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(54) Title: MEDICAMENTS AND METHODS FOR TREATING MESOTHELIOMA

(57) Abstract: The present invention relates to the use of at least one attenuated measles virus for the manufacture of a medicament intended for treating malignant mesothelioma in an individual.

# MEDICAMENTS AND METHODS FOR TREATING MESOTHELIOMA

## Field of the invention

The present invention relates to methods and medicaments intended to cure cancers, such as malignant mesothelioma.

## Background of the invention

Malignant mesothelioma (MM) are relatively rare and highly aggressive neoplasms, arising from the uncontrolled proliferation of mesothelial cells lining serosal cavities, most commonly the pleura (Malignant Pleural Mesothelioma or MPM) (Robinson *et al*. (2005) *Lancet* **366**:397-408). Epidemiologic studies have established that exposure to asbestos is one of the most important MPM etiologic factor in industrialized countries (Gruber (2005) *Lung Cancer* **49S1**:S21-S23; Bartrip (2004) *Postgrad Med. J*. **80**:72-76). Although worldwide usage of asbestos has been considerably reduced, the incidence of mesothelioma is expected to rise in the next two decades, because of a long latency period (20 to 40 years) between asbestos exposure time and clinical symptoms apparition.

Today, cancer diagnosis is usually established at an advanced stage because of the absence of overt symptoms in the early period of the disease, thus making poor the prognosis for mesothelioma patients. Consequently, MPM is actually considered as a cancer relatively refractory to all conventional treatment modalities. Accordingly, there is a pressing need for the development of new therapeutic approach.

During the past decade, there has been an increasing interest in virotherapy, partly related to the growing knowledge in the production of recombinant viral vectors for human gene therapy. Numerous RNA replicating viruses are now considered as potential cancer therapeutics. As such, therapy of MPM using engineered replication-competent Herpex Simplex Viruses (HSV) has been proposed, based on *in vitro* studies and results obtained on a murine model of MPM (Adusumilli *et al*. (2006) *J. Gene Med*. **8**:603-615). However, the long term safety of these engineered viral vectors in humans is not known and extensive clinical trials will be necessary to document this aspect of HSV usage.

Accordingly, there is a need for viral vectors with recognized safety liable to be used in the frame of mesothelioma treatment.

MV (Measles Virus) is an enveloped, negative single strand RNA virus belonging to the *Paramyxoviridae* family, genus *Morbilli* virus. Various replication-competent live attenuated strains of MV have been developed for producing vaccines against measles. By way of example, Schwartz, Moraten, or Zagreb (which are derived from MV samples isolated from an Edmonston patient) are safe and well-documented MV vaccine strains.

It has been shown recently that *in vivo* administration of a replication-competent Edmonston MV strain resulted in growth slowing or sometimes regression of tumors established animal models of lymphoma and myeloma cancer (Grote *et al*. (2001) *Blood* **97**:3746-3754; Peng *et al*. (2001) *Blood* **98**:2002-2007). Besides, Anderson *et al*. (2004) *Cancer Res*. **64**:4919-4926, have shown in *in vitro* experiments that high CD46 expression by tumor cells was necessary for the infection and the killing of these cells by a live attenuated Edmonston MV strain. However, it is known that CD46 is variably expressed by human carcinomas (Niehans *et al*. (1996) *American J. Pathol.* 149:129-142), thereby casting doubts on the general applicability of live attenuated MV strains for treating cancers.

## Summary of the invention

The present invention arises from the unexpected finding, by the present inventors, that attenuated measles virus could efficiently infect and kill mesothelioma cells. Furthermore, the present inventors have shown that dendritic cells contacted with lysate from attenuated measles virus-infected mesothelioma cells could activate anti-mesothelioma CD8 T cells.

Thus, the present invention relates to an attenuated measles virus for use in the treatment of malignant mesothelioma in an individual.

The present invention also relates to the use of at least one attenuated measles virus for the manufacture of a medicament intended for treating malignant mesothelioma in an individual.

The present invention also relates to a method for treating malignant mesothelioma in an individual, wherein a therapeutically effective quantity of at least one attenuated measles virus is administered to said individual.

The present invention further relates to a method for preparing vaccinal dendritic cells intended for treating cancer in an individual, comprising the following steps:

- *in vitro* infection of cancer cells, preferably taken from the individual, by an attenuated measles strain to yield a cell lysate;
- contacting dendritic cells with the cell lysate to yield vaccinal dendritic cells.

The present invention also relates to vaccinal dendritic cells liable to be obtained by the above-defined method of preparation, to a pharmaceutical composition comprising said vaccinal dendritic cells as active ingredient, in association with a pharmaceutically acceptable carrier, to said vaccinal dendritic cells for use in the treatment of cancer in an individual, and to the use of said vaccinal dendritic cells, for the manufacture of a medicament intended for treating cancer in an individual.

The present invention further relates to a method for treating cancer in an individual, wherein a therapeutically effective quantity of vaccinal dendritic cells liable to be obtained by the above-defined method of preparation are administered to said individual.

**Detailed description of the invention**

As intended herein, the individual is preferably a mammal, more preferably a human. Preferably also, the individual has been exposed to asbestos.

As intended herein, the expression “attenuated measles virus” designates any virus derived from a measles-causative virus and presenting a decreased virulence with respect to said measles-causative virus. As intended herein the attenuated measles virus can be derived from measles-causative virus by any technique known to the man skilled in the art, such as serial passages on cultured cells and/or genetic engineering. In particular, the attenuated measles virus may be a recombinant virus, optionally expressing additional genes. More particularly, the attenuated measles

virus may be a measles virus wherein the expression of one or more proteins, preferably the accessory C protein, is abolished. It is preferred that the attenuated measles virus causes essentially no measles symptoms when administered to a human. Besides, the attenuated measles virus is preferably alive and replication-competent.

Preferably, the attenuated measles virus is an Edmonston strain. Edmonston strains of attenuated measles virus are well-known to one of skill in the art and are notably described in Griffin (2001) *Field's Virology 4th Edition vol. 2* Knipe and Howley (ed.) Lippincott-Raven Publishers, Philadelphia, 1401-1441; Hilleman (2002) *Vaccine* **20**:651-665). More preferably, the attenuated measles virus is selected from the group constituted of a Schwartz strain and a Moraten strain. These strains, which genomes have been shown to be identical, are well-known to the man skilled in the art and are widely used for the production of vaccines against measles. They are notably described in Schwarz (1962) *Am. J. Dis. Child* **103**:216-219; Parks *et al.* (2001) *J. Virol.* **75**:921-933 and Parks *et al.* (2001) *J. Virol.* **75**:910-920. Most preferably, the attenuated measles virus is produced from the pTM-MVSchw plasmid (SEQ ID NO: 1) described by Combredet *et al.* (2003) *J. Virol.* **77**:11546-11554.

Cancers to be treated within the frame of the present invention are preferably malignant mesotheliomas, more preferably malignant pleural mesotheliomas or peritoneal mesotheliomas, most preferably malignant pleural mesotheliomas. Such cancers are notably described in Kazan-Allen (2005) *Lung cancer* **49S1**:S3-S8 and Robinson *et al.* (2005) *Lancet* **366**:397-408.

Where the attenuated measles virus is administered to an individual, it can be administered through the intrapleural cavity or by the intranasal, intramuscular, intravenous or subcutaneous routes. Where the attenuated measles virus is administered through the intrapleural cavity, it is preferably administered in close proximity or directly into the tumors to be treated. If necessary, the attenuated measles virus can be associated to any suitable pharmaceutically acceptable carriers. The therapeutically effective quantity of attenuated measles virus to be administered is preferably in the range of from $10^3$ to $10^6$ 50% tissue culture infective doses

(TCID50). TCID50 determination is well known to one of skill in the art and is notably described by Karber (1931) *Arch. Exp. Path. Pharmak.* **162**:840-483.

The step of taking the cancer cells from the individual to be treated by the vaccinal dendritic cells is preferably not included in the above-defined method of preparation of vaccinal dendritic cells. This step can proceed according to any technique known to one of skill in the art for taking cells, such as biopsies and effusions (*e.g.* pleural effusions). After being taken, the cancer cells can be maintained in culture according to classical techniques, or frozen (*e.g.* at -80℃) for conservation, for instance. Where the cancer cells do not originate from the individual to be treated by the vaccinal dendritic cells, they can notably derive from allogenic human mesothelioma cell lines.

In the above-defined method of preparation, infection of the cancer cells by the attenuated measles virus can proceed by directly contacting cells and virus, for instance at a Mutliplicity Of Infection (MOI) of 1, with an incubation of 2 hours at 37℃. After infection, death of the infected cells proceeds spontaneously due to virus action. A syncitia is usually first formed followed by lysis of the cells. This phenomenon can be evidenced by direct microscopic observation of infected cells. As intended herein "cell lysate" encompasses both whole (or total) cell lysate, or fractions of the cell lysate, such as membrane fractions (*e.g.* cytoplasmic inclusion bodies or apobodies). As will be well-understood by those skilled in the art, the cell lysate obtained in the first step of the above-defined method of preparation corresponds to a virus infected cancer cell lysate.

Dendritic cells can be obtained by numerous ways well known to the man skilled in the art. The dendritic cells preferably originate from the individual to be treated. It is presently preferred that the dendritic cells are monocyte-derived dendritic cells. The obtention of monocyte-derived cells is particularly well known to one of skill in the art. Preferably, monocyte-derived cells can be obtained following the general methodology described in **Example 4** or by Spisek *et al.* (2001) *Cancer Immunology Immunotherapy* **50**:417-427, or by Royer *et al.* (2006) *Scand. J. Immunol.* **63**:401-409. Where the monocyte-derived dendritic cells originate from the individual to be treated, monocytes can be obtained from leukapheresis of said individual.

As will be apparent to one skilled in the art, contacting of the dendritic cells and of the cell lysate should be maintained for a time sufficient to enable an effective loading of the dendritic cells by antigens present in the cell lysate. Once loaded (or pulsed), vaccinal dendritic cells according to the invention are obtained. Loading can proceed by following the general methodology described in **Example 4**. An exemplary contact period between dendritic cells and the cell lysate sufficient to enable efficient loading of the dendritic cells is of about 24 hours. In particular, the contact period can be maintained until the dendritic cells are in an activated state. The activated state is usually reached after the dendritic cells have been loaded. The activated state (or mature state) of dendritic cells can be evidenced by numerous markers well known to one of skill in the art, such as membrane or cytokine markers. Such markers of activated dendritic cells are notably listed in **Example 5**.

Thus, vaccinal dendritic cells obtainable according to the method of preparation of the invention are particularly advantageous since they are potent stimulators of anti-cancer CD8 T cells. Equally advantageous, the method of preparation according to the invention allows the preparation of vaccinal dendritic cells in an activated state.

## Brief description of the figures

**Figures 1, 2, 3, 4 and 5: Mesothelioma susceptibility to attenuated Measles Virus (MV).**

Figure 1 - Selective Oncolytic activity of Schwarz MV vaccine strain. A panel of human epithelioid mesothelioma cell lines (M11, M13, M47, M56 & M61) and an immortalized normal mesothelial cell line (Met5A) were infected with non-recombinant MV (MOI 1.0) and microscope observations of infected cultures morphology were performed 72 hours later.

Figures 2-3 - Higher surface expression level of CD46 receptors for tumoral cells in comparaison with their normal counterparts. Cells were stained with FITC-conjugated CD46-specific antibodies (grey histogram) or related isotype Ig control (white histogram) (Figure 2). Numbers indicate the mean fluorescence index and histogram

shows mean values of CD46 expression obtained for mesothelial (white bar) and mesothelioma (hatched bar) cell lines (Figure 3).

Figures 4-5 - Schwarz MV vaccine strain preferentially infects transformed tumoral cells. Equal numbers of M13 and Met5A cells were cultured separately (Figure 4) or co-cultured (Figure 5) overnight, allowing cellular adherence, and infection was done at MOI of 1.0 with eGFP-recombinant MV. In separate cultures, analysis of eGFP expression was performed at different times post-infection (24, 48, & 72 hours) by flow cytometry (Figure 4). In co-culture model, the same experiment was conducted along with HLA-A2 staining, as HLA alleles differential expression allowed distinction between two cell lines. Histogram shows % eGFP-positive cells for Met5A (white bar) and M13 (black bar) cells from co-culture (Figure 5).

**Figure 6: Immunogenicity of MV-infected mesothelioma cell line.**

Figure 6 - Cellular death induced by MV- and UV- treatments. Flow cytometry analysis of M13 tumoral cells apoptosis triggered by UV exposure (5 kJ/cm$^2$) or MV infection (MOI=1.0) at the indicated time points (D1 = 24h, D2 = 48h, D3 = 72h, and D4 = 96h) (hatched bars) *vs.* untreated control cells (white bars).

**Figures 7 and 8: Phagocytosis of apobodies by monocyte-derived DCs.**

Figure 7 - UV- or MV-treated M13 tumor cells were labelled with PKH-26 and co-cultured with immature DCs for 24 hours. Harvested DCs were subsequently stained with FITC-conjugated anti HLA-DR antibodies and analysed by flow cytometry. One representative experiment of three with similar results is shown. The number of double-positive DCs, that is the percentage of PKH-26 positive DCs gated on basis of HLA-DR expression (FITC-conjugated antibodies, clone B8.12.2, Immunotech), indicates the phagocytosis efficiency of apoptotic cells.

Figure 8 - The histogram represents mean values of phagocytosis yield obtained for each loading condition tested.

**Figures 9, 10 and 11: DC maturation induced by co-culture with MV-infected mesothelioma cells.**

Figures 9 and 10 - Immature DCs and M13 tumoral cells were cultured in the indicated combinations (ratio 1/1) for 24 hours. As controls, DCs were incubated with TLR3 ligand, polyinosinic:polycytidylic acid (50 µg/ml; Sigma), or directly infected with MV (MOI=1.0). Subsequently DCs were harvested and stained with a PE-conjugated antibody panel specific for the indicated cell surface molecules (Figure 9 - HLA molecules; Figure 10 - Maturation Markers). DCs were gated according to their morphology characteristic, and dead cells were excluded on basis of TOPRO-3 staining (Molecular Probes). DCs surface phenotype was analysed by three-colors flow cytometry. Histogram shows means values obtained from four independent donors.

Figure 11 - DC cytokine secretion pattern was investigated on 24 hours supernatant co-culture by CBA (for IL-6, IL-1β, TNFα, IL-12 & IL-10) and ELISA (for IFNα) assays.

**Figure 12: DCs loaded with MV-infected mesothelioma cells induce MSLN-specific CD8 T cell priming.**

Figure 12 - Number of MSLN-specific CD8 T cells, derived from one week sensitization co-culture with unpulsed or UV-M13 or MV-M13 pulsed DCs, was analysed by flow cytometry. Histogram indicates the percentage of PE-tetramer positive cells among T cells gated on basis of human CD8 expression (PE-Cy5-conjugated antibodies, clone RPA-T8, BD Biosciences). One representative experiment is shown.

## Examples

### Example 1

**Mesothelioma susceptibility to MV infection and oncolytic activity.**

To compare MV-related cytopathic effect on tumoral and non-tumoral cells, a panel of five epithelioid mesothelioma cell lines (M11, M13, M47, M56, and M61) and mesothelial cells (Met5A) were infected with a Schwarz vaccine strain at a Multiplicity Of Infection (MOI) of 1.0.

The mesothelioma cell lines (M11, M13, M47, M56, and M61) were established from pleural effusion collected by thoracocentesis of cancer patients. Diagnosis of epithelioid mesothelioma was established by biopsies immunohistochemical staining. The control mesothelial cell line (Met5A) was isolated from pleural fluids of cancer-free patients and immortalized by transfection with the pRSV plasmid encoding SV40 T-antigen (ATCC-LGC Promochem, Molsheim, France). Cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated Foetal Calf Serum (FCS from Biowest, Nuaille, France), 1% L-glutamine and 1% penicillin/streptomycin antibiotics (all purchased from Sigma, St Quentin Fallavier, France). Cellular cultures were routinely checked for *Mycoplasma* contaminations using Hoechst 33258 staining (Sigma).

Attenuated MV Schwarz vaccine strains were obtained from F. Tangy (Pasteur Institut, France). Schwarz MV was rescued from the pTM-MVSchw (SEQ ID NO: 1) cDNA by use of the helper-cell-based rescue system described by Radecke *et al.*(1995) *EMBO J.* **14**:5773-5784 and modified by Parks *et al.* (1999) *J. Virol.* **73**:3560-3566. Briefly, 293-3-46 helper cells were transfected with 5 µg of pTM-MVSchw and 0.02 µg of pEMC-Lschw expressing the Schwarz MV-L gene (Combredet *et al*. (2003) *J. Virol*. **77**:11546-11554) (SEQ ID NO: 2). After overnight incubation at 37℃, a heat shock was applied for 2 h at 43℃, and transfected cells were transferred onto a Vero cell monolayer. Syncytia that appeared in 15 days coculture were transferred to 35-mm wells and then expanded in 75-$cm^2$ and 150-$cm^2$ flasks of Vero cells culture in 5% FCS DMEM. When syncytia reached 80-90% confluence, the cells were scraped into a small volume of OptiMEM and frozen-

thawed once. After low-speed centrifugation to pellet cellular debris, virus-containing supernatant was stored at -80℃. The titer of recom binant MV stock was determined by an endpoint limit-dilution assay on Vero cells. The TCID50 was calculated by use of Kärber method (Karber (1931) *Arch. Exp. Path. Pharmak*. **162**:480-483).

Viral infections of the mesothelioma cell lines were performed at a MOI=1.0 for 2 hours incubation at 37℃. Three days following MV infection, typical morphological modifications of MV-infected cells were observed, that is development of an important cytopathic effect (CPE) on most tumoral MPM lines (4/5), by contrast with non cancerous Met5A cells (**Figure 1**). CPE was evidenced through development of more or less important syncitia, which finally led to shedding in culture supernatant of cytoplasmic inclusion bodies of dead tumoral cells (**Figure 1**). The development of these multinucleated giant syncitia is characteristic of measles infection and is produced by fusion of $HA^+$ infected cells with neighbour $CD46^+$ culture cells.

A significant upregulated expression of live-attenuated MV strains receptor CD46 by mesothelioma cells could be evidenced **(Figures 2-3)**.

In order to quantify susceptibility to MV infection, Met5A and M13 cell lines were infected with eGFP-recombinant MV stock (Combredet *et al*. (2003) *J. Virol*. **77**:11546-11554). The GFP-transgene expression was used as a marker of viral infection, thus allowing determination of infected cells percentage by flow cytometry. MV infection yield of both culture cells was dose-dependent (MOI ranging from 0.01 to 5.0), indicating the specificity of eGFP signal. Whereas Met5A was infected by the MV strain (for MOI ranging from 0.5), M13 was also significantly infected by MV, but always at lowest MOI (for MOI ranging from 0.1). A significant increased infection yield of tumour cells in comparison to normal cells (for MOI 1.0), was also observed both in cellular separate culture (**Figure 4**) and co-culture (**Figure 5**) systems (ratio 1:3) at 48 hours post-infection. Moreover, virus infection could also be evidenced by down-regulation of CD46 surface expression observed in infected cellular cultures.

Thus, according to these *in vitro* results, mesothelioma tumors present a more important susceptibility both to MV-mediated infection and MV-related cytolytic activity than mesothelial tissue. Consequently, MPM appears as a relevant candidate for virotherapy approach based on measles virus administration.

**Example 2**

**Tumoral cell-death induced by MV and UV treatments.**

After demonstrating that MV is able to infect mesothelioma cells, the inventors verified if virus infection could also lead to apoptosis-mediated cell death.

Sub-confluent monolayer M13 cells culture were either MV-infected (MOI 1.0), or UV-B-irradiated (312 nm - 5 kJ/$m^2$) using an UV Stratalinker2400 (Stratagene Europe, Amsterdam, Netherlands), as positive control for apoptosis. Cells were collected at different times post-treatment, and cellular death was quantified as described by Ebstein *et al.* (2004) *Am. J. Respir. Crit. Care Med.* **169**:1322-1330 by concomitant phosphatidylserine and Annexin-V stainings.

As shown in **Figure 6**, 24 hours exposition of M13 cells to UV-B irradiation and 72 hours infection of M13 cells with MV yielded an equivalent rate of tumoral cell death (comprised between 70% and 80% of Annexin-V positive cells), which indicates that MV induces apoptosis in infected tumor cells. The thus-defined M13 cell death-induced conditions were used in following experiments.

Moreover, virus-related cell killing was also confirmed by observation of an important cytopathic effect, leading to complete dislocation of M13 cellular layer 72-96 hours post-infection (**Figure 1**).

**Example 3**

**Follow-up of viral replication cycle in MV-infected tumoral cells.**

In order to follow viral growth kinetic in infected M13 cells culture (MOI=1.0), RT-PCR specific for viral dsRNA potential receptors (Mda-5, TLR-3, RIG-I and PKR) were performed. Specific primers for the β-actin gene were used as an internal experiment control.

Briefly, M13 cells were either incubated with polyinosinic:polycytidylic acid ligand (10 µg/ml) or MV (MOI=1.0) and cellular pellets were collected at different times. Whole cellular RNA was then extracted using RNeasy kits (Qiagen, Courtaboeuf, France) according to manufacturer's instructions, and reverse-transcribed using RTase (Invitrogen, Paisley, UK). Resulting cDNA was used as

template for PCR amplification using primers specific for Mda-5, TLR-3, RIG-I, PKR, IFNβ, and β-actin. PCR primers sequences are listed in **Table 1**. PCR products were visualized by agarose gel electrophoresis.

| Primer | Sequence | Fragment size (bp) | SEQ ID NO: |
|---|---|---|---|
| β-actin | Forward ATCTGGCACCACACCTTCTACAATGAGCTGCG | 837 | 3 |
| | Reverse CGTCATACTCCTGCTTGCTGATCCACATCTGC | | 4 |
| TLR-3 | Forward ATTGGGTCTGGGAACATTTCTCTTC | 319 | 5 |
| | Reverse GTGAGATTTAAACATTCCTCTTCGG | | 6 |
| Mda-5 | Forward GAGCAACTTCTTTCAACCAC | 633 | 7 |
| | Reverse GAACACCAGCATCTTCTCCA | | 8 |
| RIG-I | Forward GAACGATTCCATCACTATCC | 580 | 9 |
| | Reverse GGCATCATTATATTTCCGCA | | 10 |
| PKR | Forward CTTCTCAGCAGATACATCAG | 689 | 11 |
| | Reverse GTTACAAGTCCAAAGTCTCC | | 12 |

**Table 2: primer sequences**

It could thus be shown that a viral replication peak occurred between 1 day to 4 days post-infection of mesothelioma M13 cells. Besides, PCR products corresponding to viral dsRNA potential receptors (Mda-5, TLR-3, RIG-I and PKR) could also be evidenced.

**Example 4**

**Efficient uptake of apoptotic mesothelioma cells by immature DCs.**

The uptake by dendritic cells (DCs) of apobodies from MV-infected (72-hours) was then studied and compared to that of UV-irradiated (24-hours) M13 tumoral cells.

Dendritic cells were derived from monocytes generated from leukapheresis harvests of HLA-A0201 healthy donors (EFS, Nantes, France), after obtaining informed consent. Monocytes-enriched fraction (>85% purity) was first separated by Ficoll density gradient centrifugation (PAA Laboratories, Les Mureaux, France).

Monocytes were then enriched by elutriation (counterflow centrifugation) using a Beckman Avanti J20 centrifuge equipped with a JE5.0 rotor and a 40-ml elutriation chamber. Routinely, purity of elutriated monocytes was over 80%, as assessed by flow cytometry based on the detection of the CD14 marker. Monocytes were cultured at $2x10^8$ cells/ml with 500 IU/ml GM-CSF and 200 IU/ml IL-4 (Cell Genix Technology, Freiburg, Germany). Cells were then allowed to differentiate for 6 days.

On day 6, monocytes-derived DCs were collected from culture supernatant and seeded in culture for subsequent loading. Immature DCs were incubated with $2.10^8$ cells/ml of apoptotic material, derived from UV-treated or MV-infected allogenic M13 tumoral cells, for additionally 24 hours co-culture (ratio 1:1). DC phagocytosis yield analysis was assessed both by flow cytometry and confocal laser microscopy, as previously described (Massé *et al.* (2002) *Cancer Research* **32**:1050-1056). Briefly, UV- or MV- treated M13 cells were labelled with PKH-26 membrane dye colorant, according to the manufacturer's protocol (Sigma, St Quentin Fallavier, France). After 24 hours co-culture, DCs were stained with FITC-conjugated anti HLA-DR antibodies (Immunotech, Marseilles, France). After PBS washes, cells were harvested and analysed either on a FACSCalibur (BD Biosciences, Grenoble, France), or with a TCS NT microscope (Leica Instruments, Heidelberg, Germany). DCs that have ingested apoptotic cells were identified as HLA-DR/PKH-26 double positive cells (**Figure 7**).

As shown in **Figure 8**, it could be evidenced that DCs efficiently engulfed UV- and MV-treated mesothelioma cells at the same rate, as illustrated by a similar percentage of PKH26-positive DCs gated on basis of HLA-DR expression (65% and 74% for DCs loaded respectively with UV- or MV-treated M13 cells).

Confocal laser-scanning microcopy experiments further confirmed an efficient internalization of apoptotic M13 cells by immature DCs within 24 hours co-culture, irrespective of the death-induced strategy used (MV-infected or UV-irradiated).

**Example 5**

**Tumor cells infected with MV induce spontaneous DC maturation, by contrast with UV radiation-induced apoptotic M13 cells.**

The inventors next examined whether cell material derived from MV-infected M13 tumoral cells could efficiently stimulate DC maturation.

DC maturation status was assessed within 24 hours following engulfment of tumoral cells killed either by radiation exposition or virus-mediated cytolytic activity.

Phenotype of viable DCs (gated on basis of TOPRO-3 positive staining exclusion) was investigated by surface expression of Class I and II MHC molecules (**Figure 9**) and of maturation markers CD80, CD86, CD83 and CD40 (**Figure 10**), completed by cytokines secretion pattern analysis performed on co-culture supernatant (**Figure 11**). As controls, DCs were left alone, or matured with a combination of TLR3 ligand and one pro-inflammatory cytokine (polyinosinic:polycytidylic acid/IFNα, as a mimick of viral infection), or directly primed by measles virus contact (MV).

Briefly, immunostaining was performed with a panel of monoclonal antibodies (all purchased from Immunotech, Marseilles, France) specific for HLA-ABC (clone B9.12.1), HLA-DR (clone B8.12.2), CD80 (clone MAB104), CD83 (clone HB15a), CD86 (clone HA5.2B7), and CD40 (clone MAB89). DCs were incubated with each of the above antibodies (1 µg/ml) at 4℃ for 30 min prior to flow cytometry. Cytokines pattern secretion was assayed in supernatants collected 24 hours after engulfment. IL-10, IL-12, IL-6, IL-1β and TNFα concentrations were measured using commercially available Cytometric Beads Array kits (BD Biosciences, Le Pont de Claix, France), according to the manufacturer's protocol. Quantification of IFNα was performed with an ELISA test (Biosource, Camarillo, USA).

A spontaneous maturation program could be observed only for DCs loaded with apobodies derived from mesothelioma cells infected with MV, at a level essentially equivalent to the positive control maturation cocktail used in the experiment (PolyI:C/IFNα). Spontaneous maturation was evidenced by significant up-regulation of co-stimulation molecules expression (for CD80, CD83, CD86, CD40 and

HLA-ABC), and production of numerous pro-inflammatory cytokines (for IL-6, IL-1β, TNFα, and IFNα).

However, in line with previous reports, pulsing DCs with UV-irradiated apoptotic tumoral cells, as well as direct infection of DCs by measles virus (MV), did not lead to this effect.

Overall these data strongly support an increased immunogenicity of MV-infected tumoral cells with respect to UV-irradiated tumoral cells.

**Example 6**

**Cross-Priming of MSLN-specific CD8 T-cell response.**

Finally, the inventors tested whether DCs loaded with apobodies derived from mesothelioma cells infected with MV could stimulate an effector CD8 response specific for an MPM-associated tumor antigen, such as Mesothelin (MSLN).

In order to assess this question, tetramer immunostaining was performed on CD8 T-lymphocytes sensibilized for one-week with autologous DCs loaded with apoptotic material derived from UV- or MV- treated M13 cells. As controls, a similar experiment was conducted with the Jurkat lymphoma T-cell line, chosen on the basis of its susceptibility to MV and its MSLN-negative expression characteristics (**Figure 12**). As internal experiment controls, MelanA/Mart-1-specific tetramer staining (MelanA26-35L) was achieved in complement of those specific for the two selected MSLN-derived CTL epitopes. These peptides (MSLN 531-539 and MSLN 541-550) were identified by scanning MSLN amino-acid sequence (GenPept NP 005814) for matches to consensus motifs for HLA-A0201 binding, using two computer algorithms BIMAS and SYFPEITHI (**Table 2**):

| Tetramer name | Localisation | Sequence | HLA-A0201 binding score SYFPEITHI | BIMAS |
|---|---|---|---|---|
| HLA-A2 VLP9 | 531-539 | VLPLTVAEV (SEQ ID NO: 13) | 29/30 | 272/285 |
| HLA-A2 KLL10 | 541-550 | KLLGPHVEGL (SEQ ID NO: 14) | 30/31 | 312/312 |

**Table 2: tetramer characteristics**

Briefly, CD8 T lymphocytes were prepared from HLA-A0201 healthy donors PBMCs by positive selection with the MACS column systems using CD8 multisort kit (Miltenyi Biotec, Paris, France). Purified naïve CD8 T cells (>90% purity) were stimulated with autologous DCs loaded with each apoptotic preparation or unloaded DCs as a control. The co-culture was performed in round bottom 96-well plates (BD Falcon), by mixing $2.10^4$ mature DCs with $2.10^5$ responder T cells (ratio 1:10) in 200 µl of 8% human serum RPMI 1640 medium, supplemented with 10 ng/ml IL-12 for the first 3 days (AbCys SA, Paris, France) and with 10 U/ml IL-2 (Proleukin, Chiron Therapeutics, USA) for the next days. IL-2 was added every three days, allowing regular culture medium renewal. After 7-8 days culture, T cells were harvested and stained with MSLN-specific tetramers as follows.

The selected CD8 epitope peptides (synthesis performed by Eurogentec, Liege, Belgium) were used for monomers production (Recombinant Proteins Production Platform, U601-IFR26, Nantes, France) as previously described (Labarrière *et al*. (2002) *Int. J. Cancer* **101**:280-286). HLA-A2 VLP9 and HLA-A2 KLL10 monomers were oligomerized with PE-labeled streptavidin (BD Biosciences). Staining and washing were performed in 0.1% BSA-PBS. T cells were stained successively with 10 µg/ml of PE-labeled pMHC multimers at 4℃ for 30 min, and with 1 µg/ml diluted PE-Cy5-conjugated anti-CD8 antibodies (clone RPA-T8, BD Biosciences) for additionally 30 min at 4℃. Cells were washed and immediately analysed on a FACSCalibur.

Interestingly, a significant increase of MSLN-specific T-cells percentage among the CD8-positive gated population could be observed for co-cultures with DCs loaded with apoptotic material derived from MV-treated M13 cells with respect to co-cultures with DCs loaded with apoptotic material derived from UV-treated M13 cells.

## CLAIMS

**1.** Attenuated measles virus for use in the treatment of malignant mesothelioma in an individual.

**2.** Attenuated measles virus according to claim 1, wherein the attenuated measles virus is an Edmonston strain.

**3.** Attenuated measles virus according to claim 1 or 2, wherein the attenuated measles virus is selected from the group constituted of a Schwartz strain and a Moraten strain.

**4.** Attenuated measles virus according to any of claims 1 to 3, wherein the malignant mesothelioma is a malignant pleural mesothelioma.

**5.** Attenuated measles virus according to any of claims 1 to 4, wherein the individual has been exposed to asbestos.

**6.** A method for preparing vaccinal dendritic cells intended for treating cancer in an individual, comprising the following steps:
- *in vitro* infection of cancer cells taken from the individual by an attenuated measles strain to yield a cell lysate;
- contacting dendritic cells with the cell lysate to yield vaccinal dendritic cells.

**7.** The method according to claim 6, wherein the dendritic cells originate from the individual.

**8.** The method according to claim 6 or 7, wherein the dendritic cells are monocyte-derived dendritic cells.

**9.** The method according to any of claims 6 to 8, wherein the cancer is malignant mesothelioma.

**10.** The method according to any of claims 6 to 9, wherein the cancer is malignant pleural mesothelioma.

**11.** The method according to any of claims 6 to 10, wherein the attenuated measles virus is an Edmonston strain.

**12.** The method according to any of claims 6 to 11, wherein the attenuated measles virus is selected from the group constituted of a Schwartz strain and a Moraten strain.

**13.** Vaccinal dendritic cells liable to be obtained by the method according to any of claims 6 to 12.

**14.** A pharmaceutical composition comprising vaccinal dendritic cells liable to be obtained by the method according to any of claims 6 to 12 as active ingredient, in association with a pharmaceutically acceptable carrier.

**15.** Vaccinal dendritic cells liable to be obtained by the method according to any of claims 6 to 12 for use in the treatment of cancer in an individual.

**16.** Vaccinal dendritic cells according to claim 15, wherein the cancer is malignant mesothelioma.

**17.** Vaccinal dendritic cells according to claim 15 or 16, wherein the cancer is malignant pleural mesothelioma.

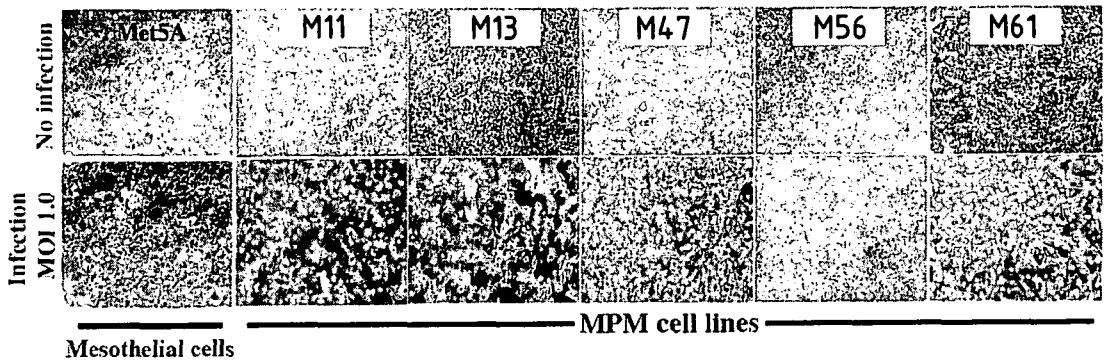



FIG.1

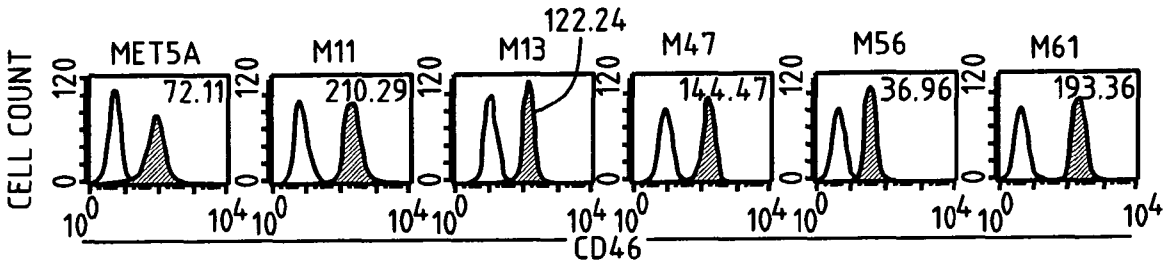



FIG.2

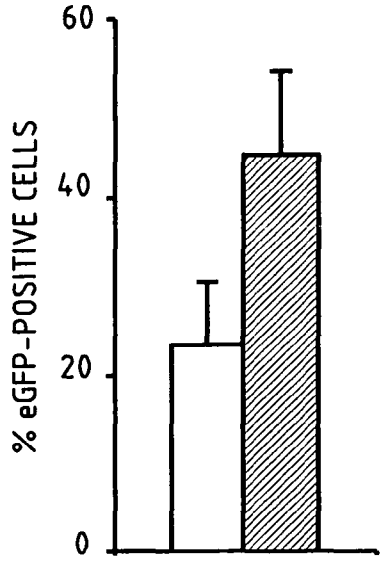



FIG.3

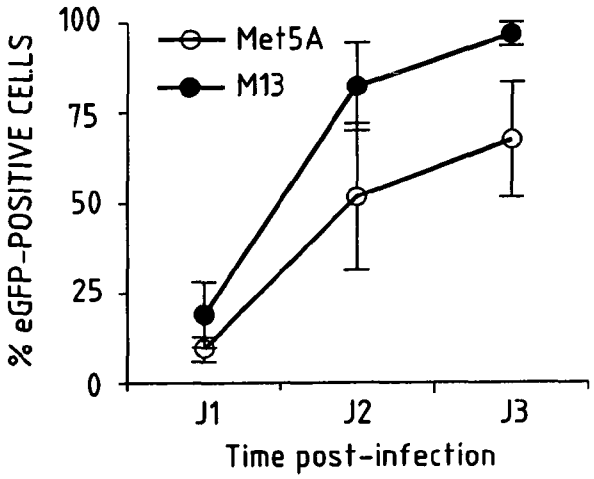



FIG.4

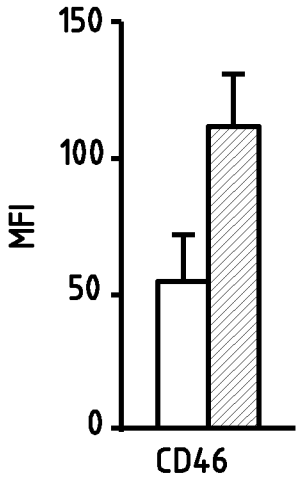

150
100
50
0
MFI
CD46

FIG.5

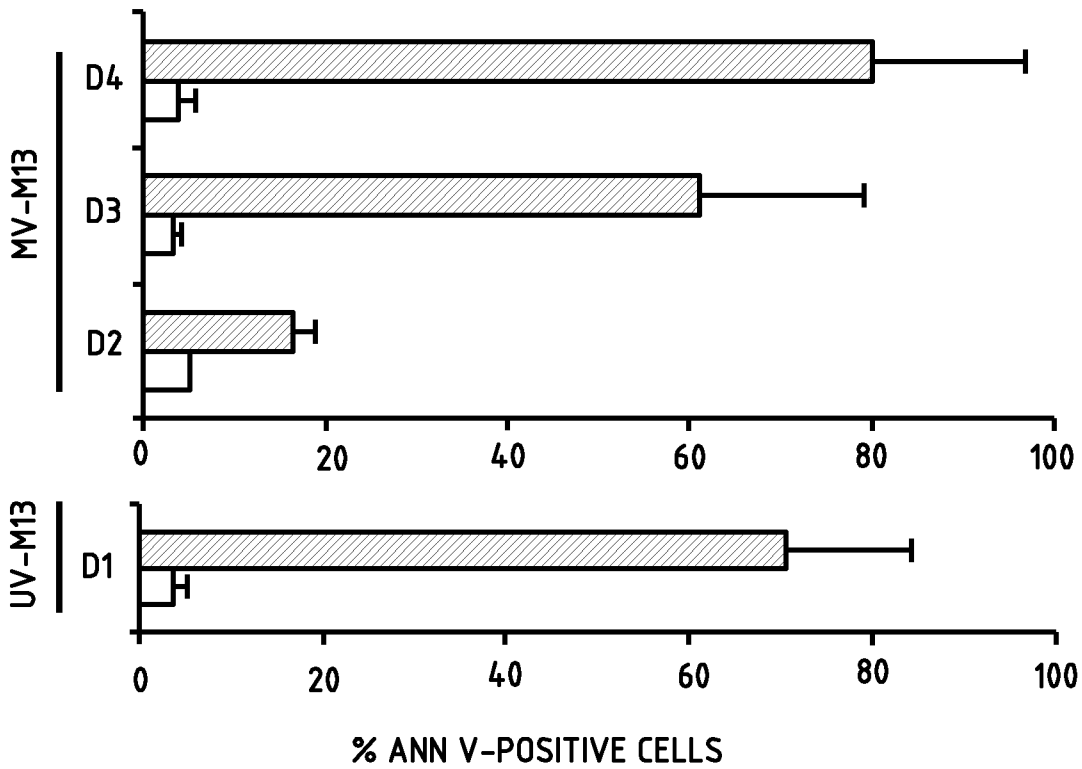

MV-M13
D4
D3
D2
UV-M13
D1
0
20
40
60
80
100
% ANN V-POSITIVE CELLS

FIG.6

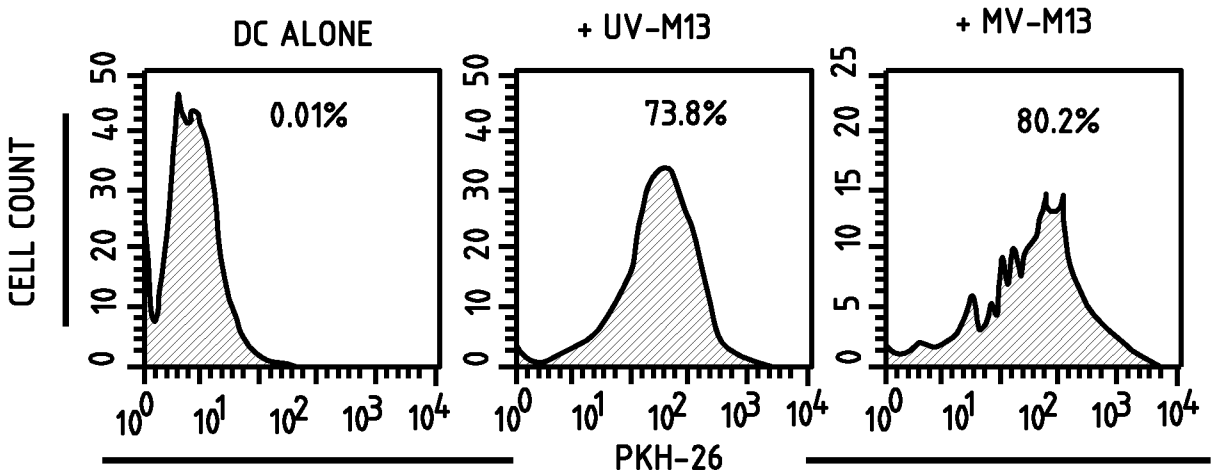



FIG.7

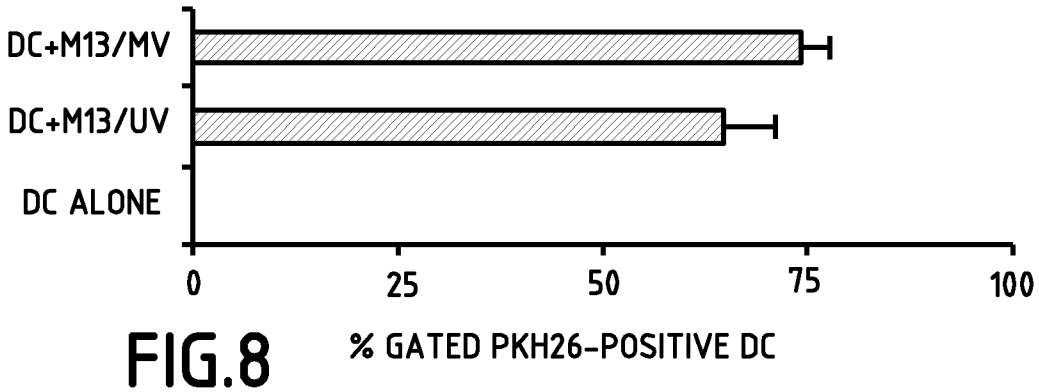



FIG.8

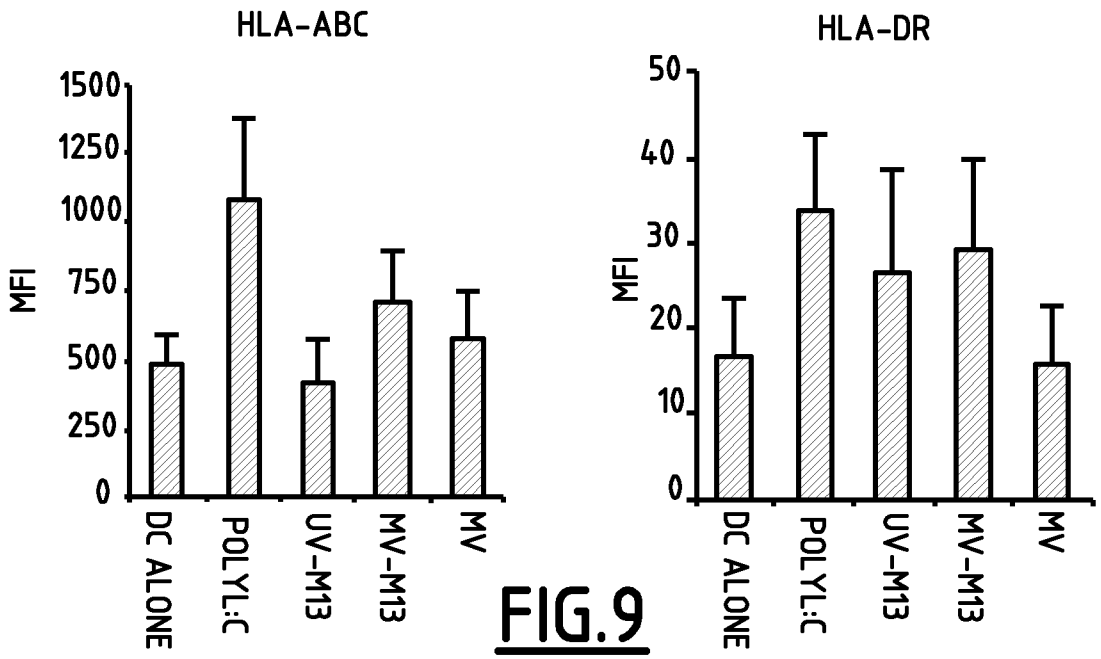



FIG.9

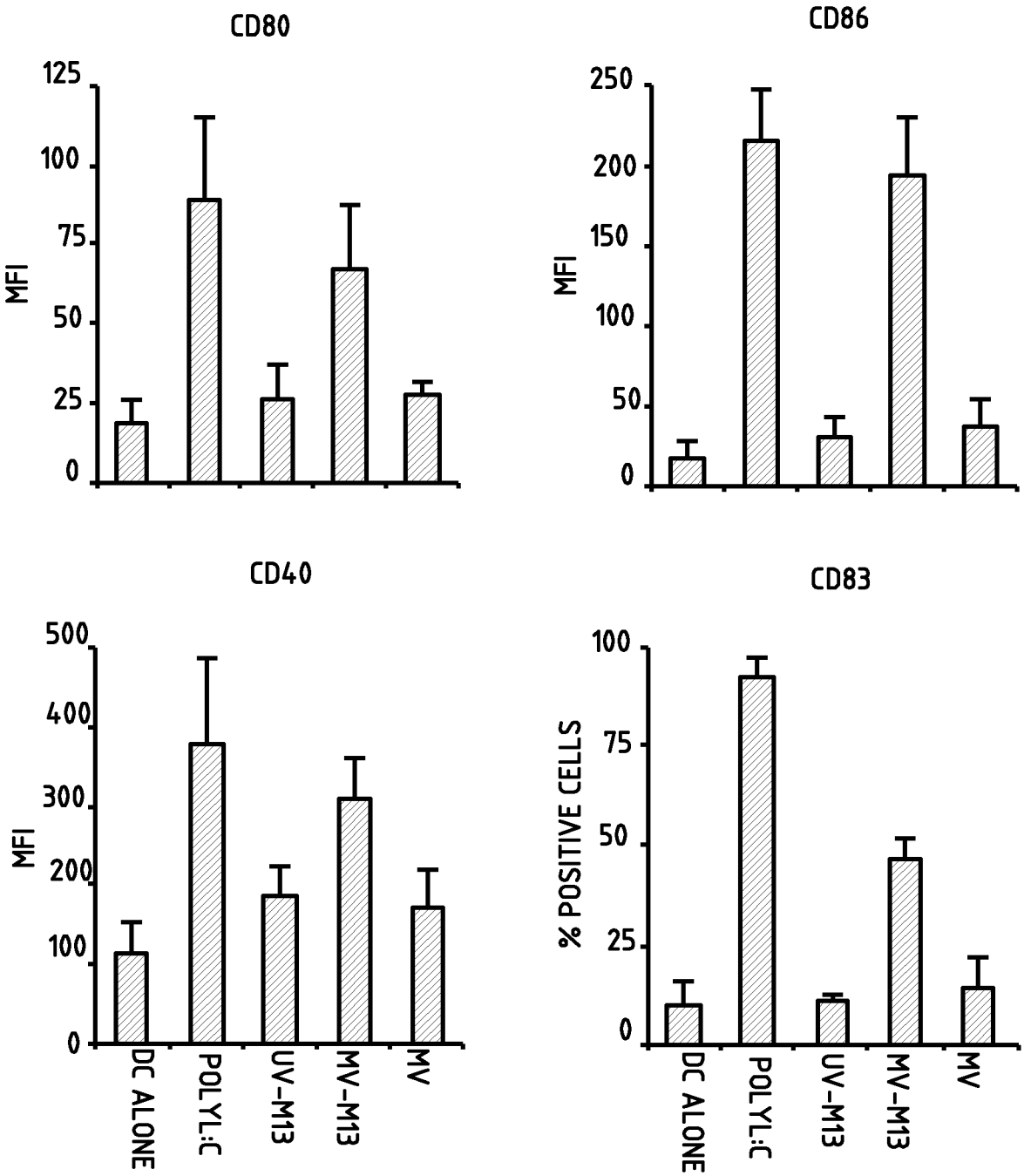

CD80
MFI
125
100
75
50
25
0
CD86
MFI
250
200
150
100
50
0
CD40
MFI
500
400
300
200
100
0
CD83
% POSITIVE CELLS
100
75
50
25
0
DC ALONE
POLY I:C
UV-M13
MV-M13
MV

FIG.10

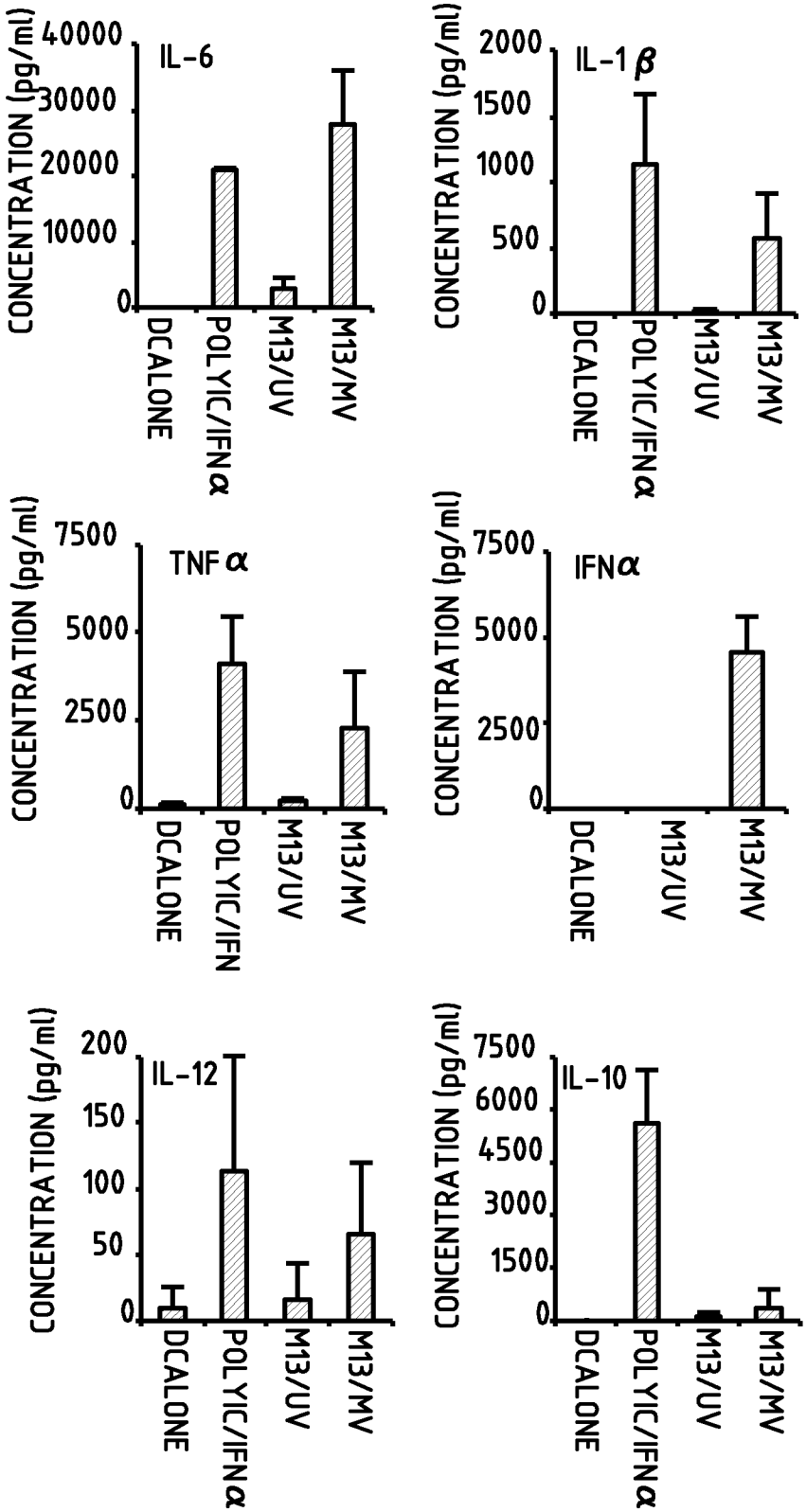

IL-6
CONCENTRATION (pg/ml)
40000
30000
20000
10000
0
DCALONE
POLYIC/IFNα
M13/UV
M13/MV
IL-1β
2000
1500
1000
500
TNFα
7500
5000
2500
IFNα
IL-12
200
150
100
50
IL-10
6000
4500
3000
1500

