

New adjuvantField of the invention

The invention relates to a new adjuvant and to its use as a vaccine in combination
5 with an antigen.

Background of the invention

Adjuvants are defined as substances whose role is to boost or direct antigen-specific immune responses when used in combination with specific antigens (Wack, A.,
10 et al (2005) Curr. Opin. Immunol. 17, 411–418.). Usually, adjuvants combined with antigens, as is the case with all currently available commercial vaccines, do not induce immune responses against themselves. Due to the poor immunogenic properties of most antigens, adjuvants are used to enhance, activate and direct the innate and adaptive immune responses to those antigens. The concept of adjuvants has been
15 extended to carriers that interact with surface molecules on specific cells of the immune system that operate at the interface between the immune system of the host and the administered antigen (Segal BH, et al, Drug Discov Today. 2006 Jun;11(11-12):534-40.). In doing so adjuvants help to stimulate the immune system and increase the response to the co-administered antigen. Therefore, adjuvants have been widely used
20 for the development of vaccines.

Adjuvants can be classified according to their physiochemical properties or mechanisms of action. The two major classes of adjuvants include compounds that directly act on the immune system such as bacterial toxins that stimulate immune responses, and molecules able to facilitate the presentation of antigens in a controlled
25 manner and behaving as a carrier. At present, a large number of adjuvants are used to increase the immunological features of antigens including oils, aluminium salts, proteins and nucleic acid (Steven G. Reed, et al (2003) Expert Rev. Vaccines 2, 167–188.).

In principle, due to the fact that the response against the antigen and the quality
30 of the immune response will depend to a large extent on the purity and nature of the adjuvant. The ideal adjuvant should be chemically and physically well defined in such a way as to facilitate quality control. Since in most cases the antigens are well defined, the control of the adjuvant specificity will ensure reproducible development of the final

antigen-specific immunological response. In this context, the adjuvants may not only elicit an immunological response against the antigen but also direct the immune response that the antigen elicited in the host. If the immune response goes in the appropriate direction, the nature of the adjuvant will substantially influence the value of the antigen as a therapeutic product. In addition to helping the induction of an immunological (humoral or cell-mediated) response against antigens, the objective of the adjuvant is to elicit immune effectors that result in the production of specific cytokines. Moreover, since the specificity and magnitude of immune responses induced by the antigen-adjuvant construct may largely depend on the nature of the host immune cells, the potency of the adjuvants cannot be analyzed without reference to the host. Thus, the immune responses induced by an antigen may vary depending on the nature of the adjuvant and of the nature of the host immune system.

There is always a need for new adjuvants since new vaccines are being developed and adjuvants are almost always needed in order to get an efficient induction of an immune response. New adjuvants may also confer new attractive properties to vaccines. For example, they can influence the type and direction of the immune response induced.

Summary of the Disclosure

In one embodiment, there is provided a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:

- i. nucleotide sequences encoding a polypeptide comprising an amino acid sequence that has at least 70% sequence identity with the amino acid sequence of SEQ ID NO:1 over its entire length,
- ii. nucleotide sequences comprising a nucleotide sequence that has at least 70% sequence identity with the nucleotide sequence of SEQ ID NO:2 over its entire length,
- iii. nucleotide sequences the complementary strand of which hybridizes to a nucleic acid molecule of sequence of (i) or (ii) and
- iv. nucleotide sequences which differ from the nucleotide sequences of (iii) due to the degeneracy of the genetic code,

for use as an adjuvant in the treatment or prevention of a disease or a condition, wherein said nucleotide sequence is operably linked to a nucleotide sequence encoding an antigen, wherein said antigen is associated or linked with said disease or condition,

wherein the nucleic acid molecule encodes a polypeptide which is able to elicit an immune response in a subject, said immune response being specific for said antigen.

In one embodiment, there is provided a polypeptide encoded by a nucleic acid molecule as identified above.

5 In one embodiment, there is provided a nucleic acid construct comprising a nucleic acid molecule as identified above.

In one embodiment, there is provided a composition comprising a nucleic acid molecule as identified above and/or a polypeptide as identified above and/or a nucleic construct as identified above.

10 In one embodiment, there is provided a nucleic acid molecule as defined above, a polypeptide as defined above a nucleic acid construct as defined above and/or a composition as defined above for use as a medicament, preferably wherein the medicament is a vaccine.

In one embodiment, there is provided the use of a nucleic acid molecule as identified above, a polypeptide as identified above, a nucleic acid construct as identified above and/or a composition as defined above for the manufacture of a medicament for treating a disease associated with an antigen or a condition associated with an antigen, said disease associated with an antigen or condition associated with an antigen being identified above, wherein said antigen:

- 20 i) is specific for said disease or condition, or
- ii) originates from an organism that causes said disease or condition in the subject, or
- iii) comprises an allergen causing an allergy if said disease or condition is said allergy, or
- 25 iv) comprises a cancer antigen associated with a cancer if said disease or condition is said cancer.

Description of the invention

We surprisingly show that the genetic fusion of particular protein fragments originating from a *Leishmania* species to a defined antigen is able to significantly increase the immunogenic potentiality of the fused antigen when the resulting chimeric protein is administered *in vivo* to mice. We also show that the protein resulting from the *in vivo* expression of the chimeric gene present in a DNA plasmid also induces a high

humoral response against the genetically fused antigen while the administration of the plasmid containing the gene coding for the antigen alone does not.

Nucleic acid molecule

- 5 In a first aspect there is provided a nucleic acid molecule represented by a nucleotide sequence selected from the group consisting of:
 - i. nucleotide sequences encoding a polypeptide or a peptide comprising an amino acid sequence that has at least 50% sequence identity or similarity with the amino acid sequence of SEQ ID NO:1,

ii. nucleotide sequences comprising a nucleotide sequence that has at least 50% sequence identity or similarity with the nucleotide sequence of SEQ ID NO:2 ,

iii. nucleotide sequences the complementary strand of which hybridizes to a nucleic acid molecule of sequence of (i) or (ii) and

5 iv. nucleotide sequences which differ from the sequence of a nucleic acid molecule under (iii) due to the degeneracy of the genetic code.

Said nucleic acid molecule is preferably for use as an adjuvant when said nucleic acid molecule is operably linked to a nucleotide sequence encoding an antigen as later defined herein.

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A nucleic acid molecule described in this invention is attractive since the encoded protein can be used as an adjuvant. An adjuvant is defined herein as a molecule which is able to present an antigen to the immune system in such a way that an immune response, or an increase thereof, is elicited against said antigen when the antigen is administered in combination with the adjuvant. To analyze the antigen-specific elicited immune response, said immune response is compared to the immune response induced in presence of the antigen without the adjuvant. The induction is assessed in a subject or in cells from a subject.

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20 In this context, the antigen-specific elicited immune response is synonymous with the induced immune response against said antigen or the increase in the induction of an immune response against said antigen or a detectable immune response against said antigen. Eliciting an antigen-specific immune response may be replaced with inducing, enhancing, or increasing an immune response against an antigen.

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An immune response may be a B and/or a T cell response. An immune response may be a B cell response, i.e. production of an antibody specifically directed against said antigen. An antibody is preferably an IgG antibody, more preferably an IgG2a and/or an IgG1 antibody. An immune response may be a T cell response, preferably a Th1 response, a Th2 response or a balanced Th2/Th1 response. The skilled person knows that depending on the disease, a B and/or T cell response may be required to be induced to control it. The production of said antibody could be assessed by ELISA, preferably as carried out in the examples. Alternatively said immune response may be detected by measuring the production of cytokines such as, for example, IFN γ , IL-6,

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TNFalpha, or IL-10. The production of such cytokines could be assessed by ELISA, preferably as carried out in the examples.

In a preferred embodiment, the detection of the antigen-specific elicited immune response means that said detection occurs after at least one, two, three, four, five, six, 5 seven, eight, nine, ten, eleven, twelve hours or more or after at least one day of administration of said adjuvant and antigen, or at least two days, or at least three days, or at least four days or more. The detection is assessed in a subject or in cells from a subject, preferably as carried out in the examples.

In the context of the invention, the antigen-specific elicited immune response 10 preferably means a detectable immune response against said antigen. A detectable increase is preferably an increase of at least 5% of the amount of an antibody and/or of a cytokine as already identified herein, or 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 150%, 200% or more after at least one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve 15 hours or more or after at least one day of administration of said adjuvant and antigen, or at least two days, or at least three days, or at least four days or more. The detection is assessed in a subject or in cells from a subject, preferably as carried out in the examples.

20 Preferably, said amino acid sequence and/or or nucleotide sequence having at least 50%, 60%, 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity or similarity with a specific identified amino acid and/or nucleotide sequence as defined earlier herein (SEQ ID NO:1 respectively SEQ ID NO:2) are said to be functional when the encoded 25 polypeptide qualifies as an adjuvant. Said polypeptide, represented by said amino acid sequence, is capable of eliciting, inducing, enhancing, or increasing an immune response against an antigen when used with said antigen to at least some extent. To "at least some extent" preferably means that at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100% of the antigen-specific immune response detected 30 using SEQ ID NO:1. Eliciting, inducing, enhancing, or increasing an immune response against an antigen has been earlier defined herein.

A nucleic acid molecule as defined herein is preferably a molecule comprising at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27,

28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 or more contiguous nucleotides of SEQ ID NO:2, and whose encoded polypeptide is able to elicit, induce, enhance, or increase an immune response against an antigen when used with an antigen as earlier defined herein. In a preferred embodiment, said nucleic acid molecule as defined herein is preferably a molecule comprising at least 762, 765, 770, 780, 790, 800, 810, 820, 830, 840 850, 860, 870, 880, 890, 900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000 or 1110 contiguous nucleotides of SEQ ID NO: 2. In a preferred embodiment, said nucleic acid molecule as defined herein is preferably a molecule comprising at most 1110, 1000, 990, 980, 970, 960, 950, 940, 930, 920, 910, 900, 890, 880, 870, 860, 850, 840, 830, 820, 810, 800, 790, 780, 770 or 765 contiguous nucleotides of SEQ ID NO: 2.

A polypeptide as defined herein is preferably a polypeptide comprising at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 or more contiguous amino acids of SEQ ID NO:1 and which is able to elicit, induce, enhance, or increase an immune response against an antigen when used with an antigen as earlier defined herein. In a preferred embodiment, said nucleic acid molecule as defined herein is preferably a molecule comprising at least 254, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360 or 370 contiguous amino acids of SEQ ID NO: 1. In a preferred embodiment, said nucleic acid molecule as defined herein is preferably a molecule comprising at most 370, 360, 350, 340, 330, 320, 310, 300, 290, 280, 270, 260 or 254, contiguous amino acids of SEQ ID NO: 1. The polypeptide consisting of SEQ ID NO:1 is also called AAP (Augmentor and Activator Protein).

An amino acid or nucleotide sequence, encompassed by the present invention, may be derived from one of the sequences as identified herein by substituting, inserting, deleting, or adding one, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides or amino acids, respectively. An amino acid sequence, encompassed by the present invention, may be derived from one of the sequences as identified herein by adding additional N- or C-terminal amino acids or chemical moieties to increase stability, solubility and immunogenicity. In an embodiment, an amino acid sequence encompassed by the present invention is derived from SEQ ID NO:1 by conservative substitution of at least one amino acid present in SEQ ID NO:1. Said amino acid that may be replaced may be a histidine. The skilled person knows that histidine may be substituted by asparagine or

glutamine (i.e. conservative substitution as later defined herein). Therefore in an embodiment, an amino acid sequence encompassed by the invention is derived from SEQ ID NO:1 and comprises 1, 2, 3, 4, 5, or 6 histidines at the most. Accordingly, in an embodiment, a nucleic acid sequence encompassed by the invention is derived from
5 SEQ ID NO:2 and encodes for an amino acid sequence that comprises 1, 2, 3, 4, 5, or 6 histidines at the most. In an embodiment, said amino acid sequence does not comprise any histidine and/or said corresponding nucleic acid sequence codes for an amino acid sequence that does not comprise a histidine.

Accordingly, in an embodiment, a nucleic acid molecule is represented by a nucleotide
10 sequence selected from the group consisting of:

i. nucleotide sequences encoding a polypeptide or a peptide comprising an amino acid sequence that has at least 50% sequence identity or similarity with the amino acid sequence of SEQ ID NO:1,

ii. nucleotide sequences comprising a nucleotide sequence that has at least 50%
15 sequence identity or similarity with the nucleotide sequence of SEQ ID NO:2 ,

iii. nucleotide sequences the complementary strand of which hybridizes to a nucleic acid molecule of sequence of (i) or (ii) and

iv. nucleotide sequences which differ from the sequence of a nucleic acid molecule under (iii) due to the degeneracy of the genetic code

and wherein said nucleic acid molecule codes for an amino acid sequence that
20 comprises 1, 2, 3, 4, 5 or 6 histidines at the most. Preferably said nucleic acid molecule codes for an amino acid sequence that does not comprise a histidine. Preferably in said nucleic acid molecule, at least one codon coding for histidine or at least 2, 3, 4, 5 or 6 codon coding for histidines have been substituted by a codon coding for asparagine or
25 glutamine.

In a preferred embodiment, a nucleic acid molecule of the invention being represented by a nucleotide sequence as defined earlier herein further comprises a nucleotide
30 sequence encoding an antigen. We found that the immune response elicited by an adjuvant of the invention was optimal when the corresponding nucleotide sequence encoding an antigen was comprised within, or fused to, or operably linked to, a nucleic acid molecule encoding the adjuvant as earlier defined herein. Therefore the adjuvant

of the invention is preferably used in such a way that its encoding nucleotide sequence is fused to or operably linked with a nucleotide sequence encoding an antigen.

In a preferred embodiment, said nucleic acid molecule of the invention is free of or does not comprise a sequence encoding for a polypeptide is identical or is at least 99%,
5 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%,
83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75% to SEQ ID NO: 3 over its entire length.

An antigen is defined herein as a molecule which is able to be recognized by an
10 antibody raised against said antigen when said molecule is present in a subject. An antigen which is able to induce a specific immune response from a subject when said antigen is present in said subject is said to be immunogenic or to be an immunogen. An immune response is preferably as defined earlier herein. An antigen is preferably a polypeptide or a peptide. A peptide may comprise 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,
15 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 amino acids or more. An antigen may be a protein fragment or a full length protein originating from an organism as identified later herein. It is also possible to use several antigens from the same organism in order to be more effective in combating the organism. An antigen may also be defined by reference to an encoding nucleic acid molecule represented by a nucleic
20 acid sequence.

The source of an antigen may be a protein, a digest of the protein and/or a fragment thereof, which may be in a purified form or may be comprised within a crude composition, preferably of biological origin, such as a bacterial lysate, parasitic lysate, yeast lysate, viral lysate, fungal lysate, sonicate or fixate. Alternatively, an antigen may
25 be chemically synthesized or enzymatically produced *in vitro*. The source of a protein, or fragment thereof as antigen, may also be a nucleic acid encoding said, or fragment thereof, from an RNA or DNA template. The RNA or DNA molecules may be 'naked' DNA, preferably comprised in vesicles or liposomes, or they may be comprised in a nucleic acid construct or a vector. The vector may be any (recombinant) DNA or RNA
30 vector known in the art, and preferably is a plasmid; wherein genes encoding latency antigens are operably linked to regulatory sequences conferring expression and translation of the encoded messengers. The vector may also be any DNA or RNA virus, such as, but not limited to, Adenovirus, Adeno-Associated Virus (AAV), a retrovirus, a

lentivirus, modified Vaccinia Ankara virus (MVA) or Fowl Pox virus, or any other viral vector capable of conferring expression of a polypeptide into a chosen subject. DNA vectors may be non-integrating, such as episomally replicating vectors, or may be vectors integrating in the host genome by random integration or by homologous recombination.

DNA molecules comprising genes encoding an antigen protein, or fragments thereof according to the current invention, optionally embedded in a vector such as a virus or plasmid, may be integrated in a genome of a subject. In a preferred embodiment of the invention, such a host may be a micro-organism. Preferably such a recombinant micro-organism is a *Mycobacterium*, for instance of the species *M. tuberculosis*, *M. smegmatis* or *M. bovis* and most preferably *M. bovis* Bacillus Calmette Guerin (BCG) or *M. smegmatis*, capable of delivering to a host the polypeptides or fragments thereof according to the invention (as described in Yue. Y. et al, (2007), J. Virol. Meth., 141: 41-48, Cayabiyab Y. et al, (2006), J. Virol., 80: 1645-1652). Recombinant BCG and methods for recombination are known in the art; for instance, in WO2004094469. Such a recombinant micro-organism may be formulated as a live recombinant and/or live attenuated vaccine, as shown in Jacobs et al. 1987, Nature, 327(6122):532-5. The vector may also be comprised in a host of bacterial origin, such as, but not limited to, live-attenuated and/or recombinant *Shigella* or *Salmonella* bacteria.

In the context of the invention, a subject means a human or an animal. An animal which is encompassed within the scope of the invention includes a mammal, preferably a dog.

An antigen may originate from any organism known to be associated with a disease or a condition in a subject. An antigen may originate from a microorganism such as a bacterium, a yeast, a fungus, a parasite. Alternatively an antigen may originate from a virus. An antigen may also be a self or auto antigen as, for example, those associated or linked with cancer or a tumor antigen. Antigens may also be associated with or linked to allergic diseases.

A disease may be a parasitic disease. In this case, an antigen may originate from a protozoan belonging to the apicomplexa (such as *Plasmodium*) and/or kinetoplastidae phylum, and in particular members of the trypanosomatid family, more in particular different species of the trypanosomatal protozoan *Leishmania*. There are over 20 known species of *Leishmania*, including species of the subgenus *Leishmania*, comprising the complex *L. major*, including *L. major*, the complex *L. Donovan*, including *L. chagasi*, *L. donovani* and *L. infantum*, the complex *L. Mexicana*, including *L. amazonensis* and *L. mexicana*, as well as the subspecies *Viannia*, comprising the complex *L. braziliensis*, including *L. braziliensis* and *L. peruviana* and the complex *L. guyanensis*, including *L. guyanensis* and *L. panamensis*. In a preferred embodiment, an antigen originates from a *Leishmania* species, preferably *Leishmania major* and/or *Leishmania infantum*. In another preferred embodiment, an antigen originates from a *Plasmodium* species. *Plasmodium* species of particular interest are *Plasmodium falciparum* and *Plasmodium vivax*.

For example, if an antigen originates from a parasite, preferably from a *Leishmania* species causing leishmaniasis, said compounds could be selected from a group consisting of a source of other proteins from a parasite causing a parasitic disease as described in the publication by Iborra, S. et al. (Iborra, S., et al 2004. *Vaccine* 22:3865-76). A preferred protein source of antigens is in this context a histone such as a H2A, H2B, H3, H4, or a ribosomal protein such as LiP0, L2, L7, L8, L16, S6, L3, L5 and S4. A preferred H2A protein is represented by SEQ ID NO:3. A preferred nucleic acid encoding a H2A is represented by SEQ ID NO:4. A preferred H2B protein is represented by SEQ ID NO:5. A preferred nucleic acid encoding a H2B is represented by SEQ ID NO:6. A preferred H3 protein is represented by SEQ ID NO:7. A preferred nucleic acid encoding a H3 is represented by SEQ ID NO:8. A preferred H4 protein is represented by SEQ ID NO:9. A preferred nucleic acid encoding a H4 is represented by SEQ ID NO:10. A preferred LiP0 protein is represented by SEQ ID NO:11. A preferred nucleic acid encoding a LiP0 is represented by SEQ ID NO:12. A preferred L2 protein is represented by SEQ ID NO:13. A preferred nucleic acid encoding L2 is represented by SEQ ID NO:14. A preferred L7 protein is represented by SEQ ID NO:15. A preferred nucleic acid encoding a L7 is represented by SEQ ID NO:16. A preferred L8 protein is represented by SEQ ID NO:17. A preferred nucleic acid encoding a L8 is

represented by SEQ ID NO:18. A preferred L16 protein is represented by SEQ ID NO:19. A preferred nucleic acid encoding a L16 is represented by SEQ ID NO:20.. A preferred S4 protein is represented by SEQ ID NO:21. A preferred nucleic acid encoding a S4 is represented by SEQ ID NO:22. A preferred S6 protein is represented by SEQ ID NO:23 . A preferred nucleic acid encoding a S6 is represented by SEQ ID NO:24. A preferred L3 protein is represented by SEQ ID NO:25. A preferred nucleic acid encoding a L3 is represented by SEQ ID NO:26. A preferred L5 protein is represented by SEQ ID NO:27. A preferred nucleic acid encoding a L5 is represented by SEQ ID NO:28.

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Another example is the use of poly-proteins containing several parasite antigens as seen in Stober et al and Aebischer, et al and Poot et al. (Stober C.B.U.G., et al (2006), *Vaccine*, 24: 2602-2616; Aebischer T., et al, (2000) *Infection and Immunity*, 68: 1328-1336; and Poot J et al, (2009), *Vaccine*, 27: 4439-4446).

15 In coming paragraph, a histone protein may also be used as a protein source of antigens. Preferred compounds include a histone protein or fragment thereof, or a nucleic acid molecule encoding said histone or said histone fragment. More preferably, a histone protein is H2A, H2B, H3 and/or H4 as identified in EP 1 687 023. Histones H2A, H2B, H3 and H4 are well-conserved nuclear proteins and their sequences are well-known in the art (Requena, J. M., et al 2000; Parasitol Today 16:246-50).

20 Preferably, the histones are obtained from an organism which is close to the disease causing organism in the evolutionary tree. Therefore, of particular interest as a source of histones to be used in the treatment of parasitic diseases such as Leishmaniasis are protozoans, as for example plasmodium. Additionally, of interest are members of the trypanosomatid family, more in particular different species of the trypanosomatal protozoan *Leishmania*.

25 Other preferred compounds include other ribosomal protein or fragment thereof or a nucleic acid molecule encoding said protein or fragment thereof. Examples of other ribosomal proteins include L19 and S4.

30 Other preferred compounds include a Ribosomal Protein Extract as identified in WO 2009/090175.

A disease may include, but is not limited to, allergy or a cancer. Any type of antigen known to be associated or be specific with a cancer may be used in the context of the

invention. Such type of antigen may also be named a tumor antigen. A tumor antigen may be a protein product of a mutated oncogene or a mutated tumor suppressor gene, or a protein product of any gene or mutated gene known to be expressed in a tumor or in a cancer. A tumor antigen may be a protein product of an overexpressed or aberrantly expressed gene. A tumor antigen may be a protein product of an oncogenic virus. A tumor antigen may be a protein product of an oncofetal gene. Examples of tumor antigen include protein product of the following genes: alphafetoprotein (AFP), carcinoembryonic antigen (CEA), epithelial tumor antigen (ETA), Melanoma-associated antigen (MAGE), p53 or CD20. A tumor antigen as defined herein may also be part of a protein product, i.e. a polypeptide, a peptide derived from a protein product of a gene as identified herein.

A preferred cancer antigen include CD20 or a fragment thereof. CD20 is expressed in some B cell malignancies. A preferred amino acid sequence representing CD20 is identified as SEQ ID NO:37. A preferred antigen in this context comprises 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 contiguous amino acids or more of SEQ ID NO:37 and/or has at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % identity or similarity with SEQ ID NO:37.

A cancer may be gastric adenoma and/or breast tumour. A preferred cancer antigen includes a S100A2 protein or a fragment thereof. The S100A2 protein is a calcium-binding protein that is up regulated in association with human gastric adenocarcinoma (1) and breast (2) tumour progression. A preferred amino acid sequence representing S100A2 is identified as SEQ ID NO: 42. A preferred nucleic acid sequence coding for a preferred S100A2 is identified a SEQ ID NO: 43. A preferred antigen in this context comprises 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 contiguous amino acids or more of SEQ ID NO:42 and/or has at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % identity or similarity with SEQ ID NO:42.

A disease may be allergy. An antigen may also be associated with or linked to an allergic disease. Examples of preferred allergic antigens include an allergen from a *Cupressus* species, preferably *Cupressus arizonica* (Cupa4 or Cupa1). A preferred nucleic acid sequence representing coding for a preferred Cupa4 is identified as SEQ ID NO:38. A preferred nucleic acid sequence representing coding for a preferred Cupa1 is identified as SEQ ID NO:39. A preferred amino acid sequence representing a

preferred Cupa4 is identified as SEQ ID NO:40. A preferred amino acid sequence representing a preferred Cupa1 is identified as SEQ ID NO:41. A preferred antigen in this context comprises 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 contiguous amino acids or more of an amino acid sequence encoded by SEQ ID NO:38 or 39 and/or has at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % identity or similarity with an amino acid sequence encoded by SEQ ID NO:38 or 39.

Another preferred antigen in this context comprises 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 contiguous amino acids or more of an amino acid sequence represented by SEQ ID NO:40 or 41 and/or has at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 99 % identity or similarity with an amino acid sequence represented by SEQ ID NO:40 or 41.

However, the skilled person will understand that the invention is not limited to this specific antigen.

Each of the antigens identified herein (i.e. proteins, parts thereof, polypeptide or peptide) is preferably used with or fused to a polypeptide being an adjuvant as earlier defined herein. It means that in a preferred embodiment, a nucleic acid molecule encoding an antigen is operably linked with a nucleic acid molecule of the invention as earlier defined herein whose encoded polypeptide functions as an adjuvant. In a more preferred embodiment, said nucleic acid molecule encoding an antigen being operably linked with a nucleic acid molecule of the invention as earlier defined herein encoding a polypeptide which functions as an adjuvant is one single nucleic acid molecule, encoding one single polypeptide. Said polypeptide may be called a chimeric polypeptide. This chimeric polypeptide comprises or consists of an antigen fused to an adjuvant of the invention. This chimeric polypeptide may comprise one or more additional amino acids at the 5' and/or at the 3' and/or between the antigen and the adjuvant.

The invention therefore provides a nucleic acid molecule as earlier defined herein encoding a polypeptide which is able to behave as an adjuvant for a given antigen, when this nucleic acid sequence is operably linked to a nucleotide sequence encoding

said antigen. In a preferred embodiment, this nucleic acid molecule encodes a polypeptide which is able to induce an antigen-specific immune response in a subject.

Therefore the invention encompasses two types of nucleic acid molecules:

- one comprising or consisting of a nucleic acid molecule encoding an adjuvant,
- 5 -one comprising or consisting of a nucleic acid molecule encoding an adjuvant fused to a nucleic acid molecule encoding an antigen.

Depending on the type of source used (protein-based or nucleic acid-based), the skilled person will know which type of formulation is suited. An antigen may be administered
10 as such (naked protein or nucleic-acid). Alternatively, a nucleic acid-based source may be administered using a nucleic acid construct as defined herein. Preferably a protein-based formulation is chosen. More preferably, a chimeric polypeptide as earlier identified herein is used.

- 15 In another embodiment, an antigen may originate from a virus. Any virus that causes a disease in humans from which antigens are known is encompassed within the scope of the present invention.

In an other embodiment, an antigen may originate from a yeast, a fungus, an allergen or
20 a cancer cell or any other pathological cell. Any yeast or fungus that causes a disease in humans from which antigens are known is encompassed within the scope of the present invention.

Accordingly, a nucleic acid molecule of the invention encodes a polypeptide,
25 preferably a chimeric polypeptide as identified herein which is able to induce an antigen-specific immune response when said nucleic acid molecule comprises a nucleotide sequence encoding an antigen.

Polypeptide

- 30 In a further aspect, there is provided a polypeptide encoded by a nucleic acid molecule as earlier identified herein. This polypeptide comprises an adjuvant and preferably an antigen as defined in the previous section.

"Polypeptide" as used herein refers to any peptide, oligopeptide, polypeptide, gene product, expression product, or protein. A polypeptide is comprised of consecutive amino acids. The term "polypeptide" encompasses naturally occurring or synthetic molecules.

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Nucleic acid construct

In a further aspect, there is provided a nucleic acid construct comprising a nucleic acid molecule as identified in the previous section. This nucleic acid construct may comprise a nucleic acid molecule encoding a polypeptide defined as an adjuvant in the previous section. This nucleic acid construct may comprise a nucleic acid molecule encoding a polypeptide defined as an adjuvant and a nucleic acid molecule encoding an antigen as defined in the previous section.

The invention also relates to an expression vector comprising a nucleic acid construct of the invention. Preferably, an expression vector comprises a nucleotide sequence of the invention, which is operably linked to one or more control sequences, which direct the production or expression of the encoded polypeptide in a cell, a subject, or a cell-free expression system. An expression vector may be seen as a recombinant expression vector.

Accordingly, a nucleic acid molecule as defined herein encoding a polypeptide comprising or consisting or composed of an adjuvant, is preferably for use as a medicament, more preferably as an adjuvant. Accordingly, said polypeptide is preferably for use as a medicament, more preferably as an adjuvant.

Accordingly, a nucleic acid molecule as defined herein encoding a polypeptide comprising or consisting or composed of an adjuvant and an antigen is preferably for use as a medicament, more preferably as a vaccine against said antigen. Accordingly, said polypeptide is preferably for use as a medicament, more preferably as a vaccine against said antigen.

A vaccine of the invention may function as a therapeutic vaccine. Typically, there is a time period between contact with an antigen, i.e. infection and apparition of the first symptom of a disease associated with said antigen. In this case, a vaccine would act as a pharmacological immune product that would prevent and/or treat the disease and/or delay its progression by eliciting in the host an immune response that counteracts the

pathological effect of the disease. A therapeutic vaccine differs from a prophylactic vaccine in that a therapeutic vaccine will induce protection in a subject who already has the infection or the disease. In another embodiment, a vaccine is a prophylactic vaccine. A prophylactic vaccine may be administered to a subject before said subject
5 has been contacted with said antigen.

A medicament as defined herein is preferably administered parenterally, e.g. by injection or infusion by intravenous, subcutaneous, intraperitoneal, intramuscular, intraarterial or intralesional route. A preferred administration mode is subcutaneous. A
10 medicament may be combined with a pharmaceutically acceptable medium or delivery vehicle by conventional techniques known in the art. For example, a medicament may be dissolved in Phosphate buffer saline (PBS). Methods for preparing parenterally administrable compositions are well known in the art and described in more detail in various sources, including, for example, Remington's Pharmaceutical Sciences, Ed. AR
15 Gennaro, 20th edition, 2000, Williams & Wilkins, PA, USA. A medicament is preferably administered in a therapeutically effective dose, i.e. one that will increase the ability of the human or animal immune system to fight a disease or condition as defined herein. Preferably, a therapeutically effective dose of a medicament of the invention will prevent and/or delay the development of said disease or condition.
20 Depending on the disease or the condition, the skilled person will know which parameter or symptom associated with the development of said disease to choose in order to follow the development of the disease or condition.

The invention further encompasses the use of another distinct known adjuvant in
25 addition to a nucleic acid molecule or a polypeptide of the invention. Any known adjuvant may be used in the present invention. The skilled person knows several suitable adjuvants. Adjuvants are most preferably selected from the following list of adjuvants: cationic (antimicrobial) peptides, saponine and Toll-like receptor (TLR) ligands such as, but not limited to, poly(I:C), CpG motifs, LPS, lipid A, lipopeptide
30 Pam3Cys and bacterial flagellins or parts thereof, and their derivatives having chemical modifications. Other preferred adjuvants for use in the method and in compositions according to the invention are: mixtures with live or killed BCG, immunoglobulin complexes with the said latency antigens or parts thereof, IC31 (from

www.intercell.com; in WO03047602), QS21/MPL (US2003095974), DDA/MPL (WO2005004911), DA/TDB (WO2005004911; Holten-Andersen et al, 2004 Infect Immun. 2004 Mar;72(3):1608-17.) and soluble LAG3 (CD223) (from www.Immunotep.com; US2002192195). In addition, another preferred adjuvant

- 5 includes the use of *Corynebacterium parvum* or *Propionobacterium acnes* (Aebischer T., et al, (2000) Infection and Immunity., 68: 1328-1336, Poot J et al, (2009), Vaccine, 27: 4439-4446 and Ferreira J.H. et al, (2008), Vaccine, 26: 67-685).

Particularly preferred adjuvants are those that are known to act via the Toll-like receptors. Adjuvants that are capable of activation of the innate immune system, can be
10 activated particularly well via Toll like receptors (TLR's), including TLR's 1 – 10 and/or via a RIG-1 (Retinoic acid-inducible gene-1) protein and/or via an endothelin receptor. Compounds capable of activating TLR receptors, and modifications and derivatives thereof, are well documented in the art. TLR1 may be activated by bacterial lipoproteins and acetylated forms thereof; TLR2 may, in addition, be activated by
15 Gram positive bacterial glycolipids, LPS, LPA, LTA, fimbriae, outer membrane proteins, heatshock proteins from bacteria or from the host, and Mycobacterial lipoarabinomannans. TLR3 may be activated by dsRNA, in particular of viral origin, or by the chemical compound poly(I:C). TLR4 may be activated by Gram negative LPS, LTA, Heat shock proteins from the host or from bacterial origin, viral coat or envelope
20 proteins, taxol or derivatives thereof, hyaluronan containing oligosaccharides and fibronectins. TLR5 may be activated with bacterial flagellae or flagellin. TLR6 may be activated by mycobacterial lipoproteins and group B Streptococcus heat labile soluble factor (GBS-F) or Staphylococcus modulins. TLR7 may be activated by imidazoquinolines and derivatives. TLR9 may be activated by unmethylated CpG DNA
25 or chromatin – IgG complexes. In particular TLR3, TLR4, TLR7 and TLR9 play an important role in mediating an innate immune response against viral infections, and compounds capable of activating these receptors are particularly preferred for use in the invention. Particularly preferred adjuvants comprise, but are not limited to, synthetically produced compounds comprising dsRNA, poly(I:C), unmethylated CpG
30 DNA which trigger TLR3 and TLR9 receptors, IC31, a TLR9 agonist, IMSAVAC, a TLR4 agonist. In another preferred embodiment, an adjuvant is physically linked to a nucleic acid molecule as earlier defined herein. Physical linkage of adjuvants and costimulatory compounds or functional groups to the HLA class I and HLA class II

epitope comprising peptides, provides an enhanced immune response by simultaneous stimulation of antigen presenting cells, in particular dendritic cells, whose role is to internalize, metabolize and display antigens. Another preferred immune modifying compound is a T cell adhesion inhibitor, more preferably an inhibitor of an endothelin receptor such as BQ-788 (Buckanovich R.J., et al, (1994), Proc. Natl. Acad. Sci. USA, 91:4892.). BQ-788 is N-cis-2,6-dimethylpiperidinocarbonyl-L-gamma-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine. However any derivative of BQ-788 or modified BQ-788 compound is also encompassed within the scope of this invention.

Other adjuvants include MPL-SE (Glaxo Smithkline Biologicals, Belgium) or EM005 (IDRI, USA).

In a preferred embodiment, an adjuvant is a Th₁-promoting adjuvant (like an adjuvant comprising a CpG ODN motif). A Th₁-promoting adjuvant has been defined in the literature (Liu N., et al (2003), Nature Immunology, 687-693) as an adjuvant which is able to promote, or trigger, or induce, or induce an increase of, a Th₁ immune response against a given antigen when used together with this antigen as detected in supernatants of splenocytes of a treated subject when cultured with the antigen. As control, the promotion, or triggering, of a Th₁ immune response is assessed in a splenocyte population of the same subject which has not been treated with the antigen and the adjuvant, or with same population only treated with the antigen. Triggering or promoting a Th₁ immune response is preferably defined by the induction of IFN γ as detected by culturing splenocytes of a treated subject with the antigen and/or by inducing the production of antigen-specific IgG2a immunoglobulines. The assessment of the induction of this cytokine and of IgG2a has already been defined herein. In a preferred embodiment, a Th-1 promoting adjuvant is, or comprises, or consists of, an oligodeoxynucleotide. More preferably, an oligodeoxynucleotide (ODN) comprises, or consists of, CpG in which the C is non-methylated (CpG ODN): 3'purine-CpG-5'pyrimidine. A preferred oligodeoxynucleotide is, or comprises, or consists of, a phosphorothioate-modified ODN sequence. The use of oligodeoxynucleotides having such modification is advantageous since the oligodeoxynucleotides hence used are more stable than non modified oligonucleotides and hence will not easily be degraded once they are in the blood stream. A preferred Th-1 promoting adjuvant consists of, or comprises, at least one CpG motif, at least two, or at least three. Preferred sequences of

the immunostimulatory ODN (5' to 3') were TCAACGTTGA (SEQ ID NO:29) and GCTAGCGTTAGCGT (SEQ ID NO:30). The skilled person is not limited to the sequences explicitly described herein. He/she may design other sequences conveying the Th-1 promoting property as defined earlier herein.

5

In a preferred embodiment, a medicine (or medical preparation or pharmaceutical composition or medicament) as defined herein is used to increase the ability of a subject's immune system to fight against an infection and/or a disease, more preferably a parasitic infection and/or a parasitic disease. In particular, it may be used for administration to a human or animal subject. A medicine as defined herein is preferably administered parenterally, e.g. by injection or infusion by intravenous, subcutaneous, intraperitoneal, intramuscular, intraarterial, intranasal, or intralesional route. A preferred administration mode is subcutaneous. The invention is not limited to a specific mode of administration of a medicament or a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide as defined herein. A preferred mode of administration is oral administration using a capsule or a tablet. Alternatively a medicament or a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide as defined herein may be locally administered via a catheter or a pump, or a suppository. Alternatively, a medicament or a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide as defined herein may be topically administered. The formulation of a medicament or a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide as defined herein or of a composition comprising said compounds depends on the intended mode of administration and (therapeutic) application. A pharmaceutical carrier can be any compatible, non toxic substance suitable to deliver said compound to a subject. E.g. sterile water, or inert solids or excipients may be used as the carrier, usually complemented with pharmaceutically acceptable adjuvants, buffering agents, dispersing agents, and the like. Compositions will either be in liquid, e.g. a stabilized suspension of said compound, or a composition comprising said compound, or in solid and/or dry forms: e.g. powder. For oral and rectal administration, said compound can be administered in solid dosage forms, such as capsules, tablets, suppositories, and powders, or in liquid dosage forms, such as elixirs, syrups, creams, ointments, enemas, and suspensions.

30

Another form may be a semi-solid or semi-liquid form wherein said compound is present as a liquid form in, or on, a solid support such as a patch.

A medicine may be combined with a pharmaceutically acceptable medium or delivery vehicle by conventional techniques known in the art. For example, a medicament or a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide as defined herein, and optionally a second adjuvant, may be dissolved in Phosphate buffer saline (PBS). Methods for preparing parenterally administrable compositions are well known in the art and described in more details in various sources, including, for example, Remington's Pharmaceutical Sciences, Ed. AR Gennaro, 20th edition, 2000, Williams & Wilkins, PA, USA. A medicine is preferably administered in a therapeutically effective dose, i.e. one that will increase the ability of the human or animal immune system to fight an infection and/or a disease as defined herein. Preferably, a therapeutically effective dose of a medical preparation of the invention is able to elicit an immune response as defined herein: a dose is therapeutically effective when it is able to elicit the proper immune response, or induce or induce an increase of the proper immune response against a specific antigen in a treated subject as defined herein. Even more preferably, the elicited or induced immune response is a protective immune response. In a preferred embodiment, a medicine as defined herein is a vaccine. In a more preferred embodiment, at least 5, 10, 15 or 20 micrograms of a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide as defined herein is being used in a vaccine. Said vaccine may be administered at least once, twice, three times, four times or more. A vaccine, as defined herein, may be a prophylactic or a therapeutic vaccine. The volume in which a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide as defined herein may be dissolved may vary from 100-500 microliters.

Composition

Additionally, there is provided a composition comprising a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide and optionally a second adjuvant, preferably a Th₁-promoting adjuvant. Each feature of said composition has already been defined herein. In a preferred embodiment, this composition consists of a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide as identified herein, a preferred Th₁-promoting adjuvant is a CpG ODN. A preferred composition

comprises or consists of a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide and optionally a second adjuvant, preferably a Th₁-promoting adjuvant dissolved in PBS or a suitable buffer. As already defined herein, an antigen may already be present as being comprised within said peptide or polypeptide or as
5 being encoded by part of said nucleic acid molecule or being encoded by part of the nucleic acid molecule present in said nucleic acid construct. In a further preferred embodiment, it is also encompassed by the present invention that a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide and optionally a second adjuvant, preferably a Th₁-promoting adjuvant are sequentially administered.
10 Therefore, each component does not need to be physically present in one single composition as long as they are both administered to a subject.
Such composition may further comprise a pharmaceutically acceptable adjuvant and/or carrier.
Such composition is preferably for use as a medicine or as a medicament. The medicine
15 is preferably a vaccine. Medicine, adjuvant and vaccine have already been extensively defined herein.
A composition may be in the liquid, solid or semi-liquid or semi-solid form as already defined herein.
In a preferred embodiment, other compounds are used sequentially or simultaneously
20 with a nucleic acid molecule or a nucleic acid construct or a peptide or a polypeptide in order to improve the specificity of the therapeutic or prophylactic treatment. It is advantageous for example to use other compounds that will further enhance the immune response of the treated subject. More preferably, such compounds are not present in a single composition together with a nucleic acid molecule or a nucleic acid
25 construct or a peptide or a polypeptide .

Use

Accordingly, there is further provided the use of a nucleic acid molecule as identified herein encoding an adjuvant and a nucleic acid molecule encoding an antigen, a
30 corresponding peptide, a corresponding polypeptide, a corresponding nucleic acid construct and/or a corresponding composition for the manufacture of a medicament for treating a disease or a condition associated with an antigen as identified earlier herein.

Accordingly, there is further provided the use of a nucleic acid molecule as identified herein comprising a nucleic acid molecule encoding an adjuvant operably linked to a nucleic acid molecule encoding an antigen, a corresponding peptide, a corresponding polypeptide, a corresponding nucleic acid construct and/or a corresponding composition for the manufacture of a medicament being a vaccine for treating a disease or a condition associated with the antigen.

Each feature of this use has already been defined herein.

Method of treatment

10 In a further aspect, there is provided a method of treatment of a disease or a condition associated with an antigen, wherein said treatment comprises a nucleic acid molecule encoding an adjuvant and a nucleic acid molecule encoding an antigen, a corresponding peptide, a corresponding polypeptide, a corresponding nucleic acid construct and/or a corresponding composition.

15 Accordingly, there is further provided a method of treatment of a disease or a condition associated with an antigen as identified herein, wherein said treatment comprises a vaccine, said vaccine comprising a nucleic acid molecule as identified herein comprising a nucleic acid molecule encoding an adjuvant operably linked to a nucleic acid molecule encoding an antigen, a corresponding peptide, a corresponding polypeptide, a corresponding nucleic acid construct and/or a corresponding composition.

Each feature of this method has already been defined herein.

Definitions

25 Sequence identity

"Sequence identity" is herein defined as a relationship between two or more amino acid (peptide, polypeptide, or protein) sequences or two or more nucleic acid (nucleotide, polynucleotide) sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences. "Similarity" between two amino acid sequences is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one peptide or polypeptide to the sequence of a second peptide or polypeptide. In a preferred

embodiment, identity or similarity is calculated over the whole SEQ ID NO as identified herein. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heine, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include e.g. the GCG program package (Devereux, J., et al., Nucleic Acids Research 12 (1): 387 (1984)), BestFit, BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Mol. Biol. 215:403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215:403-410 (1990). The well-known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992); Gap Penalty: 12; and Gap Length Penalty: 4. A program useful with these parameters is publicly available as the "Ogap" program from Genetics Computer Group, located in Madison, WI. The aforementioned parameters are the default parameters for amino acid comparisons (along with no penalty for end gaps).

Preferred parameters for nucleic acid comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0; Gap Penalty: 50; Gap Length Penalty: 3. Available as the Gap program from Genetics Computer Group, located in Madison, Wis. Given above are the default parameters for nucleic acid comparisons.

Optionally, in determining the degree of amino acid similarity, the skilled person may also take into account so-called "conservative" amino acid substitutions, as will be clear to the skilled person. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulphur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Substitutional variants of the amino acid sequence disclosed herein are those in which at least one residue in the disclosed sequences has been removed and a different residue inserted in its place. Preferably, the amino acid change is conservative. Preferred conservative substitutions for each of the naturally occurring amino acids are as follows: Ala to ser; Arg to lys; Asn to gln or his; Asp to glu; Cys to ser or ala; Gln to asn; Glu to asp; Gly to pro; His to asn or gln; Ile to leu or val; Leu to ile or val; Lys to arg; gln or glu; Met to leu or ile; Phe to met, leu or tyr; Ser to thr; Thr to ser; Trp to tyr; Tyr to trp or phe; and, Val to ile or leu.

Hybridization conditions

Hybridization conditions for a nucleic acid molecule may have low or medium or high stringency (southern blotting procedures). Low or medium or high stringency conditions means pre-hybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200pg/ml sheared and denatured salmon sperm DNA, and either 25% or 35% or 50% formamide for low or medium or high stringencies respectively. Subsequently, the hybridization reaction is washed three times for 30 minutes each using 2XSSC, 0.2%SDS and either 55 °C or 65 °C, or 75 °C for low or medium or high stringencies respectively.

Nucleic acid construct, expression vector, operably linked, expression, control sequences

A nucleic acid construct is defined as a nucleic acid molecule which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acids which are combined or juxtaposed in a manner which would not otherwise exist in nature. A nucleic acid molecule is represented by a nucleotide sequence. Optionally,
5 a nucleotide sequence present in a nucleic acid construct is operably linked to one or more control sequences, which direct the production or expression of said peptide or polypeptide in a cell or in a subject.

"Operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the nucleotide sequence coding for the
10 polypeptide of the invention such that the control sequence directs the production/expression of the peptide or polypeptide of the invention in a cell and/or in a subject.

"Operably linked" may also be used for defining a configuration in which a sequence (i.e. defined as an adjuvant) is appropriately placed at a position relative to another
15 sequence coding for an antigen such that a chimeric polypeptide (i.e. comprising an adjuvant fused to an antigen) in a cell and/or in a subject is formed.

"Operably linked" refers to the genetic fusion of a sequence encoding a protein being able to behave as an adjuvant as defined herein to a sequence encoding an antigen as defined herein resulting in a chimeric nucleic acid sequence encoding a chimeric
20 protein.

Expression will be understood to include any step involved in the production of the peptide or polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification and secretion.

Control sequence is defined herein to include all components which are necessary or
25 advantageous for the expression of a polypeptide. At a minimum, the control sequences include a promoter and transcriptional and translational stop signals. Optionally, a promoter represented by a nucleotide sequence present in a nucleic acid construct is operably linked to another nucleotide sequence encoding a peptide or polypeptide as identified herein

30 An expression vector may be any vector which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of a nucleotide sequence encoding a polypeptide of the invention in a cell and/or in a subject. As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control

the transcription of one or more genes or nucleic acids, located upstream with respect to the direction of transcription of the transcription initiation site of the gene. It is related to the binding site identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites, and any other DNA sequences, including, but
5 not limited to, transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one skilled in the art to act directly or indirectly to regulate the amount of transcription from the promoter. Within the context of the invention, a promoter preferably ends at nucleotide -1 of the transcription start site (TSS).

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition the verb "to consist" may be replaced by "to consist essentially of" meaning that a product or a composition or a nucleic acid molecule or a peptide or polypeptide of a nucleic acid construct as defined herein may comprise additional component(s) than the ones specifically identified; said additional component(s) not altering the unique characteristic of the invention. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the elements is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Description of the figures

Fig. 1. Solid phase Kmp11. Humoral response against Kmp11 and against Q. A: IgG response against Kmp11 after ip and sc administration of Kmp11 (20 μ g). B: IgG response against Kmp11 after ip and sc administration of KAAP (5 μ g) and Kmp11 (1 μ g). C: IgG response against Q after ip and sc administration of KAAP (5 μ g). D: IgG response against Kmp11 after ip and sc administration of Q(5 μ g)+Kmp11(20 μ g). E: IgG response against Q after ip and sc administration of Q(5 μ g)+Kmp11(20 μ g). The bars indicate the response variability between the animals within a group.

Fig. 2. Solid phase Kmp11. Humoral response against Kmp11 and against Q. A: IgG Humoral response against Kmp11 after administration of KAAP(1 μ g) and Kmp11 (0,25 μ g) via ip and sc. B: Humoral response against Q after administration of KAAP (1 μ g) via ip and sc. C: IgG1 and IgG2a response against Kmp11 after ip and sc administration of KAAP (1 μ g). D: IgG1 and IgG2a response against Q after ip and sc administration of KAAP (1 μ g). The bars indicate the response variability between the animals within a group.

Fig. 3. Solid phase Kmp11. Humoral response against Kmp11 and against Q. A: IgG response against Kmp11 after sc administration of pRcKmp11 (20 μ g), KAAP (3 μ g) and pRcKAAP (20 μ g). B: IgG response against Q after sc administration of KAAP (3 μ g) and pRcKAAP (20 μ g). C: IgG1 and IgG2a response against Kmp11 after sc administration of pRcKmp11 (20 μ g), KAAP (3 μ g) and pRcKAAP (20 μ g). D: IgG1 and IgG2a response against Q after sc administration of KAAP (3 μ g) and pRcKAAP (20 μ g). The bars indicate the response variability between the animals within a group.

Fig. 4. Solid phase Kmp11. Cytokine production in non-stimulated and in KAAP stimulated spleen cells from PBS, KAAP and pRcKAAP injected mice. The bars indicate the variation between triplicate determinations. The units indicated in the Y axis are given in pg/ml. In the X axis the vaccinated groups are indicated.

Fig. 5. Solid phase Kmp11. Cytokine production in non-stimulated and in Kmp11 stimulated spleen cells from PBS, KAAP and pRcKAAP injected mice. The bars indicate the variation between triplicate determinations. The units indicated in the Y axis are given in pg/ml. In the X axis the vaccinated groups are indicated.

Fig. 6. Solid phase Cup a 4. Animals were immunized with three doses of PBS, 5 μ g of CAAP; 2 μ g of Cup a 4 and 2 μ g Cup a 4+3 μ g of AAP. A week after administration of the third dose sera were collected and analyzed for reactivity against Cup a 4. 1A-: IgG reactivity. 1B-: IgG1 reactivity. 1C-: IgG2a reactivity. The sera were tested at a dilution of 1/2000 for IgG and 1/1000 for IgG1 and IgG2a. Stars indicate statistical differences.

Fig. 7. Solid phase Cup a 4. Animals were immunized with two doses of of PBS, 5 μ g of CAAP; 2 μ g of Cup a 4 and 2 μ g of Cup a 4+3 μ g of AAP. A week after administration of the second dose sera were collected and analyzed for reactivity against Cup a 4. 2A-: IgG reactivity; 2B-: IgG1 reactivity; 1C-: IgG2a reactivity. The sera were tested at a dilution of 1/1600 for IgG and 1/400 for IgG1 and IgG2a. Stars indicate statistical differences.

Fig. 8. Solid phase Cup a 4. IgG reactivity of the sera from animals immunized with a single dose of PBS, 1.25 µg CAAP; 0.5 µg of Cup a 4 and 0.5 µg of Cup a 4+0.75 µg of AAP. A week after administration of the dose sera were collected and analyzed for reactivity against Cup a 4. The sera were tested at a dilution of 1/100. Stars indicate statistical differences.

Fig. 9. Solid phase Cup a 4. Animals immunized with two doses of PBS, 1.25 µg CAAP; 0.5 µg of Cup a 4 and 0.5 µg of Cup a 4+0.75 µg of AAP. A week after administration of the second dose sera were collected and analyzed for reactivity against Cup a 4. 4A-: IgG; 4B-, IgG1; 4C-, IgG2a. The sera were tested at a dilution of 1/4000 for IgG and 1/100 for IgG1 and IgG2a. Stars indicate statistical differences.

Fig. 10. Solid phase S100A2. Reactivity against S100A2 (In O.D. units). Four groups of animals (N=5) were immunized with a single dose of S100AAP (1.05 µg), S100A2 (0.3 µg), AAP (0.75 µg of AAP) and S100A2+AAP (0.3µg+0.75µg). PBS buffer was administered to another group of animals used as controls. The sera were obtained 15 days after administration of the proteins. The sera were analyzed at a dilution of 1/100

Fig. 11. Solid phase S100A2. Reactivity against S100A2 (In O.D. units). The same group of animals indicated in Fig 1 were immunized with S100AAP (1.05 µg), S100A2 (0.3 µg), AAP (0.75 µg of AAP) and S100A2+AAP (0.3µg+0.75µg) 15 days after administration of the first dose. The sera were obtained a week after. (A) IgG reactivity. (B) IgG1 reactivity. (C) IgG2 reactivity. The sera were analyzed at a dilution of 1/200. The stars indicate high statistical differences.

25

Fig. 12. Expression vector Cup a4 AAP

Fig. 13. Expression vector S100A2 AAP

Fig. 14. Reactivity against S100A2 (In O.D. units) after a third immunization. The same group of animals indicated in Fig 1 were immunized with S100AAP (1.05 µg), S100A2 (0.3 µg), AAP (0.75 µg of AAP) and S100A2+AAP (0.3µg+0.75µg) 15 days after the administration of the second dose. The sera were obtained a week after. (A)

IgG reactivity. (B) IgG1 reactivity. (C) IgG2a reactivity. The sera were analyzed at a dilution of 1/6400. The stars indicate statistical differences ($p < 0.05$).

5

Examples

Section I

Materials and Methods

Animals and immunization. Female 6–8-week-old BALB/c mice were purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). The immunization of the animals
10 was done by an intraperitoneal (ip) or subcutaneous (sb) route as indicated in the legends of the Figures included in the Results Section. The mice were bled by orbital plexus puncture.

Construction of the DNA plasmid expressing the chimeric Kmp11AAP gene.

The DNA sequences coding for the amino and carboxyl terminal ends of the H2A
15 antigenic determinants were removed from the chimeric clone pPQ (Soto M, et al. J Clin Microbiol. 1998 Jan;36(1):58-63.) by Bam HI digestion. The nucleic acid sequence of PQ or Q is represented by SEQ ID NO: 46. The amino acid sequence of PQ or Q is represented by SEQ ID NO: 47. The resulting clone without the sequences coding for H2A determinants was named pAAP (SEQ ID NO:2). The DNA sequence
20 coding for the Kmp11 protein was obtained by PCR amplification of the DNA sequence coding for that protein present in plasmid pBLs-KMP-11 (Fuertes M.A., et al , J Biol Inorg Chem 6 (2001) 107-117.) using as primers the forward 5' CGGGATCCTTTAATGGCCACCGTACGAGGAG3' (SEQ ID NO: 31) and the reverse 5'CGGGATCCCCCCTTGATGGGTACTGCGCAGC3' (SEQ ID NO: 32)
25 oligonucleotides. Then, the PCR product coding for the Kmp11 protein was digested with Bam HI and inserted into pAAP (the Bam HI digested pPQ clone lacking the H2A determinants) (SEQ ID NO:33). The resulting clone, called pKmp11AAP, was transformed into E. coli (strain M15). The chimeric purified protein expressed by the pKmp11AAP clone was called KAAP (SEQ ID NO:34). In the 10th-12th position a
30 TTA triplet was included to avoid the selection of clones having an insertion of the sequence coding for Kmp11 in the reverse orientation. In the 9th-11th position a triplet coding for glycine was introduced to provide flexibility to the intersection between the Kmp11 protein and the protein fragment coded by pAAP.

Construction of plasmid pRcKAAP.

To construct the DNA plasmid containing the DNA sequence coding for the KAAP protein, the pKmp11AAP plasmid indicated above was PCR amplified using as primers the forward 5'-CCCAAGCTTATGGCCACCTACGAGGAG-3' (SEQ ID NO:35) and the reverse 5'-CATTACTGGATCTATCAACAGG-3' (SEQ ID NO:36). The DNA sequence was inserted into a pRc/CMV Hind III digested plasmid. Plasmid pRC is commercially available by In vitrogen. The DNA plasmids were purified by an endotoxin free Giga-preparation Kit (Qiagen, Hilden, Germany).

Protein purification.

The purification of the recombinant protein KAAP, expressed by clone pKmp11AAP, as well as the recombinant Q and Kmp11 proteins (Soto M, et al. J Clin Microbiol. (1998) Jan;36(1):58-63, and Planelles L, et al, Immunol Cell Biol. (2002);80(3):241-7.), was performed on Ninitrilotriacetic acid resin columns under denaturing conditions, according to the method provided by the supplier (Qiagen).

Expression of the KAAP and Kmp11 proteins by pRcKAAP.

COS7 cells were transfected with 20µg of the pRcKmp11AAP or pRcKmp11 plasmids using the Lipofectin® Reagent (Gibco, BRL) according to the manufacturer's protocol. Briefly, 3×10^6 competent cells were seeded on 100 mm plates in Dulbecco's modified Eagle's medium plus 5% FCS and transfected when they reached 50–75% confluence. Seventy-two hours post-transfection, the cells were harvested, washed two times with ice-cold PBS and immediately lysed by addition of Laemmli's buffer. Proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham, Aylesbury, UK). The blots were probed with a serum from mice immunized with the KAAP protein. A protein band of the expected size reacting with anti-KAAP was observed in the blots.

Analysis of the humoral response.

Serum samples were analyzed for specific antibodies against Q or Kmp11. Briefly, the wells of standard ELISA plates were coated overnight at room temperature with 100µl of PBS containing 2µg/ml Q or Kmp11. The IgG and the isotype-specific analysis was

done using horseradish peroxidase-conjugated anti-mouse immunoglobulins (Nordic Immunological Laboratories, Tilburg, The Netherlands): Dilution of anti IgG and anti-IgG1 (1:1000) and anti-IgG2a (1:500). Ortophenylenediamine dihydrochloride - OPD- (Dako, A/S, Glostrup, Denmark) was used as substrate. After 15min, the reaction
5 was stopped by the addition of 100µl of 1 M H₂SO₄. The absorbance was read at 450 nm.

Cytokine analysis.

Spleens were removed from mice one week after the last immunization under aseptic
10 conditions on a sterile dish containing DMEM medium. Single cell suspensions were prepared by grinding the spleen using an autoclaved mesh. 5–10 ml of DMEM medium was added to it and the contents were mixed to homogeneity. The clear supernatant was pipetted out slowly. Cells were pelleted by centrifugation at 4°C at 250 g (Sorvall RC-5 centrifuge, HB-4 rotor) for 10 min. The pellet containing erythrocytes and splenocytes
15 were collected. The pellet was washed once with 0.9% ammonium chloride to lyse the erythrocytes. The splenocytes from each mouse in a group were pooled and resuspended to a density of 10⁷ cells/ml in RPMI containing 10% FCS and 0.05 mM 2-mercaptoethanol, then divided into 200 µl aliquots (5×10⁵ cells) in 1.5 ml eppendorf tubes. The splenocytes were re-stimulated with 12µg/ml KAAP or Kmp11. The cells
20 were incubated for 48 hrs at 37°C in atmosphere containing 5% CO₂ and 95% humidity. The supernatants of the mice from each group (N=4) were pooled. All determinations were performed in triplicate. To control cell proliferation the amount of IFN-γ and IL-2 in the supernatants of ConA (3µg/ml) treated cells were measured. High amounts of IFN-γ (1000 fold) and IL-2 (200 fold) were detected in ConA treated
25 cells relative to untreated cells.

Cytokine determination. IL-4, IL-6, IFN-γ, TNF-α, IL-17 and IL-10 concentrations in cell culture supernatants were determined using a CBA kit (BD Biosciences, Singapore) according to manufacturer instructions. The results were acquired using
30 FACS CALIBUR (BD Biosciences, Singapore) and analyzed using FCAP software.

Results

In order to know whether the protein coded by the amino acid sequence SEQ ID

NO:1 (AAP) is able to increase or modify the humoral response to a covalently attached antigen such as Kmp11, a KAAP protein expressed by a chimeric gene formed by the genetic fusion of the AAP and Kmp11 DNAs was administered to mice. The AAP sequence is derived from the Q sequence previously described with the exception of the DNA coding for the amino and carboxyl terminal amino-acid fragments corresponding to the H2A antigens having a 75% identity. In the chimeric KAAP protein the Kmp11 protein represents 1/4 of the complete protein. An assay was designed in which two groups of 5 mice were injected each with three doses of 20µg Kmp11, 5µg KAAP and 20µg Kmp11-5µg Q on day 0, 15th and 30th. The proteins were administered to mice via a subcutaneous (sc) or an intra-peritoneal (ip) route. One week after the administration of the third dose the IgG reactivity against Q and Kmp11 was determined by an ELISA test. Fig. 1A shows that the Kmp11 protein elicited high reactivity against Kmp11 when it was administered by an ip route but that it was not able to trigger an immune response when it was administered by a sc route. It was observed that after ip administration of 5 µg KAAP the response against Kmp11 was also high and similar to that observed when 20µg of Kmp11 protein were administered alone and that, moreover, a high response was triggered after sc administration in contrast to the lack of response when 20µg of Kmp11 were administered (Fig. 1B) alone by the same route. High response was observed against Q either when the KAAP protein was ip or sc administered (Fig 1C).

Since a high response against Kmp11 was elicited when 5µg of KAAP was administered and the amount of Kmp11 in 5 µg of KAAP is equivalent to about 1µg of Kmp11, we tested whether 1 µg of Kmp11 was able to also induce a serological response. Fig 1B shows that the protein is able to induce a slight humoral response against Kmp11 when is ip administered (mean OD=0.3) but that the response was significantly lower than that elicited after the administration of 5µg of KAAP. It may be observed, moreover, that, as expected, there was not any reactivity against Kmp11 after sc administration of 1µg Kmp11 but that, in contrast, the response was high after administration of the same amount of Kmp11 present in 5µg of KAAP.

In order to know whether the increase in reactivity against Kmp11 was due to the co-administration of Kmp11 with the protein fragments present in the Q protein, the Kmp11 protein was administered mixed with Q. It was observed that the reactivity against Kmp11 decreased when the mix was administered ip and that no response was

observed against Kmp11 when the mix was sc administered (Fig. 1D), as an indication that the protein fragments present in Q do not elicit any adjuvant effect on Kmp11. High serological reactivity was observed against Q (Fig 1E).

Due to the high serological response observed after administration of 5µg KAAP, we analyzed the response against Q and Kmp11 after the administration of 1µg of KAAP. Five mice were sc injected in the footpad on day 0 and 15th. The IgG and IgG1/IgG2a response was analyzed one week after administration of the second dose. As a control, 0.25µg of Kmp11, which is the amount present in 1µg of KAAP, were administered to mice. Fig 2A shows that high response against Kmp11 was obtained after administration of 1µg KAAP either by ip or sc administration but that no response was obtained either after a sc or ip administration of 0.25µg of Kmp11. High humoral response against Q was observed when the KAAP was administered either by an ip or sc route (Fig 2B). The response was similar to that observed after administration of 3µg and 5µg of KAAP (data not shown). The type of response was predominantly of an IgG1 type either against Kmp11 or Q (Fig 2C and D) being the ratio IgG1/IgG2a of about 0.5.

In order to know whether the KAAP protein is able to elicit a humoral response after administration to mice of a plasmid DNA containing the gene coding for KAAP the IgG, IgG1 and IgG2a humoral response against Q and Kmp11 was analyzed. Groups of 7 mice were injected in the footpad with 20 µg of pRcKQ1, 20µg of pRcKmp11 and 3µg of KAAP on day 0, 15th and 30th. PBS was administered to control animals. Fig 3 shows that high IgG reactivity against Kmp11 (Fig. 3A) and Q (Fig. 3B) was detected after administration of pRcKAAP and that it was similar to that detected after administration of the KAAP protein. However, no response was obtained after administration of the DNA plasmid containing the gene coding for Kmp11 protein alone. A balanced IgG1/IgG2a ratio was detected when the reactivity was analyzed against Q (Fig.3D) either after KAAP or pRcKAAP administration. A slight predominance of the mean IgG1 reactivity was observed when tested against Kmp11 (Fig. 3C).

The cytokine production was analyzed in non-stimulated and in KAAP and Kmp11 stimulated spleen cells from KAAP and pRcKAAP injected mice. An antigen specific up-production of IFN-γ, IL-6, TNF-α and IL-10 was observed in KAAP stimulated cells isolated from pRcKAAP- immunized animals. No differences were

detected in cytokine production by KAAP stimulated cells from PBS and KAAP-immunized mice. Since a similar increase in IL-6, TNF- α and IL-10 was detected in KAAP stimulated cells from PBS and KAAP immunized, most likely the increase in cytokine production relative to non-stimulated cells is due to an unspecific stimulation of the spleen cells rather than to a stimulation of KAAP specific cells. A similar production of IL-17 was observed in spleen cells from PBS, KAAP and pRcKAAP-immunized mice stimulated with KAAP (Fig. 4). An up-production of IFN- γ and IL-17 was also observed after Kmp11 stimulation of spleen cells from pRcKAAP-immunized animals. Also an increase in IL-17 production was observed in cells from KAAP-immunized mice. In addition, an unspecific production of TNF- α , IFN- γ and IL-6 was observed in the cell cultures stimulated with Kmp11 relative to non-stimulated cells (Fig. 5).

Conclusion:

The immunogenic potential of Kmp11 is highly increased when genetically fused to a chimeric protein formed by fragments from the Lip2a, Lip2b and P0 proteins from *Leishmania infantum*. A chimeric protein containing the Lip2a, Lip2b and P0 fragments mix (but not fused to) with Kmp11 does not increase the immunogenic potential of Kmp11.

The Kmp11 protein when administered as DNA fused to the DNA fragments coding for the antigenic determinants of Lip2a, Lip3b and P0 proteins from *Leishmania infantum* elicited a high humoral response. The Kmp11 protein when administered alone as DNA does not elicit a detectable humoral response.

An antigen specific up-production of IFN- γ , IL-6, TNF- α and IL-10 was observed in KAAP stimulated cells isolated from pRcKAAP-immunized animals. Also an up-production of IFN- γ was observed after stimulation with Kmp11. This type of up-production was not detected in KAAP stimulated cells isolated from KAAP-immunized animals.

The protein resulting from in vitro translation of the AAP DNA sequence may be used as a tool for promoting the generation of antibodies against a genetically fused protein.

The AAP DNA sequence may be used as a vector to promote the immunogenic potential of an antigen when administered as DNA.

The AAP protein may be used as a carrier and as an adjuvant to elicit an immune

response against a genetically fused antigen.

Section II

Materials and Methods

5

Construction of the CAAP expression vector (Cup a 4-AAP).

Cup a 4 had been previously cloned into the pQE-30 vector and expressed and purified as a recombinant protein (Molecular cloning and characterization of Cup a 4, a new allergen from *Cupressus arizonica*, Biochem Biophys Res Commun. 2010 Oct

10 22;401(3):451-7. Yago Pico de Coaña, Nuria Parody, Miguel Ángel Fuertes, Jerónimo Carnés, Daniela Roncarolo, Renato Ariano, Joaquín Sastre, Gianni Mistrello, Carlos Alonso). The protein was, then, inserted into the AAP vector that had been prepared after digestion of the KAAP quimeric protein with Bam HI in order to liberate the K (Kmp11) fragment (This patent). The resulting expression vector contains the AAP
15 chimeric protein plus the Cup a 4 *C. arizonica* allergen (Fig. 12). The protein is called CAAP.

Expression and Purification of the CAAP protein.

The vector coding for the CAAP protein (reporter protein) was transformed into the *E.*
20 coli expression strain M15 (Qiagen). Expression was carried out in standard conditions after the addition of 1 mM IPTG during 5 hours to the bacterial culture that had been transformed with the CAAP vector. Purification of the protein was done as described previously for KAAP.

25 Results

In a previous section it was shown that the immune response of a poorly immunogenic protein is highly increased when fused to the AAP fragment. In order to know whether the AAP fragment could serve also as an adjuvant and, therefore,
30 increase the immunogenic potential of a protein that it is highly immunogenic even in the absence of adjuvant, the CAAP vector was constructed. The Cup a 4 is an allergenic protein from *Cupressus arizonica*. It has been described that in 10% of Cupressus allergic patients an intense IgGE response is triggered against Cup a 4. To test whether

AAP fused to Cup a 4 increases the immunogenicity of Cup a 4 a group of mice (N=5) was injected, each one, with 5 μ g of the recombinant CAAP protein equivalent to 3 and 2 μ g of AAP and Cup a 4, respectively. A second group of mice (N=5) was injected each one with a mix formed by 3 μ g of AAP and 2 μ of Cup a 4. A third group of mice (N=5) was injected each one with 2 μ g of the Cup a 4 protein. To a fourth group 3 μ g of AAP was administered. All proteins were dissolved in PBS and administered to mice via s.c. in the absence of any adjuvant.

The IgG reactivity against Cup a 4 was determined at a dilution of 1/2000. As expected a week after administration of a third dose of PBS or AAP no response against Cup a 4 was observed (data not shown). The results of the IgG response a week after administrations of the third dose of Cup a 4, CAAP, Cup a 4+AAP and AAP are shown in Fig. 6A. It may be observed that in the Cup a 4 immunized animals an IgG response was elicited against Cup a 4 as expected from a highly immunogenic protein. The response was variable ranging from 0.9 to 2.3 with a mean value of 1.4 OD. A small decrease in reactivity against Cup a 4 was observed in the Cup a 4+AAP immunized animals. This indicates that the administration of AAP mixed with Cup a 4 did not increase the immunogenicity of Cup a 4 but that on the contrary AAP competes with Cup a 4. The mean value was 0.95 OD. However, when the Cup a 4 protein was administered fused to AAP (CAAP) an increase in response was observed in most of the animals. The response ranged from 1.1 to 2.9 OD with a mean value of 2.2 OD, in contrast to the 1.4 OD detected in the Cup a 4 immunized animals. A similar behaviour regarding the IgG response against Cup a 4 in the sera from the Cup a 4, Cup a 4+AAP and CAAP and AAP animals was observed one week after administration of the second dose (Fig. 7A) as a further indication of the intense immunogenic character of Cup a 4. In order to know whether the AAP has any influence in the modulation of the IgG1 and IgG2a response elicited against Cup a 4 the IgG1 and IgG2a isotype response was analyzed in the animals immunized with Cup a 4, Cup a 4+AAP and CAAP (dilution of the sera 1/400) one week after administration of the second and their dose.

Fig 7B shows that one week after the administration of the second dose of Cup a 4 also a dispersed intensity in IgG1 reactivity was detected ranging from 0.18 to 2.4 OD (mean value of 1.1). The IgG1 reactivity against Cup a 4 in the Cup a 4+AAP immunized animals was somewhat lower and uniform, around the mean value of 0.7 OD. In contrast the mean value of the IgG1 reactivity against Cup a 4 in the animals

immunized with CAAP was higher (mean value of 2 OD), than that observed in the Cup a 4 and Cup a 4+AAP animals as a further indication that the AAP behaved as an adjuvant when fused to the antigen. Similar differences were also observed when the IgG1 reactivity against Cup a 4 was analyzed one week after the administration of the third dose (Fig 6B), in particular when compared with the Cup a 4+AAP mixture was administered. When the intensity of the IgG2a reactivity against Cup a 4 was analyzed in the sera from animals immunized with Cup a 4, CAAP and Cup a 4+AAP one week after the third dose it was observed that in the sera of animals immunized with Cup a 4 and Cup a 4+AAP (Fig 6C) the reactivity was uniform around a mean value of 0.8 and 0.6 OD, respectively. The IgG2a responses observed in the CAAP group, with values ranging from 1.1 to 2.9 OD and a mean value of 1.9 OD, were higher than those detected in the Cup a 4 and the Cup a 4+AAP animals. A similar observation was detected when the sera, obtained one week after the second dose, from the Cup a 4, Cup a 4+AAP and CAAP animals were analyzed (Fig. 7C).

To have a further evidence of the early adjuvant capacity of AAP a group of animals was immunized with 1/4 of the Cup a 4, CAAP and Cup a 4+AAP protein doses used in the experiment shown above (equivalent to 0.5 μ g of Cup a 4, 1.5 μ g CAAP and 0.5 μ g Cup a 4+0.75 μ g AAP, respectively). The sera were collected a week after the first dose. The IgG reactivity against Cup a 4 was determined at 1/100 dilution. The results are shown in Fig 8. It was observed that while there was no response against Cup a 4 in any of the animals immunized with Cup a 4 or Cup a 4+AAP, positive responses were observed in all of the animals immunized with CAAP. Again it was observed that the response was variable ranged from 0.16 to 1.7 OD. Thus, with the exception of one animal the response was high in most of the animals at that early stage post immunization. After administration of a second dose an interesting result was also observed. In the Cup a 4+ AAP group the response triggered by AAP competes with the response triggered by Cup a 4. In fact no reactivity against Cup a 4 was detected (Fig 9A). Interestingly, this competition did not occur when the antigen was fused to AAP. In order to discriminate in detail the adjuvant capacity of AAP the sera were analyzed at a dilution of 1/4000 (Fig 9A). The mean OD of the sera of animals immunized with CAAP was 0.45 OD while it was 0.15 OD in the sera from the Cup a 4 animals. In this group the reactivity of the sera against Cup a 4 of two animals was close to the background level. All the sera from the CAAP group of

animals were positive. The difference between the reactivity against Cup a 4 in both groups was statistically different ($p < 0.05$). When the IgG1 and IgG2a response was analyzed (dilution 1/100) the data shown above (Fig 7B and 7C) were confirmed (Fig. 9B and 9C). The AAP when fused to the antigen directs the response towards IgG2a.

- 5 The p value of the reactivity of the response against Cup a 4 due to administration of CAAP relative to Cup a 4 was 0.055 and relative to Cup a 4+AAP was $p < 0.05$).

Thus, the data presented indicate that the AAP protein fragment when administered fused to Cup a 4 is able not only to increase the immune response against a highly immunogenic protein, such as Cup a 4, but it is also able to modulate the type of response elicited, in particular towards a IgG2a response. That type of adjuvant
10 activity is not observed when AAP was administered mixed with protein Cup a 4. On the contrary, AAP seems to compete with the immune activity of the antigen.

Section III

- 15 Preparation of the S100AAP expression vector

After PCR amplification of the S100A2 DNA fragment in the Invitro gen pDEST-17 vector (Laboratory stock), using the 5'-AAGGATCCATGTGCAGTTCTCTGGAG-3' (SEQ ID NO: 44) and 5'-AACTTAAGCAGGGTCGGTCTGGGCAG-3' (SEQ ID NO:
20 45) primers, it was subcloned into a pST Blue-1 vector (the Bam HI and Afl II restriction sites are shown in *italics*). It was, then, subcloned into a modified AAP vector (in a PQE30 vector) that had been synthesized including the necessary restriction sites. The resulting expression vector contains the three fragments of the AAP chimeric protein plus the S100A2 DNA fragment (Fig. 13). This vector was
25 transformed into the *E. coli* expression strain M15 (Qiagen). Expression was carried out in standard conditions: Induction of a 0.8 O.D._{450nm} culture with 1 mM IPTG during 5 hours. The final vector was named S100AAP. The S100A2 protein was expressed in the PQE30 vector.

- 30 In previous sections it was shown that the immune response against a parasite poorly immunogenic protein (Kmp11) is highly increased when fused to the AAP DNA sequence and that the immune response against a plant highly immunogenic protein (Cup a4) is also increased when fused the AAP DNA sequence. It was also shown that

the capacity of AAP to increase the immunogenicity of the reporter protein was only efficacious when the reporter protein was genetically fused to AAP. In order to know whether the AAP fragment could also serve as an adjuvant and, therefore, increase the immunogenic potential of a mammalian protein the S100AAP vector was constructed.

