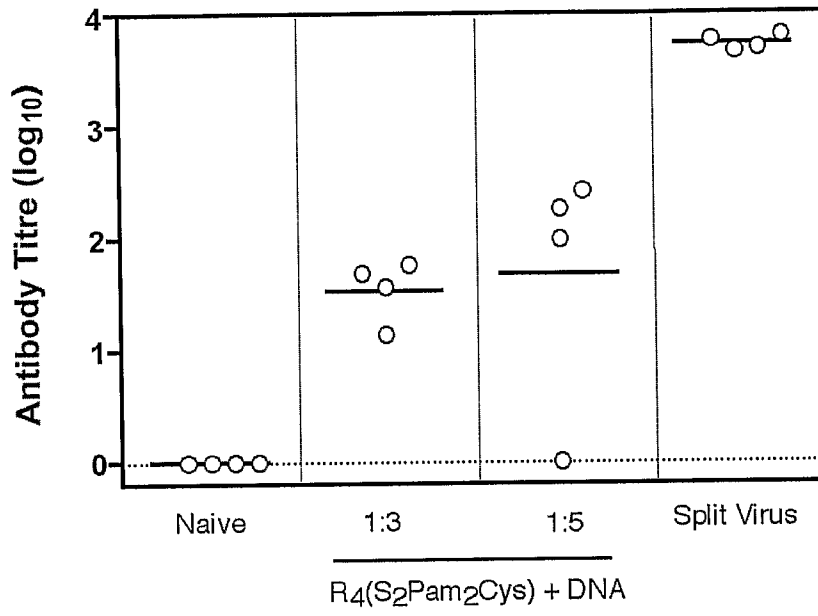
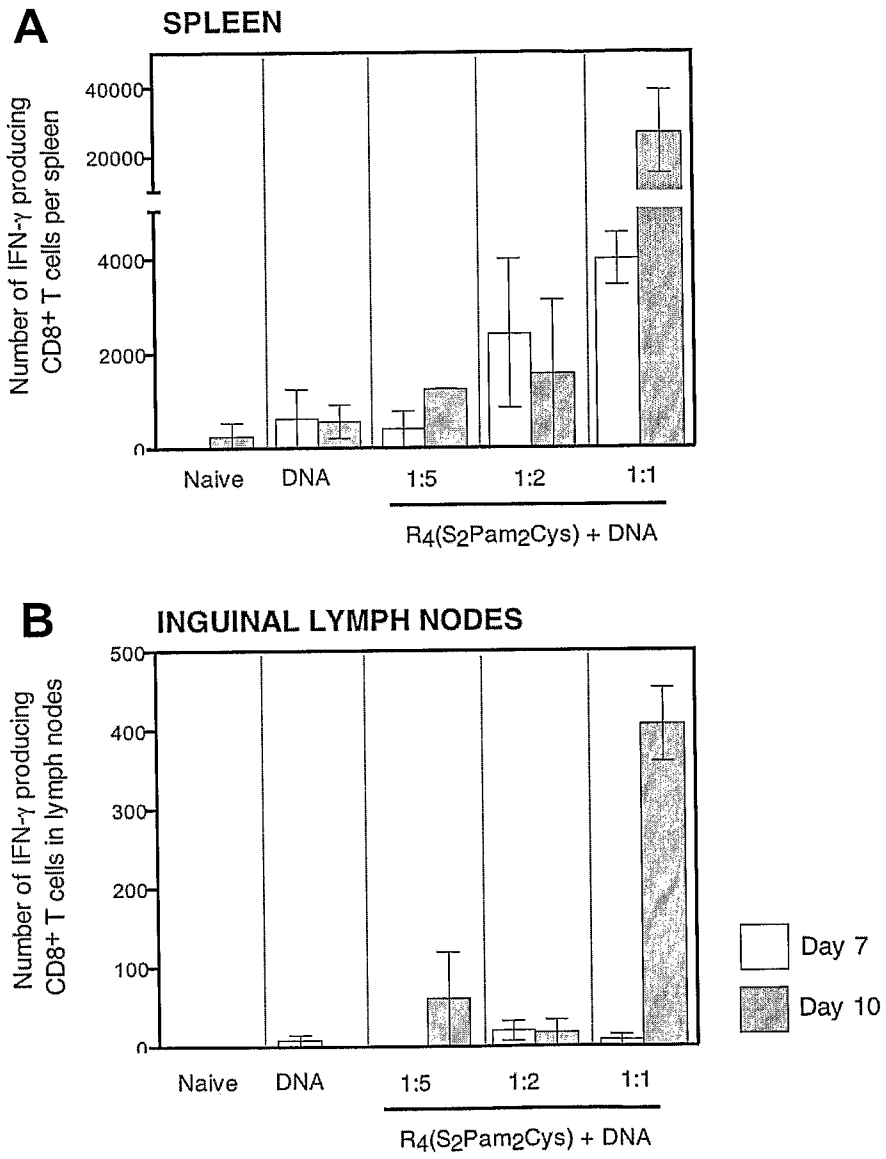


Figure 9



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2008/001501

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl. <b>A61K 39/00 (2006.01)</b> According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) <b>MEDLINE, CAPLUS, BIOSIS, WPI, EPODOC (TLR,TOLL LIKE RECEPTOR, TLR 1-10, LIPID, LIPOPEPTIDE, LIPOPROTEIN, LIPOPOLYSACCHARID, CATION, POSITIVE, CHARGE, LYSINE, ARGININE, VACCINE)</b>		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No....
X	WO 2007/103322 A2 (VAXINATE CORPORATION) 13 September 2007 (Abstract, pages 6 and 7, page 26 lines 5- page 35 line 13, pages 48-50, page 52 line 23-62 line 31, Examples, Claims)	1-11 and 17-20
X	WO 2005/070959 A2 (VIEVEX CORP) 04 August 2005 (Abstract, page 2 lines 16-25, page 19 line 4-page 10 line 4, page 31 line 1- page 33 lines 3, page 36 lines 1-28)	1-8, 10, 11 and 17-20
X	EP 1666056 A1 (BESTEWIL HOLDING B.V) 07 June 2006 (Abstract, ([0029]-[0031]))	1-7, 17-19
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "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 "T" 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 "X" 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 "Y" 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 "&" document member of the same patent family		
Date of the actual completion of the international search 01 December 2008		Date of mailing of the international search report 08 DEC 2008
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaaustralia.gov.au Facsimile No. +61 2 6283 7999		Authorized officer <b>ANITA PREMKUMAR</b> AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2515

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2008/001501

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JACKSON, D.C. et al; (2004) Proceedings of the National Academy of Sciences, Vol 101(43): 15440-45. "A totally synthetic vaccine of generic structure that targets Toll-like receptor 2 on dendritic cells and promotes antibody or cytotoxic T cell responses." (Abstract, page 15440 col 2 para 2-page 15441 col 1 para 1, Fig 1)	1-5, 7-11, 17, 19 and 20

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/AU2008/001501**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2007103322	AR	059772	AU	2007224034	CA	2638760
		EP	1991264	US	2007224205	US	2007253982
WO	2005070959	US	2006002941				
EP	1666056	NONE					

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX