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**WO 01/30847 A1**

(54) Title: MODIFIED GP100 AND USES THEREOF

(57) Abstract: A gp100 molecule useful in modulating the immune system is described. In particular what is described is a nucleotide sequence for a gp100 which has been modified so as to be useful in modulating the immune system. The isolated nucleic acid sequence is described and referred to as *gp100M*. The corresponding protein, referred to as *gp100M*, is also described. Applications of the molecules as vaccines are described.

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**Title: MODIFIED GP100 AND USES THEREOF**

This application claims the benefit of United States application serial no. 60/160,879, filed on October 22, 1999 and United States application serial no. 5 60/223,325 filed on August 7, 2000, both of which are incorporated by reference herein.

**FIELD OF THE INVENTION**

The present invention is in the field of immunotherapy and relates to the construct of novel forms of gp100 suitable for the generation of immune 10 responses *in vivo*.

**BACKGROUND OF THE INVENTION**

The incidence and mortality of cutaneous malignant melanoma have risen dramatically over the past several decades (Liu, T. et al. (1996) *Surg Clin North Am* 76:1205; Gloster, H.M. et al. (1996) *Dermatol Surg* 22:217). At present, 15 the estimated lifetime risk for an American of developing melanoma is approximately 1 in 90 (Rigel, D.S. et al. (1979) *Mayo Clin Proc* 72:367). While most early stage melanomas can be treated successfully by a simple surgical excision (Greenstein D.S. et al. (1995) *Dermatol Surg* 21:927; Whooley, B.P. et al. (1995) *Dermatol Surg* 4:187; Urist, M.M. et al. (1996) *Ann Rev Med* 47:211), patients with 20 advanced disease are rarely cured even with aggressive chemotherapy and/or immunotherapy (Falkson, C.I. et al. (1995) *Anticancer Drugs* 6:709).

Although melanoma can present as an aggressive primary lesion that metastasizes within weeks, its development typically takes place over a period of several months to years and progresses through a series of distinct pathological 25 stages. The earliest stage is the radial growth phase (RGP) melanoma that starts as an intradermal neoplasm (melanoma *in situ*). RGP melanomas may remain relatively quiescent for months or even years and are generally cured by simple excision. Eventually, most RGP melanomas develop a component of vertical growth (vertical growth phase melanoma, VGP), which denotes a more 30 aggressive tumor less likely to be cured by simple excision. Metastatic spread is

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the final stage in tumor progression and is indicative of a very poor outcome (Reintgen, D. (1997) *Ann Surg* 225:1).

The most important prognostic factor in determining survival in a patient with primary melanoma is the depth of the lesion. While the five-year survival for patients with tumours of  $\leq 0.75$  mm in thickness is approximately 96%, individuals with tumours  $> 4.5$  mm in depth have a five year survival of only approximately 38% (Whooley, B.P. et al. (1995) *Dermatol Surg* 4:187; Urist, M.M. et al. (1996) *Ann Rev Med* 47:211). Since the development of a VGP melanoma from its RGP precursor generally takes at least several months, a "window of time" exists within which surgical excision can have a dramatic effect on patient outcome. By extension, it is reasonable to propose that novel attempts to deal with residual disease would be most successful at this time.

Several lines of evidence suggest that manipulation of immune responses against melanoma may be therapeutic: 1. Clinical observations of spontaneous regression of metastatic melanoma may be caused by an anti-melanoma immune response (Nelson, C.A. et al. (1976) *Natl Cancer Inst Monogr* 44:145). 2. Regression of metastatic melanoma has also been observed in some patients given high doses of IL-2 with or without lymphokine activated killer (LAK) cells or tumor infiltrating lymphocytes (TIL's) (Rosenberg, S.A. et al. (1998) *J Natl Cancer Inst* 90:1894) 3. More recently, a number of melanoma specific and associated tumor antigens have been cloned (Van den Eynde, B. et al. (1997) *Curr Opin Immunol* 9:684). The availability of these reagents has given hope that specific vaccines may be developed to enhance the ability of tumor specific T cells to eliminate melanoma cells. These antigens can be administered in a variety of delivery vehicles, and the most effective dosing regimen for optimization of an anti-tumor response is not presently clear.

Conventional vaccines for many infectious diseases have shown that a primary infectious challenge can induce an effective protective memory immune response. A critical event required to initiate an immune response, even in the presence of circulating tumor reactive T cells, is that tumor antigens gain entry

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into the secondary lymphoid structures of the spleen and lymph nodes (Zinkernagel, R.M. et al. (1997) *Immunol Rev* 156:199). Melanoma that develops locally in the skin is hidden from the immune system as long as the tumor antigens do not reach the secondary lymphoid organs. The tumor may then  
5 grow to such a large mass that, by the time an immune response is triggered, it is rapidly overwhelmed by the tumor (Moskophiis, D. et al. (1993) *Nature* 362:758). Alternatively, melanoma cells that break off and traffic to the local lymph node may not be able to trigger an immune response because they are poor antigen presenting cells, i.e., they do not express high levels of HLA-class I  
10 molecules (Ferrone, S. (1995) *Immunology Today* 61:487) or co-stimulatory molecules, such as members of the B7 family (Bluestone, J.A. et al. (1995) *Immunity* 2:555). Again, the tumor may reach a tolerizing size before an immune response is initiated. A successful vaccination strategy in patients with deep, but not metastatic, melanoma should both increase the number of tumor reactive T  
15 cells and activate them so that they can traffic to the periphery and exert their cytotoxic effector function.

gp100 is normally found in melanosomes and expressed in melanocytes, retinal cells, and other neural crest derivatives (Kawakami, Y. et al. (1997) *Int Rev Immunol* 14:173). The function of gp100 is currently unknown (Rosenberg, S.A. et al. (1998) *Nature Med* 4:321). By mass spectrometry, three immunodominant  
20 HLA-A2 binding gp100 peptides have been identified: g9-154 (amino acids 154-162), g9-209 (amino acids 209-217); and g9-280 (amino acids 280-288) (Kawakami, Y. et al. (1995) *J Immunol* 154:3961). Notably, two of these peptides have been synthetically altered so as to induce a more vigorous immune response in the  
25 original T cell clone: the threonine at position 2 in g9-209 was changed to a methionine, and the alanine residue at position 9 in g9-280 was changed to a valine (Parkhurst, M.R. et al. (1996) *J Immunol* 157:2539). These changes increase the binding affinity of the peptides to the HLA-A2 molecule without changing the epitopes recognized by the T cell receptor (TCR). Rosenberg et al have  
30 already successfully immunized melanoma patients with one of these modified

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peptides and achieved objective clinical responses in some patients (Rosenberg, S.A. et al. (1998) *Nature Med* 4:321).

### **SUMMARY OF THE INVENTION**

The present inventors have developed a molecule useful in modulating  
5 the immune system. In particular, they have found that a nucleotide sequence for a full gp100 which has been modified is useful in modulating the immune system. The isolated nucleic acid sequence is referred herein as *gp100M* and the corresponding protein is referred to herein as gp100M. Preferably the molecule (i.e. protein or nucleic acid) or immunogenic fragments of the protein may be  
10 used to prime and boost the immune system of an animal.

Accordingly, the present invention provides an isolated nucleic acid molecule comprising a sequence encoding a modified gp100 protein, preferably the gp100M protein. Preferably, a modified gp100 protein according to the present invention is one wherein the modification results in at least one amino  
15 acid modification of the said gp100, although any other modification capable of modifying gp100 so as to enable it to modify the immune system is within the scope of the present invention.

The nucleic acid sequence of *gp100M* is shown in Figure 1 and is also known herein as SEQ.ID.NO.1. The corresponding amino acid sequence encoded  
20 by the nucleic acid sequence of *gp100M* is shown in Figure 2 which is referred to herein as SEQ.ID.NO.2.

Accordingly, in one embodiment of the invention, an isolated nucleic acid molecule is provided having a sequence as shown in Figure 1 (SEQ.ID.NO.1).

Preferably, the purified and isolated nucleic acid molecule comprises:

- 25 (a) a nucleic acid sequence as shown in SEQ.ID.NO.1 wherein T can also be U;
- (b) nucleic acid sequences complementary to (a);
- (c) nucleic acid sequences which are homologous to (a) or (b);

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(d) a fragment of (a) to (c) that is at least 15 bases, preferably 20 to 30 bases, and which will hybridize to (a) to (d) under stringent hybridization conditions; or

(e) a nucleic acid molecule differing from any of the nucleic acids of (a) to 5 (c) in codon sequences due to the degeneracy of the genetic code.

The invention further includes an isolated gp100M protein and/or immunogenic fragments thereof encoded by a nucleic acid molecule of the invention. In a preferred embodiment the gp100M has the amino acid as shown in Figure 2 (SEQ.ID.NO.2).

10 Preferred embodiments of these fragments include a fragment having an amino acid sequence according to SEQ.ID.NO.124 or according to SEQ.ID.NO.125.

The invention further provides a method of modulating an animal's immune system comprising administering, to an animal in need thereof, an 15 effective amount of a gp100 or *gp100* which has been modified to provide a molecule which modulates the immune system. Preferably the modified gp100 or *gp100* is gp100M or *gp100M*, respectively, most preferably the modified gp100 or *gp100* have the sequences of SEQ. ID. NO 1 and 2 respectively.

The invention further provides a method of modulating an animal's 20 immune system comprising administering to an animal in need thereof, an effective amount of a vector into which has been inserted a *gp100* which has been modified to provide a molecule which modulates the immune system, preferably the vector is viral, preferably the virus is an adenovirus, alphavirus or poxvirus. More preferably where the virus is poxvirus it is vaccinia, fowlpox, avipox, 25 TROVAC, ALVAC, NYVAC or MVA, preferably ALVAC. .

According to another embodiment of the present invention the modified gp100 or *gp100* is administered with a second agent, preferably a lymphokine, cytokine, or co-stimulatory molecule such as a member of the B7 30 IFN $\gamma$ 1.

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In one embodiment, the present invention provides a method of stimulating an animal's immune system comprising administering to an animal in need thereof, an effective amount of a gp100 or *gp100* which has been modified to provide a molecule which stimulates the immune system, preferably  
5 gp100M or *gp100M*, respectively, most preferably the modified gp100 or *gp100* have the sequences of SEQ. ID. NO 1 and 2 respectively

The present invention also provides a method of enhancing the efficacy of a gene vaccine in the treatment of an animal comprising administering an effective amount of a *gp100* which has been modified to provide a molecule  
10 which stimulates the immune system, preferably *gp100M*. According to a preferred embodiment, the animal has cancer.

According to a further embodiment of the present invention, there is provided a composition for modulating an animal's immune system comprising an effective amount of a gp100 or *gp100* which has been modified to provide a  
15 molecule which stimulates the immune system, preferably gp100M or *gp100M* respectively, in a pharmaceutically acceptable diluent or carrier, most preferably the modified gp100 or *gp100* have the sequences of SEQ. ID. NO 1 and 2 respectively.

According to another aspect of the present invention, provided are  
20 methods for prophylactic or therapeutic uses involving a nucleic acid sequence encoding a modified gp100, preferably gp100M, more preferably a gp100M having and amino acid sequence according to SEQ ID NO. 2.

According to a further aspect of the present invention, there is provided a melanoma vaccine comprising a nucleic acid sequence encoding a modified  
25 gp100 for preventing or treating cancer preferably gp100M, more preferably a gp100M having and amino acid sequence according to SEQ ID NO. 2..

According to a further aspect of the present invention, there is provided a melanoma vaccine comprising a nucleic acid sequence encoding a modified  
30 gp100 for preventing or treating melanoma preferably gp100M, more preferably a gp100M having and amino acid sequence according to SEQ ID NO. 2..

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According to yet another aspect of the present invention, there is provided a modified gp100 protein sequence which is modified to increase its immunogenicity or to enhance its induction of an anti-melanoma immune response by enhancing the binding to MHC molecules, for use in a prophylactic or therapeutic methods described herein.

According to another aspect of the present invention, there is provided a vaccine comprising a modified gp100 nucleic acid sequence or its corresponding protein capable of eliciting the production of antibodies in a mammal to corresponding antigens preferably *gp100M* or *gp100M*, respectively, more preferably a *gp100M* having and nucleic acid sequence according to SEQ ID NO. 1 and a *gp100M* having and amino acid sequence according to SEQ ID NO. 2..

According to another aspect, the present invention relates to an antigenic, immunological or vaccine composition or a therapeutic composition for inducing an antigenic or immunological response in a host animal inoculated with the composition, said vaccine including a modified recombinant virus having inactivated nonessential virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety.

In yet another aspect, the present invention relates to an immunogenic composition containing a modified recombinant virus having inactivated nonessential virus-encoded genetic functions so that the recombinant virus has attenuated virulence and enhanced safety.

In a still further aspect, the present invention relates to a modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the virus has attenuated virulence, and wherein the modified recombinant virus further contains DNA from a heterologous source in a nonessential region of the virus genome.

According to another embodiment of the invention there is provided a recombinant virus comprising a virus into which is inserted a nucleic acid according to to the invention wherein the nucleic acid encodes for a polypeptide,

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the recombinant virus causing the expression of the polypeptide in an infected cell.

In another embodiment, there is provided a recombinant virus into which is inserted a nucleic acid according to the present invention wherein the  
5 nucleic acid encodes for a modified gp100 polypeptide, wherein cells infected with the said recombinant virus are capable of eliciting an immune response directly against a member selected from the group consisting of:

- (1) the polypeptide;
- (2) a fragment of the polypeptide;
- 10 (3) a cell expressing the polypeptide or a fragment thereof; or
- (4) cells binding the protein or fragment thereof, preferably the virus is adenovirus, alphavirus, or poxvirus, preferably where the virus is poxvirus it is vaccinia, fowlpox, avipox, TROVAC, ALVAC, NYVAC or MVA, preferably the virus is ALVAC.

15 In an embodiment such recombinant virus' are part of a composition comprising the particular recombinant virus and a pharmaceutically acceptable diluent or carrier.

The invention in yet a further aspect relates to the product of expression of a recombinant virus including a nucleic acid encoding a modified gp100,  
20 preferably the virus is an adenovirus, alphavirus or poxvirus, more preferably where the virus is poxvirus it is vaccinia, fowlpox, avipox, TROVAC, ALVAC, NYVAC or MVA, preferably ALVAC, and uses therefor, such as to form antigenic, immunological or vaccine compositions for treatment, prevention, diagnosis or testing; and, to DNA from the recombinant poxvirus which is useful  
25 in constructing DNA probes and PCR primers.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of  
30 illustration only. Various changes and modifications within the spirit and scope

of the invention will become apparent to those skilled in the art from this detailed description.

### **DESCRIPTION OF THE DRAWINGS**

5 The invention will be better understood with reference to the drawing in which:

Figure 1 shows the nucleic acid sequence of *gp100M* cDNA.

Figure 2 shows the deduced amino acid sequence for the *gp100M* protein.

10 Figure 3 shows the nucleic acid sequence of C5H6*gp100M*, the H6 promoted human *gp100M* insertion cassette.

Figure 4 shows a schematic representation of the ALVAC(2)-*gp100M*(vCP1584) genome.

Figure 5 shows the nucleotide sequence of the oligonucleotide primers used to sequence pBS/1584.

15 Figure 6 shows immunoprecipitate results from uninfected HeLa cells or cells infected with either ALVAC parental virus, ALVAC-*gp100*, or ALVAC (2)-*gp100M*.

20 Figure 7 shows a Western blot of HeLa cells infected with one of ALVAC parental virus, ALVAC-*gp100*, or ALVAC (2)-*gp100M*, illustrating expression of full length *gp100* in ALVAC-*gp100*, or ALVAC (2)-*gp100M* infected cells.

Figure 8 is a bar graph showing the results of an IFN- $\gamma$ -ELISPOT analysis of animal receiving intranodal injection of the tumor antigen.

25 Figure 9 is a bar graph showing the results of an IFN- $\gamma$ -ELISPOT analysis of animal receiving intranodal injection of the tumor antigen.

Figure 10 is a bar graph showing the results of an IFN- $\gamma$ -ELISPOT analysis of animal receiving subcutaneous injection of the tumor antigen.

Figure 11 is a bar graph showing the results of an IFN- $\gamma$ -ELISPOT analysis of animal receiving subcutaneous injection of the tumor antigen.

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Figure 12 is a graph showing the antibody response after a regiment of intranodal (group 2) and subcutaneous (group 3) administration of ALVAC-modified gp100/modified gp100 peptide immunogens.

### **DETAILED DESCRIPTION OF THE INVENTION**

5           As already mentioned, the present inventors have developed a molecule useful in modulating the immune system of an animal. In particular, they have found that a nucleotide sequence for a full gp100, which has been modified, is useful in modulating the immune system. The term "animal" as used herein includes all members of the animal kingdom including mammals, preferably  
10 humans.

#### **I. NUCLEIC ACID MOLECULES OF THE INVENTION**

As mentioned above, the inventors have isolated and characterized the gene (*gp100M*) and its gene product (gp100M).

Broadly stated, the present invention provides an isolated nucleic acid  
15 molecule comprising a sequence encoding a protein with the activity of a gp100 which has been modified to provide a molecule which stimulates the immune system. As used herein a gp100 which has been modified to provide a molecule which stimulates the immune system includes those gp100 sequences with modified sequences at about amino acids 209 and/or at about 280 (as set out in  
20 Parkhurst, M.R., et al. J. Immunol. 157:2539-2548 (1996)) and/or immunogenic fragments thereof.

Accordingly, the present invention includes any isolated nucleic acid molecule encoding a modified gp100 capable of modulating the immune system. The term "isolated" refers to a nucleic acid substantially free of cellular material or  
25 culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded. In an embodiment of the invention, an isolated nucleic acid molecule is provided having a sequence as shown in Figure 1.

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Preferably, the purified and isolated nucleic acid molecule comprises:

- (a) a nucleic acid sequence as shown in Fig. 1, wherein T can also be U;
- (b) nucleic acid sequences complementary to (a);
- (c) nucleic acid sequences which are homologous to (a) or (b);
- 5 (d) a fragment of (a) to (c) that is at least 15 bases, preferably 20 to 30 bases, and which will hybridize to (a) to (c) under stringent hybridization conditions; or
- (e) a nucleic acid molecule differing from any of the nucleic acids of (a) to (c) in codon sequences due to the degeneracy of the genetic code.

10 It will be appreciated that the invention includes nucleic acid molecules encoding truncations of the proteins of the invention, and analogs and homologs of the proteins of the invention and truncations thereof, as described below. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a  
15 cDNA of the invention are encompassed by the invention.

Further, it will be appreciated that the invention includes nucleic acid molecules comprising nucleic acid sequences having substantial sequence homology with the nucleic acid sequence as shown in Figure 1 and fragments thereof. The term "sequences having substantial sequence homology" means  
20 those nucleic acid sequences which have slight or inconsequential sequence variations from these sequences, i.e., the sequences function in substantially the same manner to produce functionally equivalent proteins. The variations may be attributable to local mutations or structural modifications.

Generally, nucleic acid sequences having substantial homology include  
25 nucleic acid sequences having at least 70%, preferably 80-90% identity with the nucleic acid sequence as shown in Figure 1.

Another aspect of the invention provides a nucleic acid molecule, and fragments thereof having at least 15 bases, which hybridize to the nucleic acid molecules of the invention under hybridization conditions, preferably stringent  
30 hybridization conditions. Appropriate stringency conditions which promote

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DNA hybridization are known to those skilled in the art (for example, Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6). Hybridization procedures are also well known to those skilled in the art (for example, as described in Ausubel *et al.*, Current Protocols in Molecular Biology, 5 John Wiley & Sons Inc. (1994), Silhavy *et al.*, Experiments with Gene Fusion, Cold Spring Harbor Laboratory Press (1984); Davis *et al.* Methods in Enzymol. 65:404 (1980)). Important parameters that can be considered for optimizing hybridization conditions are reflected in a formula that allows calculation of a critical value, the melting temperature above which two complementary DNA 10 strands separate from each other (Davis *et al.* Methods in Enzymol. 65:404(1980)). This formula is as follows:  $T_m = 81.5 + 0.41 \times (\% \text{ G+C}) + 16.6 \log (\text{actual ion concentration}) - 0.63 \times (\% \text{ formamide}) - 600/\text{base number}$ . Under appropriate stringency conditions, hybridization temperature ( $T_h$ ) is approximately 20 to 40°C, 20 to 25°C, or, preferably 30 to 40°C below the calculated  $T_m$ . Those 15 skilled in the art will understand that optimal temperature and salt conditions can be readily determined empirically in preliminary experiments using conventional procedures.

For example, stringent conditions can be achieved, both for pre-hybridizing and hybridizing incubations, (i) within 4 to 16 hours at 42°C, in 6 x 20 SSC containing 50% formamide or (ii) within 4-16 hours at 65°C in an aqueous 6 x SSC solution (1 M NaCl, 0.1 M sodium citrate (pH 7.0)). Typically, hybridization experiments are performed at a temperature from 60 to 68°C, e.g. 65°C. At such a temperature, stringent hybridization conditions can be achieved in 6xSSC, preferably in 2xSSC or 1xSSC, more preferably in 0.5xSSC, 0.3xSSC or 25 0.1xSSC (in the absence of formamide). 1xSSC contains 0.15 M NaCl and 0.015 M sodium citrate.

For polynucleotides containing 30 to 600 nucleotides, the above formula is used and then is corrected by subtracting (600/polynucleotide size in base pairs). Stringency conditions are defined by a  $T_h$  that is 5 to 10°C below  $T_m$ .

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Hybridization conditions with oligonucleotides shorter than 20 to 30 bases do not exactly follow the rules set forth above. In such cases, the formula for calculating the  $T_m$  is as follows:  $T_m = 4 \times (G+C) + 2 (A+T)$ . For example, an 18 nucleotide fragment of 50% G+C would have an approximate  $T_m$  of 54°C.

5 Nucleic acid molecules from a modified *gp100* gene such as the *gp100M* gene can be isolated by preparing a labeled nucleic acid probe based on all or part of the nucleic acid sequence as shown in Figure 1, and using this labeled nucleic acid probe to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). Nucleic acids isolated by screening of a cDNA or  
10 genomic DNA library can be sequenced by standard techniques.

Nucleic acid molecules of the invention can also be isolated by selectively amplifying a nucleic acid using the polymerase chain reaction (PCR) method and cDNA, genomic DNA or other source of DNA. It is possible to design synthetic oligonucleotide primers from the nucleic acid molecule as shown in Figure 1 for  
15 use in PCR. These synthetic oligonucleotide primers may also be further modified to incorporate specific changes from the normal nucleic acid sequence so as to be utilized for site directed mutagenesis. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an  
20 appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., (Biochemistry, 18, 5294-5299 (1979)). cDNA is then synthesized from the mRNA using reverse transcriptase  
25 (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

An isolated nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding a novel protein of the invention into an  
30 appropriate vector which allows for transcription of the cDNA to produce an

RNA molecule which encodes the gp100M protein. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g., a T7 promoter) in a vector, cDNA can be transcribed *in vitro* with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

5 A nucleic acid molecule of the invention may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., U.S. Patent No. 4,598,049; U.S. Patent No. 4,458,066; 10 and U.S. Patent Nos. 4,401,796 and 4,373,071).

The initiation codon and untranslated sequences of the nucleic acid molecules of the invention may be determined using currently available computer software designed for the purpose, such as PC/Gene (IntelliGenetics Inc., Calif.). Regulatory elements can be identified using conventional 15 techniques. The function of the elements can be confirmed by using these elements to express a reporter gene which is operatively linked to the elements. These constructs may be introduced into cultured cells using standard procedures. In addition to identifying regulatory elements in DNA, such constructs may also be used to identify proteins interacting with the elements, 20 using techniques known in the art.

The invention also provides nucleic acids encoding fusion proteins comprising a novel protein of the invention and a selected protein, or a selectable marker protein (see below).

## 25 II. METHODS OF EXPRESSING NUCLEIC ACID SEQUENCES OF THE INVENTION

A polynucleotide molecule of the invention, containing RNA, DNA, or modifications or combinations thereof, can have various applications. For example, a polynucleide molecule can be used (i) in a process for producing the encoded polypeptide in a recombinant host system, (ii) in the construction of 30 vaccine vectors (such as poxviruses) used for modulating immune systems; (iii)

as vaccine vectors which are further used in methods and compositions for preventing and/or treating melanoma, (iv) as a vaccine agent (as well as an RNA molecule), in a naked form or formulated with a delivery vehicle and, (v) as detection reagents/hybridization probes in molecular and/or therapeutic assays.

5           According to further aspects of the invention, there are provided (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required for expression, in particular under the control of an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a procaryotic or eucaryotic cell transformed or  
10 transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention, which involves culturing a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, under conditions that allow expression of the DNA  
15 molecule of the invention and, recovering the encoded polypeptide or polypeptide derivative from the cell culture.

A recombinant expression system can be selected from procaryotic and eucaryotic hosts. Eucaryotic hosts include yeast cells (*e.g.*, *Saccharomyces cerevisiae* or *Pichia pastoris*), mammalian cells (*e.g.*, COS1, NIH3T3, or JEG3 cells),  
20 arthropods cells (*e.g.*, *Spodoptera frugiperda* (SF9) cells), and plant cells. Preferably, a procaryotic host such as *E. coli* is used. Bacterial and eucaryotic cells are available from a number of different sources to those skilled in the art, *e.g.*, the American Type Culture Collection (ATCC; 10801 University Blvd., Manassas, VA 20110-2209, USA).

25           The choice of the expression system depends on the features desired for the expressed polypeptide. For example, it may be useful to produce a polypeptide of the invention in a particular lipidated form or any other form.

The choice of the expression cassette will depend on the host system selected as well as the features desired for the expressed polypeptide. Typically,  
30 an expression cassette includes a promoter that is functional in the selected host

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system and can be constitutive or inducible; a ribosome binding site; a start codon (ATG) if necessary; a region encoding a signal peptide (*e.g.*, a lipidation signal peptide); a DNA molecule of the invention; a stop codon; and optionally a 3' terminal region (translation and/or transcription terminator). The signal peptide-encoding region is adjacent to the polynucleotide of the invention and placed in proper reading frame. The signal peptide-encoding region can be homologous or heterologous to the DNA molecule encoding the mature polypeptide and can be specific to the secretion apparatus of the host used for expression. The open reading frame constituted by the DNA molecule of the invention, solely or together with the signal peptide, is placed under the control of the promoter so that transcription and translation occur in the host system. Promoters, (and signal peptide encoding regions) are widely known and available to those skilled in the art and include, for example, the promoter of *Salmonella typhimurium* (and derivatives) that is inducible by arabinose (promoter araB) and is functional in Gram-negative bacteria such as *E. coli* (as described in U.S. Patent No. 5,028,530 and in Cagnon *et al.*, Protein Eng. 4:843 (1991)), the promoter of the gene of bacteriophage T7 encoding RNA polymerase that is functional in a number of *E. coli* strains expressing T7 polymerase (described in U.S. Patent No. 4,952,496), OspA lipidation signal peptide, and the RlpB lipidation signal peptide (Cagnon *et al.*, Protein Eng. 4:843 (1991)).

The expression cassette is typically part of an expression vector, which is selected for its ability to replicate in the chosen expression system. Expression vectors (*e.g.*, plasmids or viral vectors) can be chosen from those described in Pouwels *et al.* (Cloning Vectors: A Laboratory Manual 1985, Supp. 1987). They can be purchased from various commercial sources.

Methods for transforming/transfecting host cells with expression vectors will depend on the host system selected as described in Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc. (1994).

Upon expression, a recombinant polypeptide of the invention (or a polypeptide derivative) is produced and remains in the intracellular

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compartment, is secreted/excreted in the extracellular medium or in the periplasmic space, or is embedded in the cellular membrane. The polypeptide can then be recovered in a substantially purified form from the cell extract or from the supernatant after centrifugation of the recombinant cell culture.

5 Typically, the recombinant polypeptide can be purified by antibody-based affinity purification or by any other method that can be readily adapted by a person skilled in the art, such as by genetic fusion to a small affinity binding domain. Antibody-based affinity purification methods are also available for purifying a polypeptide of the invention. Antibodies useful for purifying by

10 immunoaffinity the polypeptides of the invention can be obtained as described below.

According to further aspects of the invention, there are provided (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required for expression, in particular under the control of

15 an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention which involves culturing a procaryotic or

20 eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention under conditions that allow expression of the DNA molecule of the invention, and recovering the encoded polypeptide or polypeptide derivative from the cell culture.

### **III. NOVEL PROTEINS OF THE INVENTION**

25 The invention further includes an isolated protein encoded by nucleic acid molecules of the invention. Within the context of the present invention, a protein of the invention may include various structural forms of the primary protein which retain biological activity. As used herein "modified gp100" or "modified gp100 protein", "gp100M" or "gp100M protein", means a gp100 which

30 has been modified to provide a molecule which modulates the immune system.

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By "polypeptide" or "protein" is meant any chain of amino acids, regardless of length or post-translational modification (*e.g.*, glycosylation or phosphorylation). Both terms are used interchangeably in the present application.

The terms "modified gp100", "modified gp100 protein", "gp100M" or  
5 "gp100M protein" as used herein is intended to include analogs of a modified gp100 or gp100M, containing one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids with amino acids of similar charge, size, and/or  
10 hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent to a modified gp100M. Non-conserved substitutions involve replacing one or more amino acids with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

15 One or more amino acid insertions may be introduced into the amino acid sequence of modified gp100, preferably gp100M. Amino acid insertions may consist of single amino acid residues or sequential amino acids.

Deletions may consist of the removal of one or more amino acids, or discrete portions (*i.e.* amino acids) from the gp100M amino acid sequence. The  
20 deleted amino acids may or may not be contiguous.

Also included in the expression "gp100M", "modified gp100", "modified gp100 protein" or "gp100M protein" as used herein are homologs of gp100M. Such homologs are proteins whose amino acid sequences are comprised of the amino acid sequences of gp100M regions from other sources whose coding  
25 nucleic acid sequences hybridize under stringent hybridization conditions (which conditions are known to those skilled in the art) with a nucleic acid probe used to obtain gp100M. It is anticipated that a protein comprising an amino acid sequence which is at least 72%, preferably 75 to 90% similar, with the amino acid sequence of gp100M will exhibit gp100M activity.

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As used herein the expressions "modified gp100", "modified gp100 protein", "gp100M", or "gp100M protein" also contemplate isoforms of the modified gp100, or gp100M protein. An isoform contains the same number and kinds of amino acids as the modified gp100 or gp100M, but the isoform has a  
5 different molecular structure. The isoforms contemplated by the present invention are those having the same properties as the protein of the invention as described herein. Also included in the expression are other proteins which share similarities to a modified gp100 or gp100M.

Broadly stated, the present invention provides an isolated protein with  
10 activity equivalent to that of a modified gp100, preferably a gp100M protein. In a preferred embodiment of the invention, the protein has the amino acid sequence as shown in Figure 2.

In addition to full length amino acid sequences, the proteins of the present invention also include truncations of the protein and analogs and  
15 homologs of the protein and truncations thereof as described herein. Truncated proteins may comprise peptides of at least fifteen amino acid residues. Analogs of the protein having the amino acid sequence shown in Figure 2 and/or truncations thereof as described herein, may include, but are not limited to, an amino acid sequence containing one or more amino acid substitutions, insertions,  
20 and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of the proteins of the invention with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent.  
25 Non-conserved substitutions involve replacing one or more amino acids of the amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into the amino acid sequences shown in Figure 2. Amino acid insertions may consist of single  
30 amino acid residues or a range of sequential amino acids.

- 20 -

Deletions may consist of the removal of one or more amino acids, or discrete portions from the amino acid sequence shown in Figure 2. The deleted amino acids may or may not be contiguous. The lower limit length of the resulting analog with a deletion mutation is about 10 amino acids, preferably 100  
5 amino acids.

Analogs of a protein of the invention may be prepared by introducing mutations in the nucleotide sequence encoding the protein. Mutations in nucleotide sequences constructed for expression of analogs of a protein of the invention must preserve the reading frame of the coding sequences.  
10 Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures, such as loops or hairpins, which could adversely affect translation of the receptor mRNA.

Mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites  
15 enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site specific mutagenesis procedures may be employed to provide an altered gene having particular  
20 codons altered according to the substitution, deletion, or insertion required. Deletion or truncation of a protein of the invention may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are  
25 disclosed by Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989).

The proteins of the invention also include homologs of the amino acid sequence shown in Figure 2 and/or truncations thereof as described herein. Such homologs are proteins whose amino acid sequences are comprised of  
30 amino acid sequences whose coding nucleic acid sequences hybridize under

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stringent hybridization conditions (see discussion of stringent hybridization conditions herein) with a nucleic acid probe used to obtain a protein of the invention.

A homologous protein includes a protein with an amino acid sequence  
5 having at least 70%, preferably 80-90% identity with the amino acid sequence as shown in Figure 2. Homology is typically measured using sequence analysis software (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Similar amino acid sequences are aligned to obtain the  
10 maximum degree of homology (*i.e.*, identity). To this end, it may be necessary to artificially introduce gaps into the sequence. Once the optimal alignment has been set up, the degree of homology (*i.e.*, identity) is established by recording all of the positions in which the amino acids of both sequences are identical, relative to the total number of positions. For example, sequence alignments may be  
15 performed using the ALIGN (Trademark) or GENALIGN (Trademark) computer programs (Inteligenetics Suite 5.4, Oxford Molecular). ALIGN uses the Needleman-Wunsch algorithm (Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970)) and its later modifications to locate regions of similarity between two sequences. Finding regions of maximum similarity between two sequences can  
20 be solved in a rigorous manner using the iterative matrix calculation of the Needleman and Wunsch 1997 algorithm. The analysis is restricted to regions with no internal deletions or insertions, joined by a minimum number of loop-outs or deletions. Sellers (*J. Appl. Math (Siam)* 26:787 (1974)) developed a true metric measure of the "distance" between sequences and Waterman et al.  
25 (*Advan. Math* 20:367 (1976)) extended this algorithm to include insertions and deletions of arbitrary length. Smith (*J. Mol. Biol.* 147:195 (1981)) improved the early algorithms to find the subsequences of maximum similarity. The algorithm has been used to analyze sequences as long as 5000 bases by dividing these sequences into segments of 200 to 400 bases, and then reassembling them into a  
30 final best match. This method of dividing the sequence and then reassembling it

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has proven quite robust. The algorithm permits the size of the segment to be specified which the program searches for similarities. The program then assembles the segments after checking overlaps of adjacent subsequences. The weighting of deletions and the relative size of overlaps may be controlled. The program displays the results to show the differences in closely related sequences. GENALIGN is a multiple alignment program. Up to 99 sequences using the Martinez/Regions (Sobel and Martinez, Nucleic Acid Res 14:363 (1985)) or Needleman-Wunsch (Needleman and Wunsch, J. Mol. Biol. 48:443 (1970)) method may be analyzed for alignment. GENALIGN places the sequences in an order that puts the most closely aligned sequence pairs adjacent to each other. A consensus sequence is displayed under the multiple sequence alignments. The sequences used in developing the consensus sequence file for use in other programs. GENALIGN allows the parameters of the search to be changed so that alternate alignments of the sequences can be formed.

These programs are used employing their default settings. The default settings are as follows:

	FastDB		
	AMINO-Res-length	=	2
	Deletion-weight	=	5.00
20	Length-factor	=	0
	Matching-weight	=	1.00
	NUCLEIC-Res-length	=	4
	Spread-factor	=	50
	Findseq		
25	<u>Search Parameters:</u>		
	Similarity matrix		Unitary
	K-tuple		4
	Mismatch penalty		1
	Joining Penalty		30
30	Randomization group length		0

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Cutoff score 5

Alignment Parameters:

Window size 32

Gap penalty 1.00

5 Gap size penalty 0.33

The invention also contemplates isoforms of the proteins of the invention. An isoform contains the same number and kinds of amino acids as a protein of the invention, but the isoform has a different molecular structure. The isoforms contemplated by the present invention are those having the same  
10 properties as a protein of the invention as described herein.

Polypeptides having a sequence homologous to the sequence of a modified gp100 such as that shown in Figure 2, include naturally-occurring allelic variants, as well as mutants or any other non-naturally occurring variants that are analogous in terms of antigenicity, to a polypeptide having a sequence as  
15 shown in Figure 2.

As is known in the art, an allelic variant is an alternate form of a polypeptide that is characterized as having a substitution, deletion, or addition of one or more amino acids that does not alter the biological function of the polypeptide. By "biological function" is meant the function of the polypeptide in  
20 the cells in which it naturally occurs, even if the function is not necessary for the growth or survival of the cells. For example, the biological function of a porin is to allow the entry into cells of compounds present in the extracellular medium. The biological function is distinct from the antigenic function. A polypeptide can have more than one biological function.

Allelic variants are very common in nature. For example, a bacterial species (i.e. *C. pneumoniae*) is usually represented by a variety of strains that differ from each other by minor allelic variations. Indeed, a polypeptide that fulfills the same biological function in different strains can have an amino acid  
25 sequence that is not identical in each of the strains. Such an allelic variation may  
30 be equally reflected at the polynucleotide level.

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The present invention also includes a protein of the invention conjugated with a selected protein, or a selectable marker protein (see below) to produce fusion proteins. Additionally, immunogenic portions and/or fragments of a modified gp100 of the invention are within the scope of the invention.

5           An example of fusion polypeptides included in this invention includes a polypeptide or polypeptide derivative of the invention fused to a polypeptide having adjuvant activity (such as, for example, subunit B of either cholera toxin or *E. coli* heat-labile toxin). Several possibilities exist for achieving fusion. First, the polypeptide of the invention can be fused to the N-, or preferably, to the C-  
10 terminal end of the polypeptide having adjuvant activity. Second, a polypeptide fragment of the invention can be fused within the amino acid sequence of the polypeptide having adjuvant activity.

The proteins of the invention (including truncations, analogs, etc.) may be prepared using recombinant DNA methods. Accordingly, the nucleic acid  
15 molecules of the present invention having a sequence which encodes a protein of the invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-  
20 associated viruses), so long as the vector is compatible with the host cell used. The expression "vectors are suitable for transformation of a host cell" is defined as meaning that the expression vectors contain a nucleic acid molecule of the invention and attendant regulatory sequences selected on the basis of the host cells to be used for expression, said regulatory sequence being operatively linked  
25 to the nucleic acid molecule. "Operatively linked" is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a  
30 fragment thereof, and the necessary regulatory sequences for the transcription

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and translation of the inserted nucleotide-sequence. Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, or viral genes. (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990)). Selection of appropriate regulatory sequences is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include the following: a transcriptional promoter and enhancer, or RNA polymerase binding sequence, or a ribosomal binding sequence (including a translation initiation signal). Additionally, depending on the host cell chosen and the vector employed, other sequences (such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription) may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may also be supplied by the *gp100/gp100M* gene and/or its flanking regions in addition to the above mentioned regulatory gene sequence(s).

The invention further provides a recombinant expression vector comprising a DNA nucleic acid molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression, by transcription of the DNA molecule, of an RNA molecule which is antisense to a nucleotide sequence comprising the nucleotides as shown in Figure 1. Regulatory sequences operatively linked to the antisense nucleic acid can be chosen which direct the continuous expression of the antisense RNA molecule.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin (which confer resistance to certain drugs),  $\beta$ -galactosidase,

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chloramphenicol acetyltransferase, or firefly luciferase. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as  $\beta$ -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. As is known to one skilled in the art, cells that have incorporated the selectable marker gene will survive, while cells which do not have any such incorporated detectable marker will die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention. It will also be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and/or aids in the purification of a target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein.

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. The term "transformed host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming

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and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic  
5 host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

10 The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, J. Am. Chem. Assoc. 85:2149-2154 (1964)) or synthesis in homogenous solution (Houbenweyl, Methods of Organic Chemistry (1987), (ed. E. Wansch) Vol. 15, pts. I and II, Thieme, Stuttgart).

#### 15 **IV. VACCINES**

The present invention includes a vaccine for modulating an animal's immune system wherein the immunogen is an effective amount of a *gp100* or modified *gp100*, and/or immunogenic fragments thereof, preferably in admixture with a suitable diluent or carrier.

20 Accordingly, the present invention also includes a method of modulating an animal's immune response comprising administering an effective amount of a *gp100* or modified *gp100* and/or immunogenic fragments thereof, preferably in admixture with a suitable diluent or carrier, to an animal in need thereof.

25 The vaccines of the present invention may additionally contain suitable diluents, adjuvants and/or carriers. Preferably, the vaccines contain one or more other adjuvants which can further enhance the immunogenicity of the vaccine *in vivo*. These other one or more adjuvants may be selected from many known adjuvants in the art including, for example, the lipid-A portion of the LPS from  
30 gram negative bacteria (endotoxin), trehalose dimycolate of mycobacteria, the

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phospholipid lysolecithin, dimethyldictadecyl ammonium bromide (DDA), certain linear polyoxypropylene-polyoxyethylene (POP-POE) block polymers, aluminum hydroxide (and other aluminum compounds), and liposomes (see below).

5 Another preferred adjuvant/immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 9602555; European  
10 Patent EP 468520; Davies et al. (1998) *J. Immunol.* 160:87; McCluskie and Davis (1998) *J. Immunol.* 161:4463). In a number of studies, synthetic oligonucleotides derived from BCG gene sequences have also been shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo; Krieg, (1995) *Nature* 374:546). Detailed analyses of immunostimulatory oligonucleotide  
15 sequences has demonstrated that the CG motif must be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. (For example, the immunostimulatory sequence is often: purine, purine, C, G, pyrimidine, pyrimidine, wherein the CG motif is not methylated; however other unmethylated CpG sequences are known to be  
20 immunostimulatory and as such may also be used in the present invention.)

The vaccines may also include as adjuvants cytokines that are known to enhance immune responses (including GM-CSF, IL-2, IL-12, TNF and IFN $\gamma$ ), co-stimulatory molecules (such of those of the B7 family) and/or other lymphokines. The vaccine may also contain preservatives such as sodium azide,  
25 thimersol, beta propiolactone, and binary ethyleneimine.

The vaccine compositions of the invention are suitable for administration to subjects in a biologically compatible form *in vivo*. The expression "biologically compatible form suitable for administration in vivo" as used herein means a form of the substance to be administered in which any toxic effects are  
30 outweighed by the therapeutic effects.

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The dose of the vaccine may vary according to factors such as the disease state, age, sex, and weight of the , and the ability of the vaccine to elicit a desired response in the animal. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be  
5 administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. The dose of the vaccine may also be varied to provide optimum preventative dose response depending upon the circumstances.

The vaccines may be administered in a convenient manner such as by  
10 injection (subcutaneous, intravenous, intramuscular, intranodal etc.), oral administration, inhalation, transdermal administration (such as topical cream or ointment, etc.), or suppository applications.

As such, in one aspect of this invention, there are provided (i) a vaccine containing a modified gp100 (or immunogenic fragment thereof) of the  
15 invention; (ii) a composition of matter containing a gp100 (or immunogenic fragment thereof) of the invention, together with a diluent or carrier; (iii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a modified gp100 (or immunogenic fragment thereof) of the invention; (iv) a method for inducing an immune response against a modified  
20 gp100 (or immunogenic fragment thereof) in a mammal (for example, a human; alternatively, the method can be used in veterinary application) which involves administering the mammal an immunogenically effective amount of a modified gp100 (or immunogenic fragment thereof) of the invention to elicit an immune response (for example, a protective or therapeutic immune response to modified  
25 gp100); (v) a method for preventing and/or treating melanoma which involves administering a prophylactic or therapeutic amount of a modified gp100 (or immunogenic fragment thereof) of the invention to an individual in need. Additionally, the invention encompasses the use of a modified gp100 (or immunogenic fragment thereof) of the invention in the preparation of a  
30 medicament for preventing and/or treating of melanoma.

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A vaccine of the invention may contain a nucleic acid molecule encoding a modified gp100 protein of the invention. Such vaccines are referred to as nucleic acid vaccines but are also termed genetic vaccines, polynucleotide vaccines or DNA vaccines, all of which are within the scope of the present invention. In such  
5 an embodiment, the modified gp100 protein is produced *in vivo* in the host animal. The vaccines containing nucleic acids may be delivered using a suitable vector (ie. "vaccine vector") including, for example, retroviral vectors, alphaviral, adenoviral vectors, poxviral vectors, other viral vectors, bacterial DNA, plasmids, or free/naked DNA.

10 Accordingly, in additional aspects of the invention, there are provided (i) a vaccine vector containing a DNA molecule of the invention placed under the control of elements required for expression; (ii) a composition of matter containing a vaccine vector of the invention, together with a diluent or carrier; (iii) a pharmaceutical composition containing a therapeutically or prophylactically  
15 effective amount of a vaccine vector of the invention; (iv) a method for inducing an immune response against modified gp100 in a mammal (for example, a human; alternatively, the method can be used in veterinary applications) which involves administering to the mammal an immunogenically effective amount of a vaccine vector of the invention to elicit an immune response, (for example, a  
20 protective or therapeutic immune response to modified gp100); (v) a method for preventing and/or treating melanoma which involves administering a prophylactic or therapeutic amount of a vaccine vector of the invention to an individual in need. Additionally, the invention encompasses the use of a vaccine vector of the invention in the preparation of a medicament for preventing  
25 and/or treating of melanoma.

*i Vaccine Vector*

The vaccine vector may be a poxvirus or other viral vector, bacterial DNA, plasmid or a free/naked DNA. Preferably the vaccine vector is incapable of integration in recipient animal cells. The elements for expression from said

vaccine vector may include a promoter suitable for expression in recipient animal cells.

Live vaccine vectors available in the art include viral vectors such as alphaviruses, adenoviruses and poxviruses as well as bacterial vectors (for example, *Shigella*, *Salmonella*, *Vibrio cholerae*, *Lactobacillus*, Bacille Calmette Guérin (BCG), and *Streptococcus*).

An example of an adenovirus vector, as well as a method for constructing an adenovirus vector capable of expressing a DNA molecule of the invention, are described in U.S. Patent No. 4,920,209 (incorporated herein by reference). Poxvirus vectors that can be used include, for example, fowlpox, vaccinia and canary pox virus (as described in U.S. Patent No. 5,766,599 and U.S. Patent No. 5,364,773, U.S. Patent No. 5,756,103, (ALVAC(2), U.S. Patent No. 5,990,091, U.S. Patent No. 6,004,777); Poxvirus vectors capable of expressing a nucleic acid of the invention can be obtained by homologous recombination as is known to one skilled in the art so that the polynucleotide of the invention is inserted in the viral genome under appropriate conditions for expression in mammalian cells (see below).

In one preferred aspect the engineered poxvirus vector is ALVAC (which has been derived from canarypox virus). ALVAC does not productively replicate in non-avian hosts, a characteristic thought to improve its safety profile.

ALVAC is an attenuated canarypox virus-based vector that was a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox (Tartaglia et al., *Viol.* 188:217 (1992)) (U.S. Patent No. 5,756,103). ALVAC has some general properties which are the same as some general properties of Kanapox. ALVAC-based recombinant viruses expressing extrinsic immunogens have also been demonstrated efficacious as vaccine vectors (Tartaglia, J. et al, In *AIDS Research Reviews* (vol. 3), Koff, W., Wong-Staal, F., Kenedy, R.C. (eds), Marcel Dekker NY, pp. 361-378 (1993); Tartaglia, J. et al. *J. Virol.* 67:2370 (1993)). For instance, mice immunized with an ALVAC recombinant expressing the rabies virus glycoprotein were protected from lethal challenge with rabies virus

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(Tartaglia, J. et al. (1992), *Virology* 188:217) demonstrating the potential for ALVAC as a vaccine vector. ALVAC-based recombinants have also proven efficacious in dogs challenged with canine distemper virus (Taylor, J. et al., (1992), *Virology* 187:321) and rabies virus (Perkus, M.E. et al., In *Combined Vaccines and Simultaneous Administration: Current Issues and Perspective*, Annals of New York Academy of Sciences (1994)), in cats challenged with feline leukemia virus (Tartaglia, J. et al. *J. Virol.* 67:2370 (1993)), and in horses challenged with equine influenza virus (Taylor, J. et al., In *Proceedings of the Third International Symposium on Avian Influenza*, Univ. of Wisconsin-Madison, Madison, Wisconsin, pp. 331-335 (1993)).

ALVAC (2) is a second-generation ALVAC vector in which vaccinia transcription elements E3L and K3L have been inserted within the C6 locus (U.S. 5,990,091; U.S. 6,004,777). The E3L encodes a protein capable of specifically binding to dsRNA. The K3L ORF has significant homology to E1F-2. Within ALVAC (2) the E3L gene is under the transcriptional control of its natural promoter, whereas K3L has been placed under the control of the early/late vaccine H6 promoter. The E3L and K3L genes act to inhibit PKR activity in cells infected with ALVAC (II), allowing enhancement of the level and persistence of foreign gene expression.

Additional vaccine vector systems involve the use of naturally host-restricted poxviruses. Fowlpox virus (FPV) is the prototypic virus of the Avipox genus of the Poxvirus family. Vectors derived from fowlpox have been generated (i.e., TROVAC, see U.S. Patent No. 5,766,599) Replication of the avipox viruses is limited to avian species (Matthews, *Inter Virol.* 17:42 (1982)) and there are no reports in the literature of avipoxvirus causing a productive infection in any non-avian species including man. This host restriction provides an inherent safety barrier to transmission of the virus to other species and makes use of avipoxvirus based vaccine vectors in veterinary and human applications an attractive proposition.

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FPV has been used advantageously as a vector expressing antigens from poultry pathogens. The hemagglutinin protein of a virulent avian influenza virus was expressed in an FPV recombinant. After inoculation of the recombinant into chickens and turkeys, an immune response was induced which  
5 was protective against either a homologous or a heterologous virulent influenza virus challenge (Taylor, J. et al., (1988) *Vaccine* 6:504). FPV recombinants expressing the surface glycoproteins of Newcastle Disease Virus have also been developed (Taylor, J. et al. (1990) *J. Virol.* 64:1441; Edbauer, C. et al., (1990) *Virology* 179:901).

10 Highly attenuated strain of vaccines, designated MVA, has also been used as a vector for poxvirus-based vaccines. Use of MVA is described in U.S. Patent No. 5,185,146.

Other attenuated poxvirus vectors have been prepared by genetic modifications of wild type strains of virus. The NYVAC vector, for example, is  
15 derived by deletion of specific virulence and host-range genes from the Copenhagen strain of vaccinia (Tartaglia, J. et al. (1992) *Virology* 188:217) and has proven useful as a recombinant vector in eliciting a protective immune response against an expressed foreign antigen (U.S. 5,364,773). The TROVAC vector provides yet another example of an attenuated poxvirus which may be used  
20 (U.S. 5,766,599).

Recombinant poxviruses can be constructed in two steps known in the art and analogous to the methods for creating synthetic recombinants of poxviruses such as the vaccinia virus and avipox virus (described in U.S. Patent Nos. 4,769,330; 4,744,848; 4,603,112; 5,100,587; and 5,179,993). Clearly, based on  
25 the attenuation profiles of the NYVAC, ALVAC, and TROVAC vectors and their demonstrated ability to elicit both humoral and cellular immunological responses to extrinsic immunogens (Tartaglia, J. et al, In *AIDS Research Reviews* (vol.3), Koff, W., Wong-Staal, F. and Kenedy, R.C. (eds), Marcel Dekker NY, pp. 361-378 (1993); Tartaglia, J. et al. (1993) *J. Virol.* 67:2370;; Taylor, J. et al., (1992) *Virology*  
30 187:321), such recombinant viruses offer a distinct advantage over previously

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described vaccinia-based recombinant viruses. It can thus be appreciated that provision of a modified gp100 recombinant poxvirus, and of compositions and products therefrom (particularly ALVAC-modified gp100 recombinants and compositions and products therefrom) would be a highly desirable advance over  
5 the current state of technology.

Plasmids and/or free/naked nucleic acids (i.e. polynucleotides (DNA or RNA)) of the invention can also be administered as vaccine vectors to an animal for vaccine (*e.g.*, therapeutic or prophylactic) purpose (US Patent No. 5589466; McDonnell and Askari, NEJM 334:42-45 (1996); Kowalczyk and Ertl, Cell Mol. Life  
10 Sci. 55:751-770 (1999)). When a DNA molecule of the invention is used, it can be in a free/naked or plasmid form. Typically it is a form that is unable to replicate in a mammalian cell and unable to integrate in the mammalian genome. The DNA molecule is also typically placed under the control of a promoter suitable for expression in a mammalian cell. The promoter can function ubiquitously or  
15 tissue-specifically. Examples of non-tissue specific promoters include the early Cytomegalovirus (CMV) promoter (described in U.S. Patent No. 4,168,062) and the Rous Sarcoma Virus promoter. The desmin promoter is tissue-specific and drives expression in muscle cells. More generally, useful vectors have been described (*i.e.*, WO 94/21797).

20 For DNA/RNA vaccination, the polynucleotide of the invention can encode a precursor or mature form of the modified gp100 or immunogenic fragment thereof. When it encodes a precursor form, the precursor form can be homologous or heterologous. In the latter case, a eucaryotic leader sequence can be used, such as the leader sequence of the tissue-type plasminogen factor (tPA).

25 Standard techniques of molecular biology for preparing and purifying polynucleotides can be used in the preparation of polynucleotide aspects of the invention. For use as a vaccine, a polynucleotide of the invention can be formulated according to various methods known to those who are skilled in the art.

First, a polynucleotide can be used in a naked/free form, free of any delivery vehicles (such as anionic liposomes, cationic lipids, microparticles, (*e.g.*, gold microparticles), precipitating agents (*e.g.*, calcium phosphate)) or any other transfection-facilitating agent. In this case the polynucleotide can be simply  
5 diluted in a physiologically acceptable solution (such as sterile saline or sterile buffered saline) with or without a carrier. When present, the carrier preferably is isotonic, hypotonic, or weakly hypertonic, and has a relatively low ionic strength (such as provided by a sucrose solution (*e.g.*, a solution containing 20% sucrose))

Alternatively, a polynucleotide can be associated with agents that assist  
10 in cellular uptake. It can be, *i.a.*, (i) complemented with a chemical agent that modifies the cellular permeability (such as bupivacaine; see, for example, WO 94/16737), (ii) encapsulated into liposomes, or (iii) associated with cationic lipids or silica, gold, or tungsten microparticles.

Cationic lipids are well known in the art and are commonly used for  
15 gene delivery. Such lipids include Lipofectin( also known as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethylammonio) propane). DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine) and cholesterol derivatives such as DC-Chol (3 beta-(N-(N',N'-dimethyl  
20 aminomethane)-carbamoyl) cholesterol). A description of these cationic lipids can be found in EP 187,702, WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5,527,928. Cationic lipids for gene delivery are preferably used in association with a neutral lipid such as DOPE (dioleoyl phosphatidylethanolamine), as, for example, described in WO 90/11092.

25 Other transfection-facilitating compounds can be added to a formulation containing cationic liposomes. A number of them are described in, for example, WO 93/18759, WO 93/19768, WO 94/25608, and WO 95/2397. They include, *i.e.*, spermine derivatives useful for facilitating the transport of DNA through the nuclear membrane (see, for example, WO 93/18759) and membrane-

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permeabilizing compounds such as GALA, Gramicidine S, and cationic bile salts (see, for example, WO 93/19768).

Gold or tungsten microparticles can also be used for gene delivery (as described in WO 91/359 and WO 93/17706). In this case, the microparticle-coated  
5 polynucleotides can be injected *via* intradermal or intraepidermal routes using a needleless injection device ("gene gun"), such as those described, for example, in U.S. Patent No. 4,945,050, U.S. Patent No. 5,015,580, and WO 94/24263.

Anionic and neutral liposomes are also well-known in the art (see, for example, Liposomes: A Practical Approach, RPC New Ed, IRL Press (1990), for a  
10 detailed description of methods for making liposomes) and are useful for delivering a large range of products, including polynucleotides.

A vaccine vector of the invention can also express a cytokine (for example, such as interleukin-2 (IL-2), interleukin-12 (IL-12), granulocyte-macrophage colony stimulating factor (GM-CSF)) and/or co-stimulatory  
15 molecules (for example, such as the B7 family of molecules) and/or other lymphokines that enhance the immune response. Thus, a vaccine vector can include an additional DNA sequence encoding, for example, a cytokine and/or lymphokine and/or co-stimulatory molecule placed under the control of suitable elements required for expression in an animal cell.

20 Alternatively, a composition of the invention can include several vaccine vectors each being capable of expressing an aspect of the invention (i.e., polypeptide derivative of the invention, cytokine and/or lymphokine and/or costimulatory molecules) a polypeptide or derivative of the invention.

*ii Mode of Administration*

25 In vaccination methods for treating or preventing cancer in an animal, a vaccine vector of the invention can be administered by any conventional route in use in the vaccine field as is known to one skilled in the art. This may include, for example, administration to a mucosal (e.g., ocular, intranasal, oral, gastric, pulmonary, intestinal, rectal, vaginal, or urinary tract) surface, via the parenteral  
30 (e.g., subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal)

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route or intranodally. Preferred routes depend upon the choice of the vaccine vector. The administration can be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters understood by skilled artisans such as the vaccine vector itself, the route of administration  
5 and the condition of the animal to be vaccinated (weight, age and the like).

The administration of the vaccine or immunogen of the present invention may be for either a prophylactic or therapeutic purpose. When provided prophylactically, the immunogen is provided in advance of any evidence or in advance of any symptom due to melanoma, or in patients  
10 rendered free of disease by conventional therapies but at significant risk for reoccurrence. The prophylactic administration of the immunogen serves to prevent or attenuate melanoma in a mammal. When provided therapeutically, the immunogen is provided at (or after) the onset of the disease or at the onset of any symptom of the disease. The therapeutic administration of the  
15 immunogen serves to attenuate the disease.

A particularly preferred vaccination method encompasses a prime boost protocol. Recent studies have indicated that this protocol, whereby immunization with a poxvirus recombinant expressing a foreign gene product (or other nucleic acid encoding for a gene product) is followed by a boost using a  
20 purified subunit preparation form of that gene product (or nucleic acid coding therefor), elicits an enhanced immune response relative to the response elicited with either product alone. Accordingly, it is within the scope of the present invention to use a modified gp100 in a prime-boost protocol. Examples of methodologies teaching prime-boost protocol are described in WO 98/58956,  
25 WO 00/00216, WO 98/56919, WO 97/39771, and WO 98/58956 which are incorporated herein by reference.

The amount of naked/free DNA to be used in a vaccine recipient generally depends on the strength of the promoter used in the DNA construct, the immunogenicity of the expressed gene product, the condition of the animal  
30 intended for administration (i.e. the weight, age, and general health of the

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animal), the mode of administration, and the type of formulation. In general, a therapeutically or prophylactically effective dose from about 1 µg to about 1 mg, preferably, from about 10 µg to about 800 µg and, more preferably, from about 25 µg to about 250 µg, can be administered to human adults. The administration  
5 can be achieved in a single dose, repeated at intervals, or incorporated into prime boost protocols (as previously described).

*iii. Oral Vaccines*

Non-toxicogenic *Vibrio cholerae* mutant strains that are useful as a live oral vaccine are described, for example, in US Patent No. 4,882,278 (disclosing a  
10 strain in which a substantial amount of the coding sequence of each of the two *ctxA* alleles has been deleted so that no functional cholerae toxin is produced); WO 92/11354 (strain in which the *irgA* locus is inactivated by mutation; this mutation can be combined in a single strain with *ctxA* mutations); and WO 94/1533 (deletion mutant lacking functional *ctxA* and *attRS1* DNA sequences).  
15 These strains can be genetically engineered to express heterologous antigens, as described in WO 94/19482. (All of the aforementioned issued patent/patent applications are incorporated herein by reference.) An effective vaccine dose of a *Vibrio cholerae* strain capable of expressing a polypeptide or polypeptide derivative encoded by a DNA molecule of the invention can contain, for  
20 example, about  $1 \times 10^5$  to about  $1 \times 10^9$ , preferably about  $1 \times 10^6$  to about  $1 \times 10^8$  viable bacteria in an appropriate volume for the selected route of administration. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

Attenuated *Salmonella typhimurium* strains, genetically engineered for  
25 recombinant expression of heterologous antigens or not, and their use as oral vaccines are described, for example, in WO 92/11361. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

As will be readily appreciated by those skilled in the art, other bacterial  
30 strains useful as vaccine vectors may also include *Shigella flexneri*, *Streptococcus*

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gordonii, and Bacille Calmette Guerin (as described in WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796, and WO 92/21376; all of which are incorporated herein by reference).

In bacterial vectors, a polynucleotide of the invention may be inserted  
5 into the bacterial genome, can remain in a free state or carried on a plasmid. An adjuvant can also be added to a composition containing a vaccine bacterial vector. A number of adjuvants are known to those skilled in the art. Suitable adjuvants can readily be selected by those skilled in the art.

#### **V. COMPOSITIONS**

10 A modified gp100 protein and gene, including the gp100M gene (*gp100M*) and gp100M protein as well as the substances identified using the methods described herein, including vaccines, may be formulated into pharmaceutical compositions for administration to subjects in a biologically  
15 compatible form suitable for administration in vivo. By "biologically compatible form suitable for administration in vivo" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to animals in need thereof. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention, or an "effective amount", are defined as  
20 an amount effective, at dosages and for periods of time necessary to achieve the desired result of "modulating an animal's immune system". A therapeutically effective amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the animal, and the ability of immunogen to elicit a desired response in the animal. Dosage regima may be adjusted to  
25 provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. "Modulating an animals immune system" is defined as the ability to produce an immune response in a target animal. This response encompasses both cellular and humoral immune  
30 responses.

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The active substance may be administered in a convenient manner such as by injection (intradermal, intramuscular, subcutaneous, intravenous, intranodal etc.), or by oral administration, inhalation, transdermal application, or rectal administration, or any other route of administration that enables the modulation of an animal's immune system. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

One route of administration which is preferably used is that of intranodal injection of a modified gp100 protein/immunogenic fragment, or nucleic acid encoding said protein/fragment, or any of the compositions of the present invention.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences (1985), Mack Publishing Company, Easton, Pa., USA) or Handbook of Pharmaceutical Additives (compiled by Michael and Irene Ash, Gower Publishing Limited, Aldershot, England (1995)). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and may be contained in buffered solutions with a suitable pH and/or be iso-osmotic with physiological fluids. In this regard, reference can be made to U.S. Patent No. 5,843,456.

The utility of the substances, antibodies, and compositions of the invention may be confirmed in experimental model systems.

The following non-limiting examples are illustrative of the present invention:

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#### EXAMPLES

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ALVAC(2)-gp100M (vCP1584) is a preparation of recombinant canarypox virus expressing a modified version of human melanoma antigen gp100.

## A. MOLECULAR PROPERTIES (IDENTITY)

### 1. Recipient characterization:

#### 5 a. Parental organism:

Canarypox virus: Family-Poxviridae, Subfamily -Chordopoxviridae, Genus-Avipoxvirus. Canarypox virus is an enveloped virus containing a linear dsDNA genome of approximately 325 kbp. This virus productively replicates exclusively in avian species ((Tripathy D. Avian pox. In: Diseases of Poultry (9<sup>th</sup> edition: B.W. Calnek et al. Eds.) pp. 583-596).

#### 10 b. Description of vector:

ALVAC(2) is a modified, attenuated canarypox virus (U.S. 5,990,091). The original strain of canarypox virus (Rentschler strain) was attenuated by 200 serial passages on primary chick embryo fibroblasts (CEFs). The attenuated virus was plaque isolated and designated ALVAC. To generate ALVAC(2), the ALVAC vector was modified by the insertion of two vaccinia virus coding sequences (E3L and K3L) to enhance the overall efficiency of viral mRNA translation. The E3L-specified gene product is a dsRNA binding protein and the K3L open reading frame (ORF) shares significant sequence similarity to the amino terminal portion of eIF-2( (; Beattie et al. Virology 183:419 (1991); Beattie et al. Virology 210:254 (1995). Both K3L and E3L are capable of inhibiting the activity of a cellular protein kinase (PKR) which, when activated by dsRNA, phosphorylates the translational initiation factor eIF-2a leading to an inhibition of initiation of mRNA translation. Results from several studies have substantiated this proposed mechanism by which the vaccinia K3L and E3L gene products lead to down regulation of PKR activity and enhanced virus-specific gene expression. (Tartaglia, J. et al. 11<sup>th</sup> Colloque des Cent Gardes, Elsevier Press (1997)).

#### 25 c. Derivation of vector from parental organism:

The parental strain of canarypoxvirus (Rentschler strain) was isolated in Germany in 1970, and obtained by Institut Merieux in 1973. The virus was

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attenuated by 200 serial passages on primary chick embryo fibroblasts (CEFs). The attenuated virus was registered as a vaccine in France in 1975 under the name KANAPOX (ND). The virus was subjected to four successive plaque purifications under agarose and a single plaque isolate was selected and  
5 designated ALVAC.

The K3L coding sequence was synthesized by PCR amplification using a plasmid containing Copenhagen vaccinia *HindIII* K fragment as template and was placed under the control of the vaccinia H6 promoter. The E3L coding sequence and upstream regulatory element were obtained from a plasmid  
10 containing a clone of the *HindIII* E fragment from Copenhagen vaccinia. The H6 promoted K3L and the E3L sequences were inserted into a donor plasmid capable of directing their insertion into the C6 site of the parent vector according to the details set out in Example 1. Recombination was performed between the donor plasmid and ALVAC rescuing virus by *in vitro* recombination as  
15 previously described (Piccini *et al.*, Methods of Enzymol. 153:545 (1987)). Expression of E3L and K3L in the resultant recombinant virus, vCP1468, was confirmed and vCP1468 was designated ALVAC(2).

d. Cloning site:

The locus designated C5 was used for the insertion of the modified  
20 gp100 coding sequences into the ALVAC(2) vector. By virtue of the C5 locus existing within the extensive inverted terminal repetitions (ITRs) of the virus genome, insertion into this locus results in the occurrence of two copies of the inserted sequence. A schematic of the insertion site is shown in Figure 4. Presently, no function has been ascribed to the C5 encoded polypeptide nor does  
25 the deduced amino acid open reading frame encoded in this region share significant homology to any entry in the existing protein databases.

**2. Donor characterization**

a. Donor organism:

Plasmids pCDNA3-gp100 and PCR11-gp100 each containing the gene  
30 encoding human gp100 (melanoma rejection antigen) were used.

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b. Donor genes:(i) Expressed donor genes: human gp100 melanoma antigen.(ii) Promoter: Vaccinia virus early/late H6 promoter (PerkusM.E. *et al*, J. Virol. 63:3829 (1989)).5 c. Donor genes in the recombinant organism:

The coding sequences for a modified human gp100 have been inserted into the ALVAC(2) genome to express the modified gp100 antigen enhanced for the interaction of two CTL epitopes with HLA class I.

**EXAMPLE 1**10 Insertion of the vaccinia E3L/K3L coding sequences into the C6 site of ALVAC

The K3L coding sequences were synthesized by PCR amplification using pSD407 (containing Copenhagen vaccinia *Hind*III K fragment) as template. The oligonucleotides MPSYN 763 (5'-CCCTCT AGATCG CGATAT CCGTTA AGTTTG TATCGT AATGCT TGCATT TTGTTA TTCGT-3') (SEQ.ID.NO.109) and  
15 MPSYN 764 (5'-CCCGAA TTCATA AAAATT ATTGAT GTCTACA-3') (SEQ.ID.NO.110) were used as primers for the PCR reaction. The approximately 325bp PCR fragment was digested with *Xba*I and *Eco*RI yielding a 315bp fragment. This 315bp fragment was purified by isolation from an agarose gel and ligated with *Xba*I and *Eco*RI digested pBSSK+ vector from Stratagene (La  
20 Jolla, CA.). The nucleic acid sequence was confirmed. This plasmid was designated pBS 763\764. Digesting pBS 763/764 with *Nru*I and *Xho*I a 340bp fragment was isolated for cloning into the plasmid vector pMM154. PMM154 contains a cassette with the vaccinia H6 promoter controlling an irrelevant gene in the NYVAC tk insertion vector background. PMM154 was prepared by  
25 digestion with *Nru*I (partially) and *Xho*I such that the 340bp fragment from pBS 763 / 764 containing the K3L gene could be directionally oriented next to the H6 promoter generating pMPTKH6K3L. The plasmid pMP42GPT containing the dominant selectable marker Eco gpt gene under the control of the Entomopox 42k promoter was digested with *Sma*I and *Bam*HI to yield a 0.7 Kbp 42k- Eco gpt  
30 expression cassette. This 0.7 Kbp fragment was purified and ligated into *Sma*I and

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*Bam*HI cut pMPTKH6K3L generating the plasmid pMPTKH6K3Lgpt. This plasmid was digested with *Xho*I generating a 1.2 Kbp fragment containing the H6/K3L and the 42k/Ecogpt expression cassette which was then gel purified. The 1.2 Kbp *Xho*I fragment was inserted into the *Xho*I site of the ALVAC C6  
5 insertion plasmid pC6L generating pMPC6H6K3Lgpt.

The entire E3L gene is contained within a 2.3 Kbp *Eco*RI fragment isolated from pSD401VC which contained a clone of the *Hind*III E fragment from Copenhagen vaccinia. The 2.3 Kbp *Eco*RI fragment was then inserted into pMPC6H6K3Lgpt that had been partially digested with *Eco*RI generating the  
10 plasmid pMPC6H6K3E3gpt. The plasmid pMPC6H6K3E3gpt was digested with *Xho*I. The resulting 6.8 Kbp vector fragment was purified and self-ligated, resulting in the plasmid pMPC6E3. The plasmid pMPTKH6K3L was digested with *Psp*AI and the resulting 560bp fragment containing the H6 / K3L expression cassette was ligated into *Psp*AI digested pMPC6E3 resulting in the plasmid  
15 construct pMPC6H6K3E3. The plasmid pMPC6H6K3E3 contains the vaccinia H6/ K3L expression cassette and the vaccinia E3L gene with the endogenous promoter flanked by the ALVAC C6 insertion site sequences.

## EXAMPLE 2

### Genetic modifications of the donor genes:

20 Plasmid pCDNA3-gp100 was transformed into MN522 yielding plasmid pMEL gp100 #1. A generic C5 donor plasmid NVQH6C5LSP-18 was digested within the polylinker region with *Bam*HI, treated with alkaline phosphatase and ligated to kinased and annealed oligonucleotides SPC5PL1 (5'-GAT-CGT-CGA-CGA-GCT-CGA-ATT-CG-3') (SEQ.ID.NO.111) and SPC5PL2 (5'-GAT-CCG-AAT-TCG-AGC-TCG-TCG-AC-3') (SEQ.ID.NO.112) generating plasmid NVQH6MC5  
25 #10. Oligonucleotides MELgp01 (5'-CCC-TCG-CGA-TAT-CCG-TTA-AGT-TTG-TAT-CGT-AAT-GGA-TCT-GGT-GCT-AAA-AAG-3') (SEQ.ID.NO.113) and MELgp02 (5'-CCC-CTC-GAG-ATA-AAA-ATC-AGA-CCT-GCT-GCC-CAC-TGA-3') (SEQ.ID.NO.114) were used in PCR with plasmid pMEL gp100 #1 to generate  
30 a 2kb fragment containing part of the H6 promoter linked to the 5' end of the

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gp100 gene. This fragment was digested with *EcoRV* and *XhoI* and cloned into *EcoRV/XhoI* digested NVQH6MC5#10 generating plasmid C5H6MELgp100 #5 which contains the gp100 gene linked to the H6 promoter.

The gp100 gene in plasmid C5H6MELgp100 #5 was sequenced using  
 5 custom primers. A 65bp deletion was found in this clone and shown to be present in pCDNA3-gp100. Plasmid PCRII-gp100 was used in PCR with oligonucleotides MELgp05(5'-CCC-ATC-TGG-CTC-TTG-GTC-3') (SEQ.ID.NO. 115) and MELgp13 (5'-TGA-CAT-CTC-TGC-CAG-TGT-GGT-3') (SEQ.ID.NO. 116) to generate a 0.6kb fragment. This fragment was digested with *Bam*HI and  
 10 *Asp*718 and ligated to a 6.5kb *Asp*718/*Bam*HI (partial) fragment from C5H6MELgp100 #5 generating plasmid C5H6MELgp100 which contains the entire gp100 gene under the control of the H6 promoter.

Pre-existing plasmid pC5H6MELgp100 was used as template for site directed mutagenesis of the two CTL epitopes beginning at amino acids 209 and  
 15 280, respectively. Primers used were:

209-A

GCT CAG CCT TCA CCA TTA TGG ACC AGG TGC CTT TCT CC  
 (SEQ.ID.NO.117)

209-B

20 GGA GAA AGG CAC CTG GTC CAT AAT GGT GAA GGC TGA CG  
 (SEQ.ID.NO.118)

280-A

GAG CCT GGC CCA GTC ACT GTT CAG GTG GTC CTG CAG GC  
 (SEQ.ID.NO.119)

25 280-B

GCC TGC AGG ACC ACC TGA ACA GTG ACT GGG CCA GGC TC  
 (SEQ.ID.NO.120)

A section containing the modified epitopes was sequenced and isolated as a 440 bp *Nco*1/*Mlu*N1 fragment. This fragment was ligated into

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pC5H6MELgp100 digested with *Nco*1 and *Mlu*N1, creating a plasmid with the complete gp100 with the modified epitopes 209-2M and 280-9V.

Sequence data revealed a G to C substitution at bp# 10, changing a.a. # 4 from a Valine to a Leucine. This was corrected by PCR using the following

5 primer pair;

MEL25

GCT CCG GGA TCC CCG GCG ATG GTA GAC AGT CAC TTC CAT CGT GTG  
TGT GCC CAG CAT TG (SEQ.ID.NO.121)

MEL27

10 ATC GCG ATA TCC GTT AAG TTT GTA TCG TAA TGG ATC TGG TGC TAA  
AAA GAT GCC TTC TT (SEQ.ID.NO.122)

MEL25 changes bp# 549 from a C to a G destroying the unique *Nco*1 site for easier screening. It does not change the amino acid.

The resulting PCR fragment was digested with *Bam*H1 and *Eco*R5 and  
15 replaced the equivalent fragment correcting the error. The resulting plasmid is pC5gp100-M which is shown in Figure 3 (SEQ.ID.NO.123).

Genetic modification of the recipient:

Recombination between donor plasmid pC5gp100M and ALVAC(2) rescuing virus generated recombinant virus vCP1584, which contains the  
20 vaccinia H6 promoted modified human gp100 in the C5 locus.

EXAMPLE 3

Screening for the identification and purification of recombinant organisms:

The aspects of screening for the identification and purification of a recombinant organism of the present invention is set out below.

25 (1) Plaque purification was done using *in situ* plaque hybridization (Piccini *et al.*, Methods of Enzymol. 153:545 (1987)) was used to identify recombinant viruses and to demonstrate purity of final virus preparations. *In situ* plaque hybridization analysis was performed with radiolabelled probes specific for the gp100 construct (a 580 bp fragment) and the C5 insertion locus.

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(2) Restriction analysis: Viral genomic DNA was isolated from cells infected with ALVAC parent or ALVAC(2)-gp100M (vCP1584). The genomic DNA was digested with restriction endonucleases (*HindIII*, *Pst I* or *BamHI*). The resultant DNA fragments were fractionated by electrophoresis through an agarose gel and visualized by ethidium bromide staining. The insertion of the mod gp100 expression cassette at the C5 locus was confirmed.

(3) Immunoprecipitation analyses: These were performed using radiolabeled lysates derived from uninfected HeLa cells or cells infected with either ALVAC parental virus, ALVAC-gp100 (vCP1465) or ALVAC(2)-gp100M (vCP1584) as described previously (Taylor *et al.* J. Virol. 64:1441 (1990)). Briefly, HeLa cell cultures were infected at an m.o.i. of 10 pfu/cell in methionine-free media supplemented with [35S]-methionine (35uCi/ml). At 18 hrs. post infection, cells were lysed. Immunoprecipitation was performed using a rabbit anti-gp100 serum (AZN-LAM, received from M. Schreurs University of Nijmegen, Netherlands). Immunoprecipitates were fractionated on a 10% SDS-Polyacrylamide gel. The gel was fixed and treated for fluorography with 1M Na-salicylate for 1/2 hr. The dried gel was exposed to Kodak XAR-2 film to visualize the protein species. Results with anti-gp100 demonstrate expression of gp100 in ALVAC-gp100 infected HeLa cells but not for parentally infected cells. (See Figure 6)

(4) Western Blot. HeLa cells were infected for 18 hours at a multiplicity of 10 pfu/cell with ALVAC(2)-gp100M (vCP1584), ALVAC-gp100 (vCP1465) or ALVAC. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated with AZN-LAM (1/5000 dilution) followed by HRP conjugated swine anti-rabbit utilizing the enhanced chemiluminescence (ECL) detection method (Amersham). Results demonstrate expression of full length gp100 in ALVAC-gp100 and ALVAC(2)-gp100M infected cells. (See Figure 7).

(5) Plaque immunoscreen analysis. This was performed on vCP1584 material to determine phenotypic stability of the virus upon passaging. The

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phenotypic stability of production batch material of ALVAC-gp100M (vCP1584) was analyzed by an immunological plaque assay which measures expression of the inserted genes at the plaque level. The assay utilizing permeabilized cells for detection of intracellular as well as surface expression of Hgp100mod was chosen  
5 for this test.

Test and control reagents (ALVAC(2)-gp100M (vCP1584) and ALVAC standard and ALVAC-gp100M, respectively) were plated on CEF monolayers under agarose at dilutions resulting in 40-200 plaques per 60 mm dish. 120 hours after incubation at 37°C, the infected monolayers were processed by plaque  
10 immunoassay for detection of internal expression of gp100M. Positive and negative plaques were counted for test and control samples. The primary antibody used was Monoclonal Anti-HMB50 at 1:800 dilution. A secondary antibody used was horse radish peroxidase (HRP)-conjugated rabbit anti-mouse antiserum diluted 1:500.

15 The result of analysis of internal expression of Human modified gp100 by individual plaques produced by (vCP1584) is presented in Table 1.

The result demonstrates that 98.7% of the plaque population of ALVAC-gp100M is expressing gp100M indicating that ALVAC-gp100M is phenotypically stable.

20 Results of the plaque immunoscreen analysis demonstrate that ALVAC(2)-gp100M is phenotypically stable with respect to expression of gp100.

(6) Nucleotide sequence analysis. This was performed on vCP1584 to validate the nucleotide sequence of the H6-promoted melanoma gp100M cassette. The sequence analysis revealed no nucleotide differences relative to the  
25 expected sequence, thus no mutations were introduced during the production of vCP1584. In order to carry out this analysis, a pool of plasmid clones containing a 2.2 kb PCR-derived fragment (encompassing the H6-promoted melanoma gp100M cassette), generated from vCP1584 genomic DNA was used.

pBS/1584 was generated by pooling 9 positive clones obtained by the  
30 ligation of a 2.2 kb PCR fragment (containing the H6-promoted melanoma

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gp100M cassette from vCP1584), into pBS-sk-(Stratogene). The 2.2 kb PCR fragment was derived from vCP1584 genomic DNA with the oligonucleotide primers, IDC5-1 and IDC5-2 (Figure 5). The nucleotide sequence of the oligonucleotide primers used to sequence pBS/1584 are listed in Figure 5.

5 **EXAMPLE 4**

This example provides results from injection in cynomolgus monkeys of modified gp100 molecules.

**Methods and Experimental Design**

**Test System**

- 10 Cynomolgus monkeys (Macaca fascicularis) purpose bred animals.  
Supplier: Siconbrec "Simian Conservation Breeding & Research Center Inc.",  
Fema Building, 44 Gil Puyat Avenue Makati, Metro Manila, Philippines.  
Number of animals in the study: 12 (6 males and 6 females).  
Age at initiation of treatment: 26 to 38 months.
- 15 - Body weight range at initiation of treatment (day -1):  
- males: 1.73 to 2.34 kg  
- females: 1.71 to 2.65 kg.
- Animal Husbandry**
- Housing: one air-conditioned room;
- 20 - temperature: 19 to 25°C (target range),  
- relative humidity: >40%  
- air changes: minimum 8 air changes per hour,  
- lighting cycle: 12 hours light (artificial)/12 hours dark.  
- Caging: animals were housed singly in stainless steel mesh cages
- 25 (approximately 540 x 810 x 760 mm).  
- Diet: expanded complete commercial primate diet (Mazuri diet, Special Diet  
Services Ltd., Witham, Essex, CM8, 3AD, Great Britain) analyzed for chemical  
and bacterial contaminants.  
Quantity distributed: 100g diet/animal/day.
- 30 In addition, animals received fruit daily (apple or banana)

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Animals were fasted for at least 16 hours before blood sampling for clinical laboratory investigations and before necropsy.

- Water: drinking water *ad libitum* (via bottles).

- Contaminants: no known contaminants were present in diet or water at levels  
5 which might have interfered with achieving the objective of the study.

#### **Pre-Treatment Procedures**

- Animal health procedure: all animals received a clinical examination for ill-health on arrival and a veterinary clinical examination during the acclimatization period.

10 - Acclimatization period: at least 3 weeks between animal arrival and start of treatment.

#### **Experimental Design**

- Allocation to treatment groups was performed during the acclimatization period using a random allocation procedure based on body weight classes.

15 - Animals were assigned to the treatment groups shown in Table 2. The dose levels administered were shown in Table 3.

#### **Administration of the Test/Control Articles**

##### Group 1 and 2 Animals

- Method of administration: injection in the left inguinal lymph node. Animals  
20 were lightly anaesthetized before each administration by an intramuscular injection of ketmine hydrochloride (Imalgene® 500 - Merial, Lyon, France). The same lymph node was injected on each occasion (left side). Each injection was followed by a local disinfection with iodine (Vétédine® - Vétoquinol, Lure, France).

##### 25 Group 3

- Route: subcutaneous.

- Method of administration: bolus injection using a sterile syringe and needle introduced subcutaneously. Four injection sites were used followed by a local disinfection with iodine (Vétédine® - Vétoquinol, Lure, France). Animals were  
30 also lightly anaesthetized before each administration by an intramuscular

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injection of ketamine hydrochloride (Imalgene® 500 - Merial, Lyon, France) in order to be under the same conditions as groups 1 and 2 animals.

Four injection sites in the dorsal cervical/interscapular regions were used as shown in Table 4.

## 5 ELISPOT Analysis

An ELISPOT assay was used in order to assess the cell mediated immune response generated in the monkeys in the various treatment groups. In particular, an ELISPOT IFN $\gamma$  assay was used in order to measure IFN $\gamma$  production from T lymphocytes obtained from the monkeys in response to  
10 gp100 antigens.

### Materials and Methods

Plates: MILLIPORE Multiscreen HA plate / MAHA S45.10 (96 wells).

Capture antibodies: MABTECH monoclonal anti-IFN $\gamma$  antibodies/G-Z4 1 mg/mL.

15 Detection antibodies: MABTECH monoclonal anti-IFN $\gamma$  antibodies/7-B6-1-biotin 1 mg/mL.

Enzyme: SIGMA, Extravidin-PA conjugate/E2636

Substrate: BIORAD, NBT/BCIP - Alkaline phosphatase conjugate substrate kit/ref: 170-64 32.

### 20 Coating

Place 100  $\mu$ L per well of capture antibodies at 1  $\mu$ g/mL diluted at 1/1000 in carbonate bicarbonate buffer 0.1M pH 9.6 into the multiwell plate. Incubate overnight at 4°C. Wash 4 times in 1X PBS.

### Saturation

25 Place 200  $\mu$ L per well of RPMI supplemented with 10% FCS, non essential amino acids, pyruvate, HEPES buffer and Peni-Strepto. Incubate 2 hours at 37°C.

### Test

Cells from the immunized animals are tested against (a) medium alone; (b) pooled peptides at a concentration of 1 mg/mL; and (c) a non specific stimulus  
30 (PMA-Iono). The pooled peptides used in this Example to stimulate IFN- $\gamma$

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production were derived from gp100 and are illustrated in Tables 5 to 8. The final volume of each sample is 200  $\mu$ L. Incubate 20 hours at 37°C.

Wash 4 times in 1X PBS and 0.05% Tween 20.

#### Detection

- 5 Place 100  $\mu$ L per well of detection antibodies at 1  $\mu$ g/mL diluted in 1/1000 1X PBS, 1% BSA and 0.05% Tween 20. Incubate 2 hours at room temperature. Wash 4 times in 1X PBS and 0.05% Tween 20.

#### Reaction

- Place 100  $\mu$ L per well of Extravidin-PA conjugate diluted 1/6000 in 1X PBS, 1%  
10 BSA and 0.05% Tween 20. Incubate 45 minutes at room temperature. Wash 4 times in 1X PBS and 0.05% Tween 20.

#### Substrate Addition

- Place 100  $\mu$ L per well of substrate previously prepared. For example, for 1 plate,  
prepare: 9.6 mL of distilled water, 0.4 mL of 25X buffer, 0.1 mL of solution A  
15 (NBT) and 0.1 mL of solution B (BCIP). Incubate 30-45 minutes at room temperature. Wash in distilled water. Dry and transfer to a plastic film. The number of spots are counted using a Zeiss image analyzer. Each spot corresponds to an individual IFN- $\gamma$  secreting T cell.

#### Results

- 20 The results of the ELISPOT analysis are shown in Figures 8-11. The results demonstrate that of the animals tested, 2 out of 2 (i.e. 100%) of the animals that received the intranodal administration of the gp100 antigen, and 2 out of 4 (i.e. 50%) of the animals that received the subcutaneous administration of the gp100 antigen had a positive cell mediated immune response.

#### 25 ELISA Analysis

- The ELISA was performed utilizing standard methodology known in the art. Briefly, the human gp100 ("hgp100"; produced in Baculovirus) was diluted in coating buffer (carbonate-bicarbonate, pH9.6) and added to 96 wells at 0.5ug/well. Plates were placed at 4°C overnight. Plates were then washed and  
30 blocking buffer (phosphate buffered saline/0.5% Tween 20/1.0% BSA, pH7.2)

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was added for 2 hours at 37°C. The plates were then washed and the sera was diluted in dilution buffer (phosphate buffered saline/0.5 % Tween 20/ 0.1 BSA, pH7.2). For this study, monkey sera was diluted to 1:800 and "7" serial 3 fold dilutions were done for each sample tested. The human sera controls were  
5 diluted to 1:50 in dilution buffer and "7" serial 2 fold dilutions were performed. Each dilution was done in duplicate. The plates were incubated a further 2 hours at 37°C. The plates were washed and the horse radish peroxidase (HRP)-conjugated anti-human secondary antibody (anti-human Ig whole antibody from sheep (Amersham Life Science, NA933)) diluted 1:100 in dilution buffer was  
10 added to the wells and incubated for 1 hour at 37°C. The plates were washed and OPD (o-phenylenediamine dihydrochloride) substrate with H<sub>2</sub>O<sub>2</sub> in substrate buffer (50mM phosphate/25mM citrate, pH 7.2) was added to the wells. For a kinetics ELISA, the plate was read repeatedly (2 minute intervals for 15 minutes) unstopped (without "stop" buffer). Plates were read at 450nm.

## 15 Results

The results of the above experiment are presented in Table 9 and in Figure 12. The animals of group 2 received intranodal injections of ALVAC(2)-gp100(mod) followed by boosts with the modified gp100 peptides 209(2M) and 290(9V); the animals in group 3 received a subcutaneous injection of the  
20 ALVAC(2) construct followed by peptide boosts; the animals in group 1 received intranodal injections of saline as a control.

As can be seen from Figure 12, both types of injection of the antigens induced a significant humoral response to the antigen.

In summary, the results of this Example demonstrate that injection of a  
25 tumor antigen according to the invention induces both a significant humoral and cell mediated response.

## EXAMPLE 5

This example presents data obtained from human melanoma patients primed with ALVAC(2)-gp100M and boosted with modified gp100 peptides  
30 (g209-2M and g280-9V).

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### Immunization Protocol

5 Patients were immunized subcutaneously in a prime-boost schedule with ALVAC(2)-gp100M ("prime"; lyophilized ALVAC(2)-gp100M resuspended in 1 ml of 0.4% NaCl; 0.5 ml injections (approximately  $0.5 \times 10^{7.09}$  CCID<sub>50</sub> per injection)) and peptides g209-2M and g280-9V ("boost"; 1000µg/peptide in 1 ml total volume per week (0.2 ml/injection per day x 5 days)). All patients: 1) were HLA-A0201 positive; 2) were between 18 and 70 years of age; 3) exhibited pathologically confirmed malignant melanoma; 4) demonstrated immunocompetence by reactivity to at least 2 or more out of 7 Cell Mediated Immunity (CMI) skin tests; 5) had blood hematology and chemistry values within the following ranges:

#### I) Hematology:

Hemoglobin	> 100g/L
Granulocytes	> $2.0 \times 10^9$ /L
Lymphocytes	> $1.5 \times 10^9$ /L
Platelets	> $100 \times 10^9$ /L

#### II) Chemistry:

Serum creatinine	< 150 µmol/L
Serum total bilirubin	<30 µmol/L
AST, ALT, and ALP	Must be < 2x the normal upper limit or <5x the normal upper limit if due to liver metastases.

15

Patients "primed" with ALVAC(2)-gp100M on weeks 1, 4 and 7; "boosted" with peptides on weeks 10 and 13.

**ELISPOT Analysis:** These results are present in Tables 10 and 11. Peripheral Blood Mononuclear Cells ("PBMNC") were isolated by density centrifugation

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over Ficoll gradients. Cells were bulk-cultured at  $3 \times 10^6$ /ml in AIM-V media along with a mixture of g209-2M and g280-9V or the HLA-A\*0201 binding Flu peptide (all at 50  $\mu$ g/ml) for 8 days. IL-2 was added on days 3 and 5 of culture. On day 9, cells were harvested, counted and  $2 \times 10^5$  cells/well plus 50 U/ml IL-2, with and without the respective peptides, were plated in nitrocellulose membrane containing ELISPOT plates that had been precoated with anti-INF- $\gamma$  antibodies. The plates were developed after 48 hours of culture. The numbers reported are the differences between the average of two wells restimulated with peptide and IL-2 and two wells treated only with IL-2.

10            Responses are the number of spots (counted by the electronic ELISPOT reader but confirmed in most cases by manual counting) per  $2 \times 10^5$  PBMNC. The number of CD8+ T cells was not routinely determined but is typically 2-5-fold less than this number.

15            Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated by those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

20            All publications, patents and patent applications referred to herein, are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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**TABLE 1**

Analysis of expression of gp100 antigen by ALVAC-gp100M

	Human gp100M			
	Positive Plaques	negative plaques	total # of plaques	% positive
ALVAC std.	0	571	571	0
vCP1584	387	0	387	100
ALVAC gp100mod L	875	11	886	98.7

**TABLE 2**

Group Number	Route of administration	Treatment days and compound administered	Number of Animals
1	Intranodal	Saline (NaCl 0.9%): days 28, 42, 56 Then 70, 71, 72, 73, 74 Then 84, 85, 86, 87 and 88	4
2	Intranodal	ALVAC(2) - gp100 mod: days 28, 42, 56 *m <sub>gp</sub> 100 peptides: days 70, 71, 72, 73, 74 Then 84, 85, 86, 87 and 88	4
3	Subcutaneous	Saline (NaCl 0.9%): day 1 ALVAC(2) - gp100 mod: days 28, 42, 56 *m <sub>gp</sub> 100 peptides: days 70 and 84	4

\*209(2M)-IMDQVPFSY (SEQ.ID.NO.124); 290(9V) YLEPGPVTV (SEQ.ID.NO.125)

- 5
- Group 1 animals (control) received the control article (saline for injection (NaCl 0.9%)).
  - Group 3 animals received the control article (saline for injection (NaCl 0.9%)) on day 1 only.

**TABLE 3**

<b>Group Number</b>	<b>Dose level</b>	<b>Dose volume (ml/administration)</b>
1	Saline (NaCl 0.9%): 0	0.250
2	Dose: $0.25 \times 10^{7.4}$ CCID 50 ALVAC (2) - gp100 mod: $0.25 \times 10^{7.4}$ CCID50	0.250
	Dose: 200 $\mu$ g (Total) of peptides IMDQVPFSY (209(2M)), and YLEPGPVTV (290(9V)) (100 $\mu$ g each)	0.2
3	Saline (NaCl 0.9%)	0.250
	ALVAC(2) - gp100 mod: $0.25 \times 10^{7.4}$ CCID 50	0.250
	Dose: 200 $\mu$ g (Total) of peptides IMDQVPFSY (209(2M)), and YLEPGPVTV (290(9V)) (100 $\mu$ g each)	0.2

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**TABLE 4**

<b>Days</b>	<b>Sites used</b>
1 and 28	lower left
42	upper left
56	upper right
70	lower left
84	lower right

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**TABLE 5**

## Peptide Pool #1

Peptide	Sequence	SEQ.ID.NO.
1329	HLAVIGALLAVGATK	SEQ.ID.NO.3
1330	GALLAVGATKVPRNQ	SEQ.ID.NO.4
1331	VGATKVPRNQDWLGV	SEQ.ID.NO.5
1332	VPRNQDWLGVSRQLR	SEQ.ID.NO.6
1333	DWLGVSRQLRTKAWN	SEQ.ID.NO.7
1334	SRQLRTKAWNRQLYP	SEQ.ID.NO.8
1335	TKAWNRQLYPEWTEA	SEQ.ID.NO.9
1336	RQLYPEWTEAQRDLC	SEQ.ID.NO.10
1337	EWTEAQRDLCWRGGQ	SEQ.ID.NO.11
1338	QRDLCWRGGQVSLKV	SEQ.ID.NO.12
1339	WRGGQVSLKVSNDGP	SEQ.ID.NO.13
1340	VSLKVSNDGPTLIGA	SEQ.ID.NO.14
1344	IALNFPGSQKVLDPG	SEQ.ID.NO.15
1345	PGSQKVLDPGQVIWV	SEQ.ID.NO.16
1346	VLPDGQVIWVNNTII	SEQ.ID.NO.17
1347	QVIWVNNTIINGSQV	SEQ.ID.NO.18
1348	NNTIINGSQVWGGQP	SEQ.ID.NO.19
1349	NGSQVWGGQPVYPQE	SEQ.ID.NO.20
1350	WGGQPVYPQETDDAC	SEQ.ID.NO.21
1351	VYPQETDDACIFPDG	SEQ.ID.NO.22
1352	TDDACIFPDGGPCPS	SEQ.ID.NO.23
1353	IFPDGGPCPSGSWSQ	SEQ.ID.NO.24
1355	GSWSQKRSFVYVWKT	SEQ.ID.NO.25
1356	KRSFVYVWKTWGQYW	SEQ.ID.NO.26
1357	YVWKTWGQYWQVLGG	SEQ.ID.NO.27
1358	WGQYWQVLGGPVSGL	SEQ.ID.NO.28
1359	QVLGGPVSGLSIGTG	SEQ.ID.NO.29

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**TABLE 6**

## Peptide Pool #2

Peptide	Sequence	SEQ.ID.NO.
1360	PVSGLSIGTGRAMLG	SEQ.ID.NO.30
1361	SIGTGRAMLGTHTME	SEQ.ID.NO.31
1362	RAMLGTHTMEVTVYH	SEQ.ID.NO.32
1363	THTMEVTVYHRRGSR	SEQ.ID.NO.33
1364	VTVYHRRGSRSYVPL	SEQ.ID.NO.34
1365	RRGSRSYVPLAHSSS	SEQ.ID.NO.35
1366	SYVPLAHSSSAFTIT	SEQ.ID.NO.36
1368	AFTITDQVPFSVSVS	SEQ.ID.NO.37
1369	DQVPFSVSVSQLRAL	SEQ.ID.NO.38
1370	SVSVSQLRALDGGNK	SEQ.ID.NO.39
1372	DGGNKHFLRNQPLTF	SEQ.ID.NO.40
1373	HFLRNQPLTFALQLH	SEQ.ID.NO.41
1374	QPLTFALQLHDPSGY	SEQ.ID.NO.42
1375	ALQLHDPSGYLAEAD	SEQ.ID.NO.43
1379	DFGDSSGTLISRALV	SEQ.ID.NO.44
1380	STGLISRALVVHTY	SEQ.ID.NO.45
1381	SRALVVHTYLEPGP	SEQ.ID.NO.46
1382	VHTYLEPGPVTAQV	SEQ.ID.NO.47
1383	LEPGPVTAQVVLQAA	SEQ.ID.NO.48
1384	VTAQVVLQAAIPLTS	SEQ.ID.NO.49
1385	VLQAAIPLTSCGSSP	SEQ.ID.NO.50
1386	IPLTSCGSSPVP GTT	SEQ.ID.NO.51
1388	VP GTTDGHRPTAEAP	SEQ.ID.NO.52
1389	DGHRPTAEAPNTTAG	SEQ.ID.NO.53
1390	TAEAPNTTAGQVPTT	SEQ.ID.NO.54
1392	QVPTTEVVGTT PGQA	SEQ.ID.NO.55
1393	EVVGTT PGQAPTAEP	SEQ.ID.NO.56

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**TABLE 7**

## Peptide Pool #3

Peptide	Sequence	SEQ.ID.NO.
1394	TPGQAPTAEPSGTTT	SEQ.ID.NO.57
1395	PTAEPSGTTSVQVPT	SEQ.ID.NO.58
1396	SGTTSVQVPTTEVIS	SEQ.ID.NO.59
1397	VQVPTTEVISTAPVQ	SEQ.ID.NO.60
1398	TEVISTAPVQMPTAE	SEQ.ID.NO.61
1399	TAPVQMPTAESTGMT	SEQ.ID.NO.62
1400	MPTAESTGMTPEKVP	SEQ.ID.NO.63
1401	STGMTPEKVPVSEVM	SEQ.ID.NO.64
1402	PEKVPVSEVMGTTLA	SEQ.ID.NO.65
1403	VSEVMGTTLAEMSTP	SEQ.ID.NO.66
1404	GTTLAEMSTPEATGM	SEQ.ID.NO.67
1405	EMSTPEATGMTPEAEV	SEQ.ID.NO.68
1408	SIVVLSGTTAAQVTT	SEQ.ID.NO.69
1409	SGTTAAQVTTTEWVE	SEQ.ID.NO.70
1410	AQVTTTEWVETTARE	SEQ.ID.NO.71
1411	TEWVETTARELPIPE	SEQ.ID.NO.72
1412	TTARELPIPEPEGPD	SEQ.ID.NO.73
1413	LPIPEPEGPDASSIM	SEQ.ID.NO.74
1414	PEGPDASSIMSTESI	SEQ.ID.NO.75
1415	ASSIMSTESITGSLG	SEQ.ID.NO.76
1416	STESITGSLGPLLDG	SEQ.ID.NO.77
1417	TGSLGPLLDGTATLR	SEQ.ID.NO.78
1418	PLLDGTATLRLVKRQ	SEQ.ID.NO.79
1419	TATLRLVKRQVPLDC	SEQ.ID.NO.80
1420	LVKRQVPLDCVLYRY	SEQ.ID.NO.81
1421	VPLDCVLYRYGSFSV	SEQ.ID.NO.82
1422	VLYRYGSFSVTLDIV	SEQ.ID.NO.83

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**Table 8**

## Peptide Pool #4

Peptide	Sequence	SEQ.ID.NO.
1424	TLDIVQGIESAEILQ	SEQ.ID.NO.84
1425	QGIESAEILQAVPSG	SEQ.ID.NO.85
1426	AEILQAVPSGEGDAF	SEQ.ID.NO.86
1427	AVPSGEGDAFELTVS	SEQ.ID.NO.87
1428	EGDAFELTVSCQGGL	SEQ.ID.NO.88
1429	ELTVSCQGGLPKEAC	SEQ.ID.NO.89
1430	CQGGLPKEACMEISS	SEQ.ID.NO.90
1431	PKEACMEISSPGCQP	SEQ.ID.NO.91
1432	MEISSPGCQPPAQR	SEQ.ID.NO.92
1434	PAQRLCQPVLPSAC	SEQ.ID.NO.93
1435	CQPVLPSACQLVLH	SEQ.ID.NO.94
1436	PSPACQLVLHQILKG	SEQ.ID.NO.95
1437	QLVLHQILKGGSGTY	SEQ.ID.NO.96
1441	LADTNSLAVVSTQLI	SEQ.ID.NO.97
1442	SLAVVSTQLIMPGQE	SEQ.ID.NO.98
1443	STQLIMPGQEAGLGQ	SEQ.ID.NO.99
1444	MPGQEAGLGQVPLIV	SEQ.ID.NO.100
1445	AGLGQVPLIVGILLV	SEQ.ID.NO.101
1448	LMAVVLASLIYRRRL	SEQ.ID.NO.102
1450	YRRRLMKQDFSVPQL	SEQ.ID.NO.103
1451	MKQDFSVPQLPHSSS	SEQ.ID.NO.104
1452	SVPQLPHSSSHWLRL	SEQ.ID.NO.105
1453	PHSSSHWLRLPRIFC	SEQ.ID.NO.106
1454	HWLRLPRIFCSCPIG	SEQ.ID.NO.107
1455	PRIFCSCPIGENSPL	SEQ.ID.NO.108

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**TABLE 9**

Monkey #	DAY (mOD/min)			
	0	57	68	96
1	3	5	2	2
2	4	6	12	10
3	7	6	10	8
4	7	6	8	8
5	5	9	20	15
6	11	8	10	12
7	11	23	51	30
8	7	30	70	22
9	1	7	5	3
10	2	6	6	4
11	3	7	14	8
12	6	9	15	6

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**TABLE 10****Gp100-specific responses to g209-2M and g280-9V\***

Patient	Pre 1 <sup>st</sup> Injection	Pre 2 <sup>nd</sup> Injection	Pre 3 <sup>rd</sup> Injection	Pre 4 <sup>th</sup> Injection	Pre 5 <sup>th</sup> Injection	4 wks post vaccination
#1	0	0	0	ND	ND	2±1.4
#2	0	14±2.8	54±6.4	16±7.8	ND	ND
#3	0	0	ND	ND	ND	ND
#4	0	0	24±13.4	1±2.1	ND	ND
#5	ND	6±6.4	ND	ND	ND	ND

5

**TABLE 11**10 **Flu-peptide specific responses\***

Patient	Pre 1 <sup>st</sup> Injection	Pre 2 <sup>nd</sup> Injection	Pre 3 <sup>rd</sup> Injection	Pre 4 <sup>th</sup> Injection	Pre 5 <sup>th</sup> Injection	4 wks post vaccination
#1	>150	ND	>70	ND	ND	12.5
#2	ND	0	24	0	ND	ND
#3	23.5	7	ND	ND	ND	ND
#4	0	29	13.5	11.5	ND	ND
#5	ND	>200	ND	ND	ND	ND

\* ND signifies that the values were not determined for the sample.

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## WE CLAIM:

1. An isolated and purified modified gp100 molecule capable of modulating an immune response in an animal.  
5
2. A molecule according to claim 1 having a nucleic acid sequence shown in Figure 1 (SEQ.ID.NO.1).
3. A molecule according to claim 1 or 2 which comprises:  
10 (a) a nucleic acid sequence as shown in Figure 1 (SEQ.ID.NO.1) wherein T can also be U;  
(b) nucleic acid sequences complementary to (a);  
(c) nucleic acid sequences which are homologous to (a) or (b);  
(d) a fragment of (a) to (c);  
15 (e) a nucleic acid which will hybridize to (a) to (d) under stringent hybridization conditions; and  
(f) a nucleic acid molecule differing from any of the nucleic acids of (a) to (d) in codon sequences due to the degeneracy of the genetic code.
- 20 4. The nucleic acid of any one of claims 1-3 wherein the nucleic acid is selected from the group consisting of viral nucleic acid, plasmid, bacterial DNA, naked/free DNA, and RNA.
5. A viral nucleic acid of claim 4 wherein the virus is selected from  
25 adenovirus, alphavirus or poxvirus.
6. A poxvirus of claim 5 which is vaccinia, fowlpox, avipox, TROVAC, ALVAC, NYVAC or MVA.
- 30 7. The poxvirus of claim 6 which is ALVAC.

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8. A composition comprising the nucleic acid of any one of claims 1-7 and a pharmaceutically acceptable diluent or carrier.
- 5 9. A composition according to claim 8 further comprising an adjuvant.
10. A cell comprising a nucleic acid according to any one of claims 1-7 wherein the cell expresses a polypeptide encoded by the nucleic acid.
- 10 11. A cell according to claim 10 wherein the cell is an antigen-presenting cell.
12. A cell according to claim 10 wherein the cell is a dendritic cell.
13. A recombinant virus comprising a virus into which is inserted a nucleic acid according to any one of claims 1-7 wherein the nucleic acid encodes for a polypeptide, the recombinant virus causing the expression of the polypeptide in an infected cell.
- 15
14. A recombinant virus into which is inserted a nucleic acid according to any one of claims 1-7 wherein the nucleic acid encodes for a polypeptide, wherein cells infected with the said recombinant virus are capable of eliciting an immune response directly against a member selected from the group consisting of:
- 20
- (1) the polypeptide;
- 25 (2) a fragment of the polypeptide;
- (3) a cell expressing the polypeptide or a fragment thereof; or
- (4) cells binding the protein or fragment thereof.
15. A recombinant virus according to claim 13 or 14 selected from adenovirus, alphavirus, or poxvirus.
- 30

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16. A recombinant virus according to claim 15 wherein the poxvirus is vaccinia, fowlpox, avipox, TROVAC, ALVAC, NYVAC or MVA.
- 5 17. A recombinant virus according to claim 16 wherein the virus is ALVAC.
18. A composition comprising a recombinant virus of any one of claims 13 to 17 and a pharmaceutically acceptable diluent or carrier.
- 10 19. An isolated protein encoded by a nucleic acid molecule according to any one of claims 1-7.
20. An isolated protein having the activity of a modified gp100 protein.
- 15 21. A protein having the amino acid sequence shown in Figure 2 (SEQ.ID.NO.2).
22. A method of modulating an animal's immune system comprising administering an effective amount of a gp100 or *gp100* which has been modified.
- 20 23. A method according to claim 22 where the gp100 is gp100M.
24. A method according to claim 22 wherein the *gp100* is *gp100M*.
- 25 25. A method according to claim 24 wherein the gp100M has a nucleic acid sequence as shown in Figure 1 (SEQ.ID.NO.1).
26. A method according to claim 23 wherein the gp100M has an amino acid shown in Figure 2 (SEQ.ID.NO.2).

30

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27. A method of modulating an animal's immune system comprising administering to an animal in need thereof, an effective amount of a vector, into which has been inserted a *gp100* which has been modified, thereby modulating the animal's immune system.
- 5
28. A method according to claim 27 wherein the vector is administered with a lymphokine, cytokine, or a co-stimulatory molecule.
29. A method according to claim 28 wherein the cytokine is GM-CSF, IL-2,  
10 IL-12, TNF, or IFN $\gamma$ 1.
30. A method according to claim 28 wherein the molecule is a lymphokine.
31. A method according to claim 28 wherein the molecule is co-stimulatory  
15 molecule.
32. A method according to claim 31 wherein the co-stimulatory molecule is a molecule of the B7 family.
- 20 33. A method according to any one of claims 27-32 wherein the vector is an adenovirus, alphavirus or poxvirus.
34. A method according to claim 33 wherein the poxvirus is vaccinia, fowlpox, avipox, TROVAC, ALVAC, NYVAC or MVA.  
25
35. A method according to claim 34 wherein the poxvirus is ALVAC
36. A method for prophylactic treatment of cancer comprising administering to an animal an effective amount of a modified *gp100* or

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immunogenic fragment thereof, or a nucleic acid sequence encoding a modified *gp100* or immunogenic fragment thereof.

37. A method according to claim 36 wherein the modified *gp100* has an amino acid sequence as shown in Figure 2 (SEQ.ID.NO.2).

38. A method according to claim 36 wherein the nucleic acid sequence is as shown in Figure 1 (SEQ.ID.NO.1).

39. A method according to any one of claims 36, 37 or 38 wherein the cancer is a melanoma.

40. A melanoma vaccine comprising a nucleic acid sequence encoding a modified *gp100*.

15

41. A vaccine according to claim 40 wherein the modified *gp100* is *gp100M*.

42. A vaccine according to claim 41 wherein the *gp100M* has the amino acid sequence as shown in Figure 2 (SEQ.ID.NO.2).

20

43. A modified *gp100* protein sequence which is modified to enhance its binding to MHC molecules.

44. A modified protein sequence according to claim 43 wherein the protein is *gp100M*.

25

45. The protein of claim 44 wherein the amino acid sequence is as shown in Figure 2 (SEQ.ID.NO.2).

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46. A vaccine comprising a modified *gp100* nucleic acid sequence or its corresponding protein or protein fragment capable of eliciting the production of antibodies in a animal to corresponding antigens.
- 5 47. A vaccine according to claim 46 wherein the protein corresponding to the nucleic acid sequence is *gp100M*.
48. A vaccine according to claim 46 wherein the modified *gp100* nucleic acid sequence is *gp100M*.
- 10 49. A vaccine according to claim 48 wherein the *gp100M* nucleic acid sequence is as shown in Figure 1 (SEQ.ID.NO.1).
- 15 50. A vaccine according to claim 47 wherein the *gp100M* has an amino acid sequence as shown in Figure 2 (SEQ.ID.NO.2).
51. A vaccine comprising a modified *gp100* nucleic acid sequence or its corresponding protein or protein fragment capable of eliciting a cellular immune response.
- 20 52. A vaccine according to claim 51 wherein the protein corresponding to the nucleic acid sequence is *gp100M*.
- 25 53. A vaccine according to claim 51 wherein the modified *gp100* nucleic acid sequence is *gp100M*.
54. A vaccine according to claim 53 wherein the *gp100M* nucleic acid sequence is as shown in Figure 1 (SEQ.ID.NO.1).

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55. A vaccine according to claim 52 wherein the gp100M has an amino acid sequence is as shown in Figure 2 (SEQ.ID.NO.2).
56. An immunogenic composition containing a vaccine vector encoding for  
5 a modified gp100 molecule.
57. A composition according to claim 56 wherein the modified gp100 molecule is gp100M.
- 10 58. A composition according to claim 57 wherein the modified gp100M has an amino acid sequence according to Figure 2 (SEQ.ID.NO.2).
59. A composition according to any one of claims 56, 57 or 58 wherein the vector is an adenovirus, alphavirus or poxvirus.  
15
60. A composition according to claim 59 wherein the poxvirus is vaccinia, fowlpox, avipox, TROVAC, ALVAC, NYVAC or MVA.
61. A composition according to claim 60 wherein the poxvirus is ALVAC.  
20
62. Immunogenic fragments of an isolated gp100M protein encoded by a nucleic acid molecule having a sequence according to SED ID NO. 1.

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FIGURE 1

```

                ATGG ATCTGGTGCT AAAAAGATGC CTTCTTCATT TGGCTGTGAT
AGGTGCTTTG CTGGCTGTGG GGGCTACAAA AGTACCCAGA AACCAGGACT GGCTTGGTGT
CTCAAGGCAA CTCAGAACCA AAGCCTGGAA CAGGCAGCTG TATCCAGAGT GGACAGAAGC
CCAGAGACTT GACTGCTGGA GAGSTGGTCA AGTGTCCCTC AAGGTCAGTA ATGATGGGCC
TACACTGATT GGTGCAAATG CCTCCTTCTC TATTGCCTTG AACTTCCCTG GAAGCCAAAA
GGTATTGCCA GATGGGCAGG TTATCTGGGT CAACAATACC ATCATCAATG GGAGCCAGGT
GTGGGGAGGA CAGCCAGTGT ATCCCCAGGA AACTGACGAT GCCTGCATCT TCCCTGATGG
TGGACCTTGC CCATCTGGCT CTTGGTCTCA GAAGAGAAGC TTTGTTTATG TCTGGAAGAC
CTGGGGCCAA TACTGGCAAG TTCTAGGGGG CCCAGTGTCT GGGCTGAGCA TTGGGACAGG
CAGGGCAATG CTGGGCACAC ACACGATGGA AGTGA CTGTC TACCATCGCC GGGGATCCCG
GAGCTATGTG CCTCTTGCTC ATTCCAGCTC AGCCTTCACC ATTATGGACC AGGTGCCTTT
CTCCGTGAGC GTGTCCAGT TGCGGGCC TTGGAGACAG TAGTGGAACT CTGATCTCTC GGGCACTTGT
TCAGCCTCTG ACCTTTGCCC TCCAGCTCCA TGACCCAGT GGCTATCTGG CTGAAGCTGA
CCTCTCCTAC ACCTGGGACT TTGGAGACAG TAGTGGAACT CTGATCTCTC GGGCACTTGT
GGTCACTCAT ACTTACCTGG AGCCTGGCCC AGTCACTGTT CAGGTGGTCC TGCAGGCTGC
CATTCTCTC ACCTCCTGTG GCTCCTCCCC AGTTCCAGGC ACCACAGATG GGCACAGGCC
AACTGCAGAG GCCCCTAACA CCACAGCTGG CCAAGTGCCT ACTACAGAAG TTGTGGGTAC
TACACCTGGT CAGGCGCCAA CTGCAGAGCC CTCTGGAACC ACATCTGTGC AGGTGCCAAC
CACTGAAGTC ATAAGCACTG CACCTGTGCA GATGCCAACT GCAGAGAGCA CAGGTATGAC
ACCTGAGAAG GTGCCAGTTT CAGAGGTCAT GGGTACCACA CTGGCAGAGA TGCAACTCC
AGAGCTTACA GGTATGACAC CTGCAGAGGT ATCAATTGTG GTGCTTTCTG GAACCACAGC
TGCACAGGTA ACAACTACAG AGTGGGTGGA GACCACAGCT AGAGAGCTAC CTATCCCTGA
GCCTGAAGGT CCAGATGCCA GCTCAATCAT GTCTACGGAA AGTATTACAG GTTCCCTGGG
CCCCCTGCTG GATGGTACAG CCACCTTAAG GCTGGTGAAG AGACAAGTCC CCCTGGATTG
TGTTCTGTAT CGATATGGTT CCTTTTCCGT CACCCTGGAC ATTGTCCAGG GTATTGAAAG
TGCCGAGATC CTGCAGGCTG TGCCGTCCGG TGAGGGGGAT GCATTTGAGC TGA CTGTGTC
CTGCCAAGGC GGGCTGCCCA AGGAAGCCTG CATGGAGATC TCATCGCCAG GGTGCCAGCC
CCCTGCCCAG CGGCTGTGCC AGCCTGTGCT ACCCAGCCCA GCCTGCCAGC TGGTTCTGCA
CCAGATACTG AAGGGTGGCT CGGGGACATA CTGCCTCAAT GTGTCTCTGG CTGATACCAA
CAGCCTGGCA GTGGTCAGCA CCCAGCTTAT CATGCCTGGT CAAGAAGCAG GCCTTGGGCA
GGTCCGCTG ATCGTGGGCA TCTTGCTGGT GTTGATGGCT GTGGTCCCTG CATCTCTGAT
ATATAGGCGC AGACTTATGA AGCAAGACTT CTCCGTACCC CAGTTGCCAC ATAGCAGCAG
TCACTGGCTG CGTCTACCCC GCATCTTCTG CTCTTGTCCT ATTGGTGAGA ACAGCCCCCT
CCTCAGTGGG CAGCAGGTCT GA
    
```

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FIGURE 2

Met	Asp	Leu	Val	Leu	Lys	Arg	Cys	Leu	Leu	His	Leu	Ala	Val	Ile	Gly
1				5					10					15	
Ala	Leu	Leu	Ala	Val	Gly	Ala	Thr	Lys	Val	Pro	Arg	Asn	Gln	Asp	Trp
			20					25					30		
Leu	Gly	Val	Ser	Arg	Gln	Leu	Arg	Thr	Lys	Ala	Trp	Asn	Arg	Gln	Leu
		35					40					45			
Tyr	Pro	Glu	Trp	Thr	Glu	Ala	Gln	Arg	Leu	Asp	Cys	Trp	Arg	Gly	Gly
	50					55					60				
Gln	Val	Ser	Leu	Lys	Val	Ser	Asn	Asp	Gly	Pro	Thr	Leu	Ile	Gly	Ala
	65				70						75				80
Asn	Ala	Ser	Phe	Ser	Ile	Ala	Leu	Asn	Phe	Pro	Gly	Ser	Gln	Lys	Val
				85					90					95	
Leu	Pro	Asp	Gly	Gln	Val	Ile	Trp	Val	Asn	Asn	Thr	Ile	Ile	Asn	Gly
			100					105					110		
Ser	Gln	Val	Trp	Gly	Gly	Gln	Pro	Val	Tyr	Pro	Gln	Glu	Thr	Asp	Asp
		115					120					125			
Ala	Cys	Ile	Phe	Pro	Asp	Gly	Gly	Pro	Cys	Pro	Ser	Gly	Ser	Trp	Ser
	130					135					140				
Gln	Lys	Arg	Ser	Phe	Val	Tyr	Val	Trp	Lys	Thr	Trp	Gly	Gln	Tyr	Trp
145					150					155					160
Gln	Val	Leu	Gly	Gly	Pro	Val	Ser	Gly	Leu	Ser	Ile	Gly	Thr	Gly	Arg
				165					170					175	
Ala	Met	Leu	Gly	Thr	His	Thr	Met	Glu	Val	Thr	Val	Tyr	His	Arg	Arg
			180					185					190		
Gly	Ser	Arg	Ser	Tyr	Val	Pro	Leu	Ala	His	Ser	Ser	Ser	Ala	Phe	Thr
		195					200					205			
Ile	Met	Asp	Gln	Val	Pro	Phe	Ser	Val	Ser	Val	Ser	Gln	Leu	Arg	Ala
	210					215					220				
Leu	Asp	Gly	Gly	Asn	Lys	His	Phe	Leu	Arg	Asn	Gln	Pro	Leu	Thr	Phe
225					230					235					240
Ala	Leu	Gln	Leu	His	Asp	Pro	Ser	Gly	Tyr	Leu	Ala	Glu	Ala	Asp	Leu
				245					250					255	
Ser	Tyr	Thr	Trp	Asp	Phe	Gly	Asp	Ser	Ser	Gly	Thr	Leu	Ile	Ser	Arg
			260					265					270		
Ala	Leu	Val	Val	Thr	His	Thr	Tyr	Leu	Glu	Pro	Gly	Pro	Val	Thr	Val
		275					280					285			
Gln	Val	Val	Leu	Gln	Ala	Ala	Ile	Pro	Leu	Thr	Ser	Cys	Gly	Ser	Ser
	290					295					300				
Pro	Val	Pro	Gly	Thr	Thr	Asp	Gly	His	Arg	Pro	Thr	Ala	Glu	Ala	Pro
305					310					315					320
Asn	Thr	Thr	Ala	Gly	Gln	Val	Pro	Thr	Thr	Glu	Val	Val	Gly	Thr	Thr
				325					330					335	
Pro	Gly	Gln	Ala	Pro	Thr	Ala	Glu	Pro	Ser	Gly	Thr	Thr	Ser	Val	Gln
			340					345					350		
Val	Pro	Thr	Thr	Glu	Val	Ile	Ser	Thr	Ala	Pro	Val	Gln	Met	Pro	Thr
		355					360					365			

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FIGURE 2 (CONT'D)

Ala	Glu	Ser	Thr	Gly	Met	Thr	Pro	Glu	Lys	Val	Pro	Val	Ser	Glu	Val
	370					375					380				
Met	Gly	Thr	Thr	Leu	Ala	Glu	Met	Ser	Thr	Pro	Glu	Ala	Thr	Gly	Met
385					390					395					400
Thr	Pro	Ala	Glu	Val	Ser	Ile	Val	Val	Leu	Ser	Gly	Thr	Thr	Ala	Ala
				405					410						415
Gln	Val	Thr	Thr	Thr	Glu	Trp	Val	Glu	Thr	Thr	Ala	Arg	Glu	Leu	Pro
			420					425					430		
Ile	Pro	Glu	Pro	Glu	Gly	Pro	Asp	Ala	Ser	Ser	Ile	Met	Ser	Thr	Glu
		435					440					445			
Ser	Ile	Thr	Gly	Ser	Leu	Gly	Pro	Leu	Leu	Asp	Gly	Thr	Ala	Thr	Leu
	450					455					460				
Arg	Leu	Val	Lys	Arg	Gln	Val	Pro	Leu	Asp	Cys	Val	Leu	Tyr	Arg	Tyr
465					470					475					480
Gly	Ser	Phe	Ser	Val	Thr	Leu	Asp	Ile	Val	Gln	Gly	Ile	Glu	Ser	Ala
				485					490						495
Glu	Ile	Leu	Gln	Ala	Val	Pro	Ser	Gly	Glu	Gly	Asp	Ala	Phe	Glu	Leu
			500					505					510		
Thr	Val	Ser	Cys	Gln	Gly	Gly	Leu	Pro	Lys	Glu	Ala	Cys	Met	Glu	Ile
		515						520					525		
Ser	Ser	Pro	Gly	Cys	Gln	Pro	Pro	Ala	Gln	Arg	Leu	Cys	Gln	Pro	Val
	530					535					540				
Leu	Pro	Ser	Pro	Ala	Cys	Gln	Leu	Val	Leu	His	Gln	Ile	Leu	Lys	Gly
545					550					555					560
Gly	Ser	Gly	Thr	Tyr	Cys	Leu	Asn	Val	Ser	Leu	Ala	Asp	Thr	Asn	Ser
				565					570						575
Leu	Ala	Val	Val	Ser	Thr	Gln	Leu	Ile	Met	Pro	Gly	Gln	Glu	Ala	Gly
			580						585				590		
Leu	Gly	Gln	Val	Pro	Leu	Ile	Val	Gly	Ile	Leu	Leu	Val	Leu	Met	Ala
	595						600					605			
Val	Val	Leu	Ala	Ser	Leu	Ile	Tyr	Arg	Arg	Arg	Leu	Met	Lys	Gln	Asp
	610					615					620				
Phe	Ser	Val	Pro	Gln	Leu	Pro	His	Ser	Ser	Ser	His	Trp	Leu	Arg	Leu
625					630					635					640
Pro	Arg	Ile	Phe	Cys	Ser	Cys	Pro	Ile	Gly	Glu	Asn	Ser	Pro	Leu	Leu
				645					650						655
Ser	Gly	Gln	Gln	Val											
			660												

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# FIGURE 3

## Nucleotide Sequence of C5H6gp100M

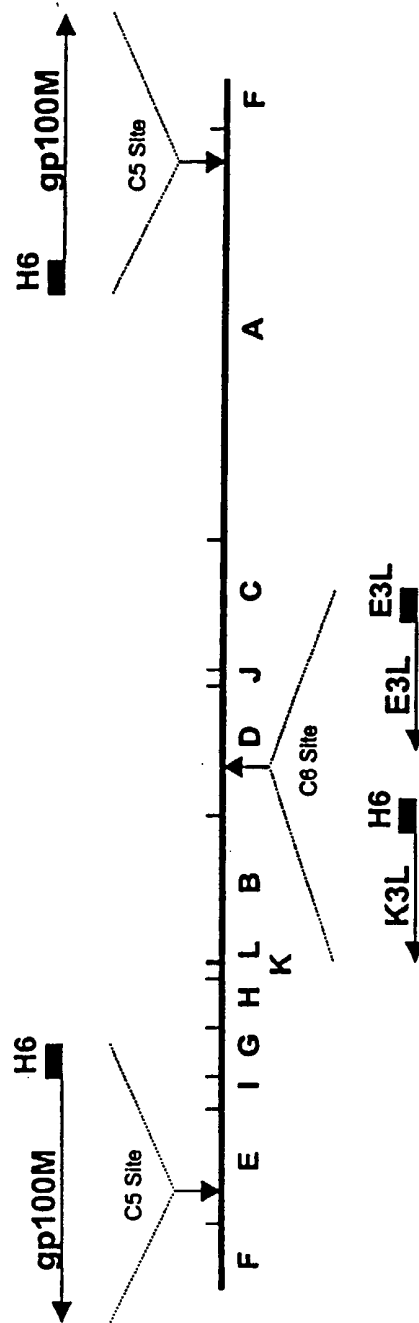
1-254 left C5 flanking arm  
 255-376 H6 promoter  
 377-2362 modified gp100 gene  
 2363-2534 right C5 flanking arm

```

1 GGCTACTTTT CAACAAAGGA GCAGATGTAA ACTACATCTT TGAAAGAAAT GGAAAATCAT
61 ATACTGTTTT GGAATTGATT AAAGAAAGTT ACTCTGAGAC ACAAAGAGG TAGCTGAAGT
121 GGTACTCTCA AAGGTACGTG ACTAATTAGC TATAAAAAGG ATCGTCGACG AGCTCGAATT
181 CGGATCCGGG TTAATTAATT AGTCATCAGG CAGGGCGAGA ACGAGACTAT CTGCTCGTTA
241 ATTAATTAGA GCTTCTTTAT TCTATACTTA AAAAGTGAAA ATAAATACAA AGGTTCTTGA
301 GGGTTGTGTT AAATTGAAAG CGAGAAATAA TCATAAATTA TTTCATTATC GCGATATCCG
361 TTAAGTTTGT ATCGTAATGG ATCTGGTGCT AAAAAGATGC CTCTTCATT TGGCTGTGAT
421 AAGTGCTTTG CTGGCTGTGG GGGCTACAAA AGTACCCAGA AACCAGGACT GGCTGGTGT
481 CTC AAGGCAA CTCAGAACCA AAGCCTGGAA CAGGCAGCTG TATCCAGAGT GGACAGAAGC
541 CCAGAGACTT GACTGCTGGA GAGGTGGTCA AGTGTCCCTC AAGGTCAGTA ATGATGGGCC
601 TACACTGATT GGTGCAAATG CCTCCTTCTC TATTGCCTTG AACTTCCCTG GAAGCCAAAA
661 GGTATTGCCA GATGGGCAGG TTATCTGGGT CAACAATACC ATCATCAATG GGAGCCAGGT
721 GTGGGGAGGA CAGCCAGTGT ATCCCCAGGA AACTGACGAT GCCTGCATCT TCCCTGATGG
781 TGGACCTTGC CCATCTGGCT CTTGGTCTCA GAAGAGAAGC TTTGTTTATG TCTGGAAGAC
841 CTGGGGCCAA TACTGGCAAG TTCTAGGGGG CCCAGTGTCT GGGCTGAGCA TTGGGACAGG
901 CAGGGCAATG CTGGGCACAC ACACGATGGA AGTGACTGTC TACCATCGCC GGGGATCCCC
961 GAGCTATGTG CCTCTGCTC ATTCCAGCTC AGCCTTCACC ATTATGGACC AGGTGCCFTT
1021 CTCCGTGAGC GTGTCCCAGT TGCGGGCCTT GGATGGAGGG AACAAGCACT TCCTGAGAAA
1081 TCAGCCTCTG ACCTTTGCCC TCCAGCTCCA TGACCCAGT GGCTATCTGG CTGAAGCTGA
1141 CCTCTCCTAC ACCTGGGACT TTGGAGACAG TAGTGGAAAC CTGATCTCTC GGGCACTTGT
1201 GGTCACTCAT ACTTACCTGG AGCCTGGCCC AGTCACTGTT CAGGTGGTCC TGCAGGCTGC
1261 CATTCTCTC ACCTCCTGTG GCTCCTCCCC AGTTCAGGC ACCACAGATG GGCACAGGCC
1321 AACTGCAGAG GCCCCTAACA CCACAGCTGG CCAAGTGCCT ACTACAGAAG TTGTGGGTAC
1381 TACACCTGGT CAGGCGCCAA CTGCAGAGCC CTCTGGAACC ACATCTGTGC AAGTGCCAAC
1441 CACTGAAGTC ATAAGCACTG CACCTGTGCA GATGCCAACC GCAGAGAGCA CAGGTATGAC
1501 ACCTGAGAAG GTGCCAGTTT CAGAGGTCAT GGGTACCACA CTGGCAGAGA TGTCAACTCC
1561 AGAGGCTACA GGTATGACAC CTGCAGAGGT ATCAATTGTG GTGCTTCTG GAACCACAGC
1621 TGCACAGGTA ACAACTACAG AGTGGGTGGA GACCACAGCT AGAGAGCTAC CTATCCCTGA
1681 GCCTGAAGGT CCAGATGCCA GCTCAATCAT GTCTACGGAA AGTATTACAG GTTCCCTGGG
1741 CCCCCTGCTG GATGGTACAG CCACCTTAAG GCTGGTGAAG AGACAAGTCC CCCTGGATTG
1801 TGTCTGTAT CGATATGGTT CCTTTTCCGT CACCCTGGAC ATTGTCCAGG GTATTGAAAG
1861 TGCCGAGATC CTGCAGGCTG TGCCGTCCGG TGAGGGGGAT GCATTTGAGC TGACTGTGTC
1921 CTGCCAAGGC GGGCTGCCCA AGGAAGCCTG CATGGAGATC TCATCGCCAG GGTGCCAGCC
1981 CCCTGCCAG CGGCTGTGCC AGCCTGTGCT ACCCAGCCCA GCCTGCCAGC TGGTTCTGCA
2041 CCAGTACTG AAGGGTGGCT CGGGGACATA CTGCCTCAAT GTGTCTCTGG CTGATACCAA
2101 CAGCCTGGCA GTGGTCAGCA CCCAGCTTAT CATGCCTGGT CAAGAAGCAG GCCTTGGGCA
2161 GGTCCGCTG ATCGTGGGCA TCTTGCTGGT GTTGATGGCT GTGGTCTTGG CATCTCTGAT
2221 ATATAGGCGC AACTTATGA AGCAAGACTT CTCCGTACCC CAGTTGCCAC ATAGCAGCAG
2281 TCACTGGCTG CGTCTACCCC GCATCTTCTG CTCTTGTCCT ATTGGTGAGA ACAGCCCCCT
2341 CCTCAGTGGG CAGCAGGTCT GATTTTTATC TCGAGTCTAG AATCGATCCC GGGTTTTTAT
2401 GACTAGTTAA TCACGGCCGC TTATAAAGAT CTAATAATGCA TAATTTCTAA ATAATGAAA
2461 AAAAGTACAT CATGAGCAAC GCGTTAGTAT ATTTTACAAT GGAGATTAAC GCTCTATACC
2521 GTTCTATGTT TATT
    
```