- 5 The S100A2 protein is a calcium-binding protein that is up regulated in association with human gastric adenocarcinoma (1) and breast (2) tumour progression. To test whether AAP fused to S100 increases the immunogenicity of the reporter protein a group of mice (N=5) was injected, each one, with 1.05 μ g of the recombinant S100AAP protein equivalent to 0.75 μ g and 0.3 μ g of AAP and S100A2, respectively.
- 10 A second group of mice (N=5) was injected each one with a mixed formed by 0,75 μ g of AAP and 0,3 μ g of S100A2. A third group of mice (N=5) was injected each one with 0,3 μ g of S100A2. To a fourth group 0,75 μ g of AAP were administered. All proteins were dissolved in PBS and administered to mice via s.c. As control another group of mice (N=5) were injected each one with a buffer solution (PBS). Fig. 10 shows the
- 15 total IgG response against S100A2 at a dilution of 1/100 one week after administration of the first dose. As expected it was observed that neither the sera from the mice immunized with AAP or PBS showed any positive reactivity against S100A2. With the exception of one mouse the reactivity against S100A2 decreased when the protein was administered mixed to AAP as previously reported for Cup a4 as an indication of the
- 20 competitive effect between two immunogenic proteins when administered as a mix (The reactivity of the serum of one animal was negative). The data from the mice immunized with the S100A2 protein alone indicated that that protein is also immunogenic in mice in the absence of adjuvant since even the low amount of protein administered was able to induce a low but positive response (mean OD= 0,41). In spite
- 25 of that it was observed that there was not any immune competition between AAP and S100A2 in mice immunized with S100AAP but that, on the other hand, the reactivity against S100A2 increased (30%) when the administered protein was fused to AAP (mean OD=0,54).

- As shown in Fig 11A, the adjuvant capacity of AAP when fused to the protein
- 30 reporter was clearly observed after the administration of a second dose of S100AAP, S100A2, AAP+S100A2 and AAP (1.05 μ g de S100AAP; 0.3 μ g de S100A2; 0,75 μ g of AAP plus 0,3 μ g of S100A2 and 0.75 μ g of AAP). A statistically significant difference in reactivity against S100A2 between the sera of the animals immunized with

S100AAP and of those immunized with the S100A2 protein alone was detected. In agreement with the observations indicated above regarding the reactivity against Kmp11 and Cup a4 after administration of AAP non fused to the reporter proteins it was detected that AAP did not have any adjuvant capacity when non fused to the reporter S100A2 protein. On the other hand an immune competition between both proteins also seems to occur. Thus, an immunologically interesting feature may be deduced from the three experimental designs since AAP needs to be fused to the reporter protein to exercise the adjuvant effect. In other words this mean that the built-in adjuvant capacity of AAP is relevant when genetically fused to the reporter protein.

10 The adjuvant effect was further observed when the type of response was analyzed (Fig. 11B). It was detected that not only the IgG reactivity against S100A2 increased after administration of the S100AAP relative to the reactivity against S100A2 after administration of S100A2 or AAP+S100A2 but that this increase in reactivity was also observed when the IgG2a type was analyzed. The IgG1/IgG2a reactivity ratio observed

15 after administration of S100A2 had a mean value of 2.1 while it was 0.95 when the animals were immunized with S100AAP as indication that the adjuvant capacity of AAP when fused to the reporter protein triggers the IgG1-IgG2a response towards IgG2a. In agreement with the observations indicated above regarding the IgG response when the animals were immunized with a protein mix it was observed that the IgG1

20 reactivity of the sera of the animals immunized with S100AAP was significantly higher than the sera from the animals immunized with AAP+S100A2. The mean of the IgG2a response in both cases was similar as a further in indication of the capacity of AAP to revert toward IgG2a only when fused to the reporter protein.

25

- 1- Identification of potential biomarkers for early and advanced gastric adenocarcinoma detection. Economescu MC, Necula LG, Dragu D, Badea L, Dima SO, Tudor S, Nastase A, Popescu I, Diaconu CC. Hepatogastroenterology. 2010 Nov-Dec;57(104):1453-64.
- 30 2- McKiernan E, McDermott EW, Evoy D, Crown J, Duffy MJ. The role of S100 genes in breast cancer progression. Tumour Biol. 2011 Jun;32(3):441-50

The adjuvant capacity of AAP in relation to S100A2 is further, even more clearly, observed after administration of the third dose as indicated:

As a further indication of the adjuvant capacity of AAP the sera of the same animals obtained after the administration of a third dose of the antigens were analyzed.

5 Fig 14 shows the response against S100A2. After a titration curve of the dilution of the sera used for analysis a dilution of 1/6400 was chosen since it is the dilution that better discriminate the adjuvant capacity of AAP. It was clearly detected that the reactivity against S100A2 of the sera of the animals immunized with S100AAP is significantly higher than that observed in the sera of the animals immunized with either the mix of
10 the S100A2 protein. In order to know whether the AAP modulates the humoral response the IgG1 and IgG2a response was analyzed. It was observed that an increase was observed in both types of responses. When the protein was administered alone the IgG2a/IgG1 mean ratio was 0.45. However, the mean ratio between IgG2a/IgG1 was 1.6 when AAP was fused to S100A2 as an indication that AAP modulates the response
15 towards IgG2a. It should be noticed, in addition that AAP also modulates the response towards IgG2a when is administered mixed to S100A2 (IgG2a/IgG1 mean ratio equivalent to 1.1).

SEQ ID NO:2

GGATCCTCTAGACCCATGTCCACCAAGTACCTCGCCGCGTACGCTCTGGCCTCCCTGAG
CAAGGCGTCCCCGTCTCAGGCGGACGTGGAGGCTATCTGCAAGGCCGTCCACATCGACG
5 TCGACCAGGCCACCCTCGCCTTTGTGATGGAGAGCGTTACGGGACGCGACGTGGCCACC
CTGATCGCGGAGGGCGCCGCGAAGATGAGCGCGATGCCGGCGGCCAGCTCTGGTGCCGC
TGCTGGCGTCACTGCTTCCGCTGCGGGTGATGCGGCTCCGGCTGCCGCCGCTGCTAAGA
AGGACGAGCCGGAGGAGGAGGCCGACGACGACATGGGCCCTCTAGAGTCGACCCCATG
CAGTACCTCGCCGCTACGCCCTCGTGGCGATGTCTGGCAAGACGCCGTGCAAGGCCGA
10 CGTTCAGGCTGTCCTGAAGGCCGCCGGCGTTGCCGTGGATGCCTCCCGCGTGGATGCCG
TCTTCAGGAGGTGGAGGGCAAGAGCTTCGATGCGCTGGTGCCGAGGGCCGCACGAAG
CTGGTGGGCTCTGGCTCTGCCGCTCTGCTGGCGCTGTCTCACTGCTGGTGCCGGCGC
TGGCGCGGTGGCCGAGGCCAAGAAGGAGGAGCCGAGGAGGAGGAGGCCGATGATGACA
TGGGCCCCGTCGACCTGCAGCCCGCCGCTGCCGCGCCGGCCGCCCTAGCGCCGCTGCC
15 AAGGAGGAGCCGGAGGAGAGCGACGAGGACGACTTCGGCATGGGCGGTCTCTTCTAA

SEQ ID NO:1

GSSRPMSTKYLAAYALASLSKASPSQADVEAICKAVHIDVDQATLAFVMESVTGRDVAT
20 LIAEGAAKMSAMPAASSGAAAGVTASAAGDAAPAAAAAKKDEPEEEADDDMGPSRVDPM
QYLAAYALVAMSGKTPSKADVQAVLKAAGVAVDASRVDAVFQEVEGKSFDAEGRTK
LVGSGSAAPAGAVSTAGAGAGAVAEAKKEEPEEEADDDMGPVDLQPAAPAAPSA
KEEPEEDEDDFGMGGLF

Claims

1. A nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:
 - i. nucleotide sequences encoding a polypeptide comprising an amino acid sequence that has at least 70% sequence identity with the amino acid sequence of SEQ ID NO:1 over its entire length,
 - ii. nucleotide sequences comprising a nucleotide sequence that has at least 70% sequence identity with the nucleotide sequence of SEQ ID NO:2 over its entire length,
 - iii. nucleotide sequences the complementary strand of which hybridizes to a nucleic acid molecule of sequence of (i) or (ii) and
 - iv. nucleotide sequences which differ from the nucleotide sequences of (iii) due to the degeneracy of the genetic code,for use as an adjuvant in the treatment or prevention of a disease or a condition, wherein said nucleotide sequence is operably linked to a nucleotide sequence encoding an antigen, wherein said antigen is associated or linked with said disease or condition, wherein the nucleic acid molecule encodes a polypeptide which is able to elicit an immune response in a subject, said immune response being specific for said antigen.
2. A nucleic acid molecule according to claim 1 for use as an adjuvant according to claim 1, wherein said nucleic acid molecule is free of a sequence encoding for a polypeptide which is identical to SEQ ID NO: 3 over its entire length.
3. A nucleic acid molecule according to any of the claims 1 – 2 for use as an adjuvant according to claim 1, wherein said antigen originates from any organism known to be associated with a disease or a condition in a subject.
4. A nucleic acid molecule according to any of the claims 1 - 3 for use as an adjuvant according to claim 1, wherein said antigen is a self or auto antigen or originates from virus or a microorganism such as a bacterium, a yeast, a fungus or a parasite.

5. A polypeptide encoded by a nucleic acid molecule as identified in claim 1.
6. A polypeptide encoded by a nucleic acid molecule as identified in any of the claims 2 to 4.
7. A nucleic acid construct comprising a nucleic acid molecule as identified in claim 1.
8. A nucleic acid construct comprising a nucleic acid molecule as identified in any of the claims 2 to 4.
9. A composition comprising a nucleic acid molecule as identified claim 1 and/or a polypeptide as identified in claim 5 and/or a nucleic construct as identified in claim 7.
10. A composition comprising a nucleic acid molecule as identified in any of the claims 2 to 4 and/or a polypeptide as identified in claim 6 and/or a nucleic construct as identified in claim 8.
11. A nucleic acid molecule according to any of the claims 2 to 4, a polypeptide according to claim 6, a nucleic acid construct according to claim 8 and/or a composition according to claim 10 for use as a medicament, preferably wherein the medicament is a vaccine.
12. Use of a nucleic acid molecule as identified in claim 1, a polypeptide as identified in claim 5, a nucleic acid construct as identified in claim 7 and/or a composition according to claim 9 for the manufacture of a medicament for treating a disease associated with an antigen or a condition associated with an antigen, said disease associated with an antigen or condition associated with an antigen being identified in claim 1, wherein said antigen:
 - i) is specific for said disease or condition, or
 - ii) originates from an organism that causes said disease or condition in the subject, or
 - iii) comprises an allergen causing an allergy if said disease or condition is said allergy, or
 - iv) comprises a cancer antigen associated with a cancer if said disease or condition is said cancer.
13. Use of a nucleic acid molecule as identified in claims 2 to 4, a polypeptide as identified in claim 6, a nucleic acid construct as identified in claim 8 and/or

a composition according to claim 10 for the manufacture of a medicament being a vaccine for treating a disease associated with an antigen or a condition associated with an antigen, said disease associated with an antigen or condition associated with an antigen being identified in claim 1, wherein said antigen:

- i) is specific for said disease or condition, or
- ii) originates from an organism that causes said disease or condition in the subject, or
- iii) comprises an allergen causing an allergy if said disease or condition is said allergy, or
- iv) comprises a cancer antigen associated with a cancer if said disease or condition is said cancer.