FIG.11

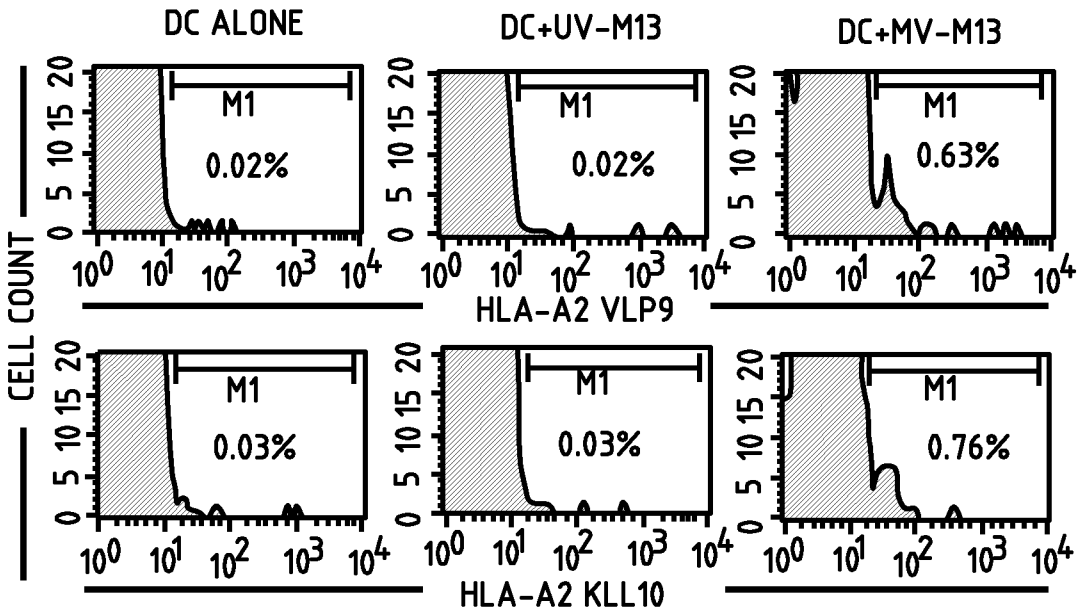

DC ALONE
DC+UV-M13
DC+MV-M13
CELL COUNT
M1
0.02%
0.02%
0.63%
HLA-A2 VLP9
M1
0.03%
0.03%
0.76%
HLA-A2 KLL10

FIG.12

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/063626

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/165

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | PENG KAH-WHYE ET AL: "Intraperitoneal therapy of ovarian cancer using an engineered measles virus" CANCER RESEARCH, vol. 62, no. 16, 15 August 2002 (2002-08-15), pages 4656-4662, XP002467554 ISSN: 0008-5472 * the whole document * page 4662, left-hand column | 1-5 |

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 November 2008 | 13/01/2009 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Schulz, Regine |

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | PENG K W ET AL: "Oncolytic measles virotherapy for ovarian cancer" CANCER GENE THERAPY, vol. 11, no. 12, December 2004 (2004-12), page 846, XP008088210 & MEETING OF THE INTERNATIONAL-SOCIETY-FOR-CANCER-GENE-THERAPY; FEBRUARY 20 -22, 2004 ISSN: 0929-1903 the whole document | 1-5 |
| A | CARI J MCDONALD ET AL: "A measles virus vaccine strain derivative as a novel oncolytic agent against breast cancer" BREAST CANCER RESEARCH AND TREATMENT, KLUWER ACADEMIC PUBLISHERS, BO, vol. 99, no. 2, 27 April 2006 (2006-04-27), pages 177-184, XP019392351 ISSN: 1573-7217 the whole document | 1-5 |
| A | WO 02/23994 A (MAYO FOUNDATION [US]; RUSSELL STEPHEN JAMES [US]; FIELDING ADELE [US];) 28 March 2002 (2002-03-28) * whole document * page 2, line 17 - page 4, line 5 | 1-5 |
| A | GREGOIRE MARC ET AL: "Immunotherapy and malignant mesothelioma: clinical perspectives" BULLETIN DU CANCER (MONTROUGE), vol. 94, no. 1, January 2007 (2007-01), pages 23-31, XP008088161 ISSN: 0007-4551 * the whole document * page 28, left-hand column - page 29 | 1-5 |
| A | BRAMWELL ET AL: "The rational design of vaccines" DDT - DRUG DISCOVERY TODAY, ELSEVIER SCIENCE LTD, GB, vol. 10, no. 22, 15 November 2005 (2005-11-15), pages 1527-1534, XP005133824 ISSN: 1359-6446 * the whole document * page 1533; table 1 | 1-5 |

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | EBSTEIN F ET AL: "Cytotoxic T cell responses against mesothelioma by apoptotic cell-pulsed dendritic cells" AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE, AMERICAN LUNG ASSOCIATION, NEW YORK, NY, US, vol. 169, no. 12, June 2004 (2004-06), pages 1322-1330, XP003003189 ISSN: 1073-449X * the whole document * page 1322 - page 1323 | 6-17 |
| A | GREGOIRE M ET AL: "Anti-cancer therapy using dendritic cells and apoptotic tumour cells: pre-clinical data in human mesothelioma and acute myeloid leukaemia" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 21, no. 7-8, 30 January 2003 (2003-01-30), pages 791-794, XP004401626 ISSN: 0264-410X * the whole document * page 794, left-hand column, paragraph 2; figure 2 | 6-17 |
| A | VIDAL L ET AL: "Reovirus and other oncolytic viruses for the targeted treatment of cancer" TARGETED ONCOLOGY, SPRINGER-VERLAG, PA, vol. 1, no. 3, 29 June 2006 (2006-06-29), pages 130-150, XP019415246 ISSN: 1776-260X page 136, right-hand column, last paragraph - page 137, left-hand column | |
| P,X | GAUVRIT ANNE ET AL: "Measles virus induces oncolysis of mesothelioma cells and allows dendritic cells to cross-prime tumor-specific CD8 response" CANCER RESEARCH, vol. 68, no. 12, June 2008 (2008-06), pages 4882-4892, XP002504976 ISSN: 0008-5472 the whole document | 1-17 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2008/063626

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

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

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

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

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

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

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

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

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1 - 5

attenuated measles virus (MV) for use of in the treatment of malignant mesothelioma in an individual.

---

2. claims: 6 - 17

method for preparing a vaccinal dendritic cell for treating cancer in an individual comprising 1) in vitro infection of cancer cells taken form the individual by an attenuated MV strain to yield cell lysate and 2) contacting dendritic cells with the cell lysate to yield vaccinal dendritic cells; as well as the so obtained dendritic cells; a pharmaceutical composition comprising them; their use for the manufacture of a medicament for the treatment of cancer in an individual.

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2008/063626

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 0223994 A | 28-03-2002 | AU 9506301 A | 02-04-2002 |
| | | US 2007071771 A1 | 29-03-2007 |
| | | US 7118740 B1 | 10-10-2006 |