FIGURE 4

**ALVAC(2)-gp100M (vCP1584)**  
(ALVAC *Xho*I Restriction Map)



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# FIGURE 5

## Oligonucleotide Primers

IDC5-1

CGT GCC ATG GCA CAC AAA AGA GGT AGC TGA A

IDC5-2

CCA GGC GGC CGC ACT AAC GCG TTG CTC ATG ATG

C5L

CAC AAA AGA GGT AGC TGA AGT

MEL 01

ATG GAT CTG GTG CTA AAA AGA

MEL 05

ACC TTG CCC ATC TGG CTC TTG

MEL 09

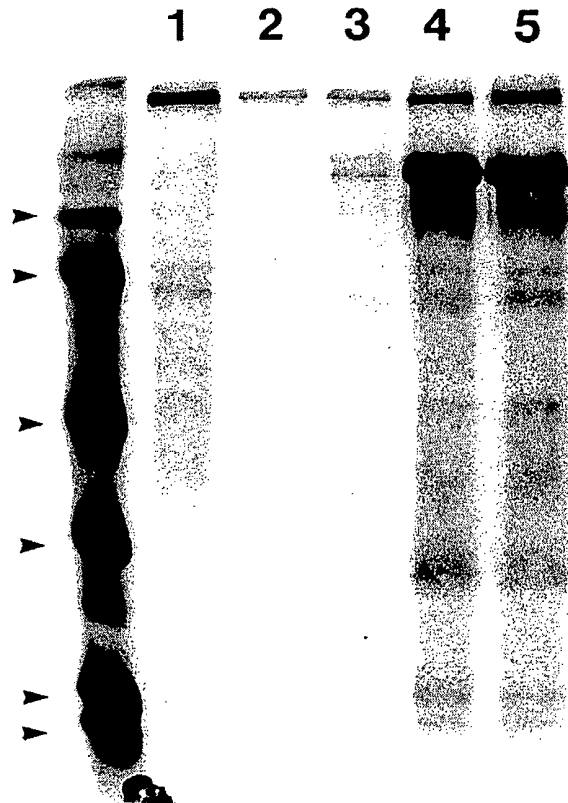
AGA TGC CAG CTC AAT CAT GTG

C5R

ATA GAT CTT TAT AAG CGG CCG

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## FIGURE 6



Molecular Weight Markers: 200, 98.6, 68, 43, 29, 18, 14 kDa

Lane 1: Uninfected HeLa cells

Lane 2: HeLa cells infected with ALVAC

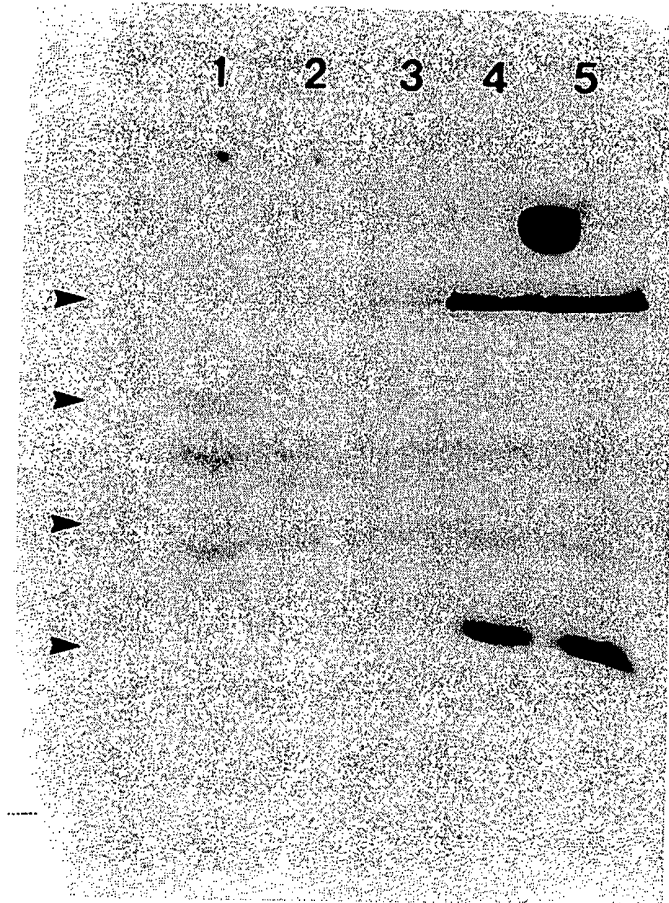
Lane 3: HeLa cells infected with ALVAC-gp100 (vCP1465)

Lane 4: HeLa cells infected with ALVAC(2)-gp100M (vCP1584)

Lane 5: HeLa cells infected with ALVAC(2)-gp100M (sister of vCP1584)

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FIGURE 7



Molecular Weight Markers: 97, 68, 43, 29 kDa

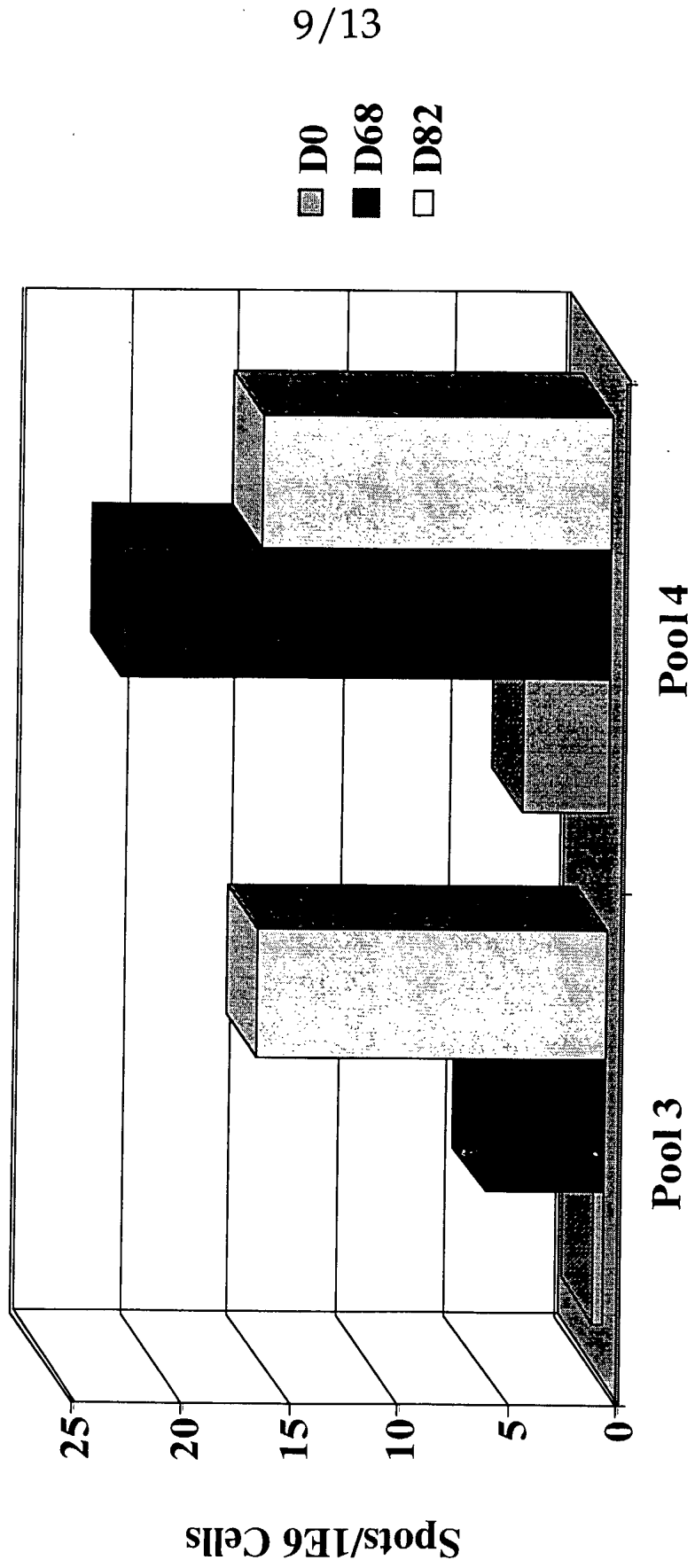
Lane 1: Uninfected HeLa cells

Lane 2: HeLa cells infected with ALVAC

Lane 3: HeLa cells infected with ALVAC-gp100 (vCP1465)

Lane 4: HeLa cells infected with ALVAC(2)-gp100M (vCP1584)

**FIGURE 8**  
**Monkey #6 (Intranodal Administration)**

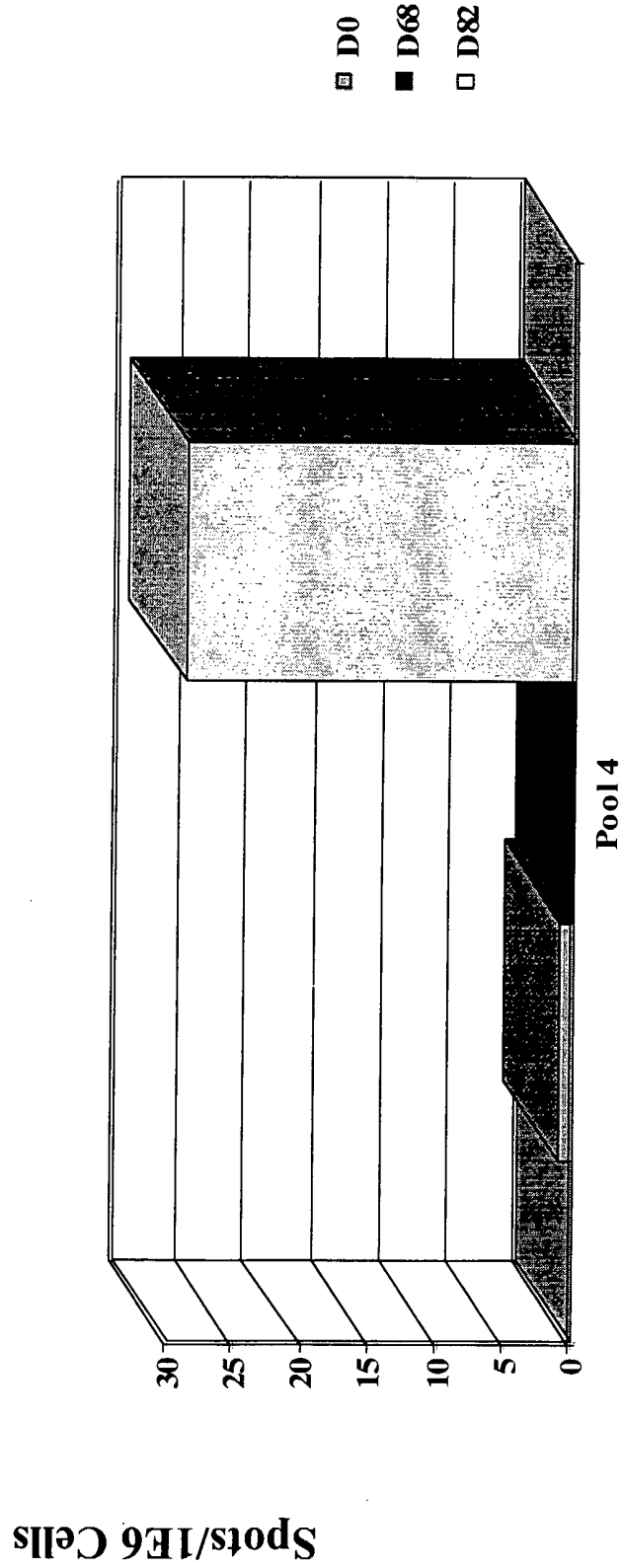


**FIGURE 9**  
**Monkey #7 (Intranodal Administration)**



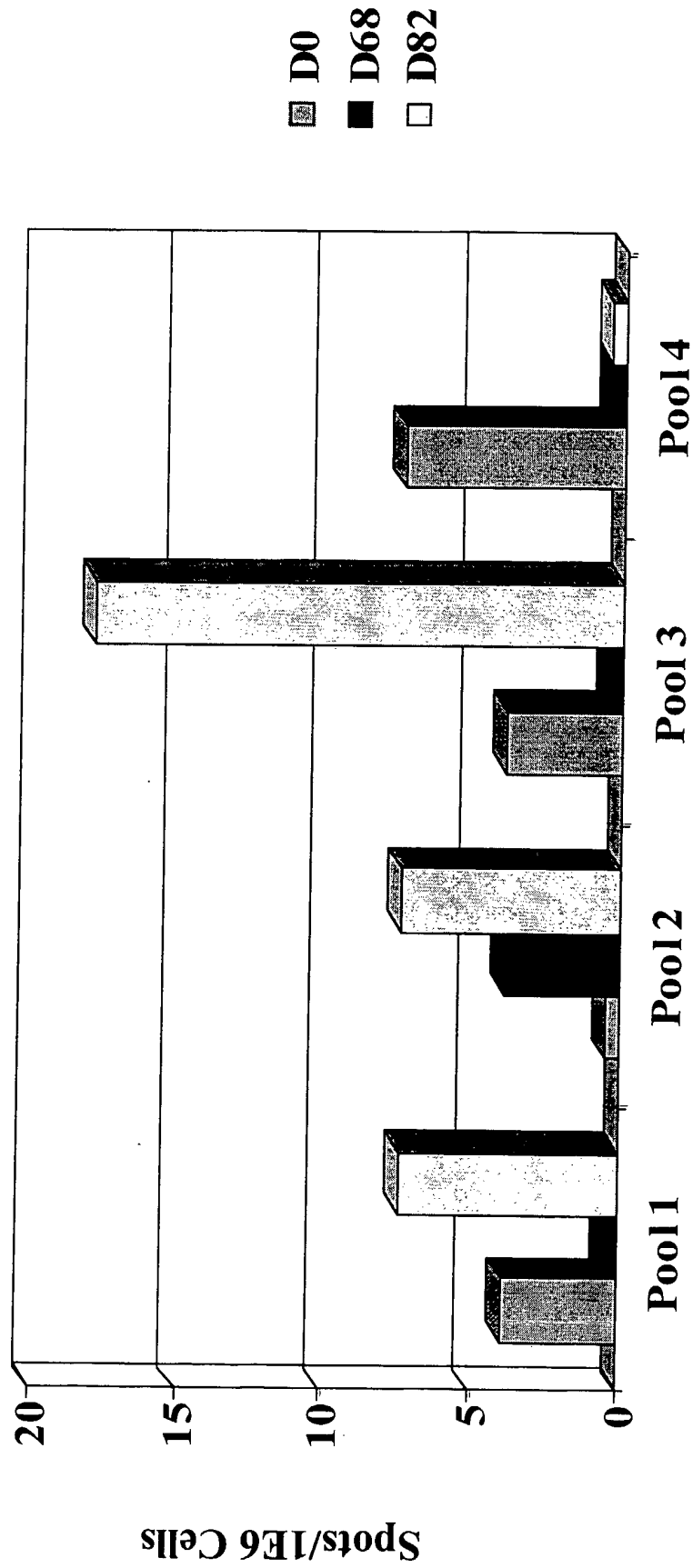
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**FIGURE 10**  
**Monkey # 11 (Subcutaneous Administration)**



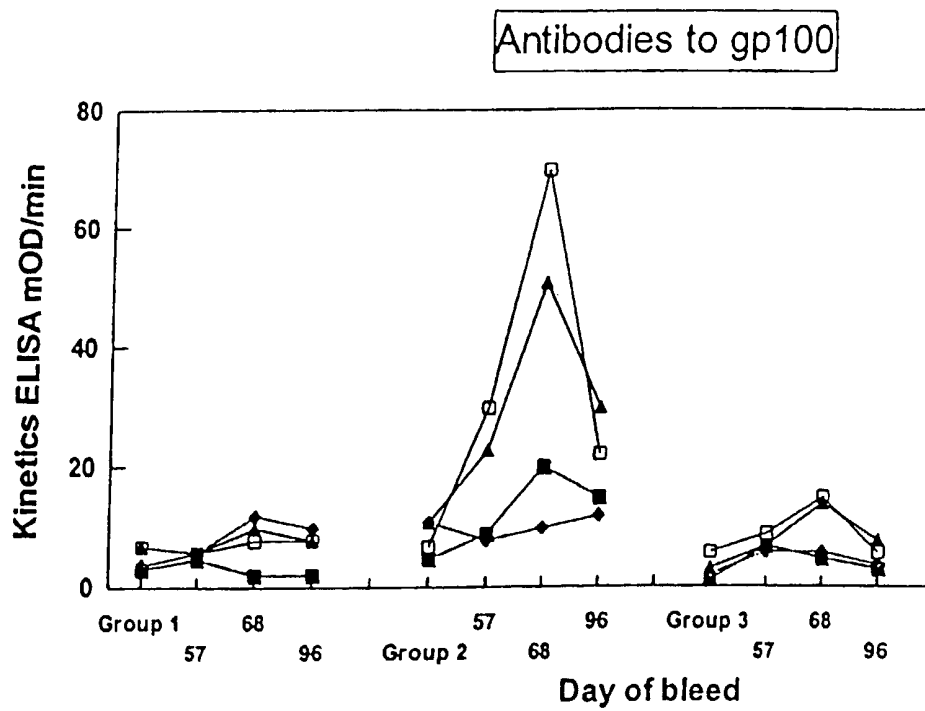
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**FIGURE 11**  
**Monkey #10 (Subcutaneous Administration)**



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FIGURE 12



## INTERNATIONAL SEARCH REPORT

 ational Application No  
 PCT/CA 00/01254

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC 7	C07K14/705	C12N9/64
	A61K35/76	A61P35/00
		A61K39/00
		A61K48/00
		C12N5/06
		C12N7/00
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 7 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
MEDLINE, CANCERLIT, LIFESCIENCES, EMBASE, CHEM ABS Data, SCISEARCH, BIOSIS, WPI Data, EPO-Internal, PAJ		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IRVINE KARI R ET AL: "Recombinant virus vaccination against "self" antigens using anchor-fixed immunogens." CANCER RESEARCH, vol. 59, no. 11, 1 June 1999 (1999-06-01), pages 2536-2540, XP002161590 ISSN: 0008-5472  page 2536, left-hand column, line 39 -right-hand column, line 14 page 2356, right-hand column, line 47 -page 2357, left-hand column, line 1 table 1  --- -/--	8,9, 13-16, 18-20, 22-24, 27-34, 36, 39-41, 43,44, 46-48, 51-53, 56,57, 59,60
<input checked="" type="checkbox"/>	Further documents are listed in the continuation of box C.	<input checked="" type="checkbox"/>
<input checked="" type="checkbox"/>	Patent family members are listed in annex.	
° Special categories of cited documents :		
<ul style="list-style-type: none"> <li>*A* document defining the general state of the art which is not considered to be of particular relevance</li> <li>*E* earlier document but published on or after the international filing date</li> <li>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</li> <li>*O* document referring to an oral disclosure, use, exhibition or other means</li> <li>*P* document published prior to the international filing date but later than the priority date claimed</li> <li>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</li> <li>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</li> <li>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</li> <li>*&amp;* document member of the same patent family</li> </ul>		
Date of the actual completion of the international search		Date of mailing of the international search report
28 February 2001		12/03/2001
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  Covone, M

## INTERNATIONAL SEARCH REPORT

 International Application No  
 PCT/CA 00/01254

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 02538 A (AKZO NOBEL N.V., NETH.; FIGDOR, CARL GUSTAV; ADEMA, GOSSE JAN) 22 January 1998 (1998-01-22) page 3, line 3-19 page 11, line 15 -page 13, line 13 examples 2-5 ---	1-62
A	WO 98 04728 A (THERION BIOLOG CORP ;US HEALTH (US)) 5 February 1998 (1998-02-05) page 6, line 9 -page 11, line 17 claims ---	1-62
A	PARKHURST M R ET AL: "Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues." JOURNAL OF IMMUNOLOGY, (1996 SEP 15) 157 (6) 2539-48. , XP002096010 page 2539, right-hand column, paragraph 2 -page 2340, left-hand column, paragraph 2 ---	1,19-21, 43-45,61
A	WO 99 46992 A (GREGORY RICHARD J ;GENZYME CORP (US); KAPLAN JOHANNE (US)) 23 September 1999 (1999-09-23) claims ---	10-12
A	KAMMULA U.S. ET AL: "Cancer immunotherapy: Is there real progress at last?." BIODRUGS, (1999) 11/4 (249-260). , XP000982586 the whole document ---	1-54
A	WO 99 46988 A (NICOLETTE CHARLES A ;GENZYME CORP (US)) 23 September 1999 (1999-09-23) figure 1 -----	26,37, 42,45, 50,55,58

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 00/01254

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9802538	A	22-01-1998	AU 3693897 A	09-02-1998
			CA 2259944 A	22-01-1998
			EP 0934405 A	11-08-1999
			JP 2000515739 T	28-11-2000
			ZA 9705858 A	01-09-1998
WO 9804728	A	05-02-1998	AU 718945 B	04-05-2000
			AU 3670697 A	20-02-1998
			EP 0951559 A	27-10-1999
			JP 2000515759 T	28-11-2000
WO 9946992	A	23-09-1999	AU 3102999 A	11-10-1999
			EP 1071333 A	31-01-2001
WO 9946988	A	23-09-1999	AU 3193499 A	11-10-1999
			EP 1071325 A	31-01-2001