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(71) Applicant(s)  
Institut Gustave Roussy; Applied Research Systems Ars Holding N.V.

(72) Inventor(s)  
Frederic Triebel

(74) Agent/Attorney  
GRIFFITH HACK, GPO Box 1285K, MELBOURNE VIC 3001

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(71) Applicants (for all designated States except US): INSTITUT GUSTAVE ROUSSY [FR/FR]; 39, rue Camille Desmoulins, F-94805 Villejuif Cedex (FR). APPLIED RESEARCH SYSTEMS ARS HOLDING N.V. [NL/NL]; John R. Gorsiraweg 6, P.O. Box 3889, Curaçao (AN).

(72) Inventor; and  
(75) Inventor/Applicant (for US only): TRIEBEL, Frédérique [FR/FR]; 10, rue Saint-Louis, F-78000 Versailles (FR).

(74) Agent: HIRSCH, Denise; Cabinet Lavoix, 2, place d'Estienne d'Orves, F-75441 Paris Cedex 09 (FR).

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(54) Title: USE OF MHC CLASS II LIGANDS AS ADJUVANT FOR VACCINATION AND OF LAG-3 IN CANCER TREATMENT

(57) Abstract

The present invention concerns a use of a MHC class II ligand, such as CD4 and LAG-3, for the manufacture of a medicament for preventing or treating pathological conditions involving an antigen specific immune response, as well as the use of LAG-3 in cancer-immunotherapy. The invention also concerns a pharmaceutical composition comprising an effective amount of an antigen able to induce an antigen-specific immune response together with an effective amount of a MHC class II ligand wherein said MHC class II ligand is present as an adjuvant-like agent.



Use of MHC class II ligands as adjuvant for vaccination  
and of LAG-3 in cancer treatment.

The present invention relates to the use of LAG-3 and CD4, and  
in a more general way, the use of MHC class II ligands or MHC class II-like  
ligands as adjuvants for vaccines, in order to boost an antigen specific immune  
response, as well as the use of LAG-3 as a therapeutical agent in cancer  
5 immunotherapy.

It is now recognized that the proteins encoded by MHC Class II  
region are involved in many aspects of immune recognition, including the  
interaction between different lymphoid cells such as lymphocytes and antigen  
presenting cells. Different observations have also shown that other  
10 mechanisms which do not take place via CD4 participate in the effector  
function of T helper lymphocytes.

The lymphocyte activation gene 3 (LAG-3) expressed in human  
CD4<sup>+</sup> and CD8<sup>+</sup> activated T-cells as well as in activated NK cells encodes a  
503 amino-acids (aa) type I membrane protein with four extracellular  
15 immunoglobulin superfamily (IgSF) domains (1) and is a ligand for MHC class  
II molecules (2). Analysis of this sequence revealed notable patches of identity  
with stretches of aminoacids sequences found at the corresponding positions  
in CD4, although the overall aminoacids sequence homology with human CD4  
is barely above background level (approximately 20 % sequence identity).  
20 There are also some internal sequence homologies in the LAG-3 molecule  
between domains 1 (D1) and 3 (D3) as well as between domains 2 (D2) and 4  
(D4) suggesting that LAG-3 has evolved like CD4 by gene duplication from a  
preexisting 2 IgSF structure (1). In addition, LAG-3 and CD4 genes are located  
in a very close proximity on the distal part of the short arm of chromosome 12  
25 (3). LAG-3 and CD4 can therefore be regarded as evolutionary "first cousins"  
within the IgSF (2).

Like CD4, hLAG-3 is composed of Ig like ectodomains with a  
WxC signature motif in domains 2 and 4 ; however a difference with CD4 is the  
presence of an extraloop sequence in domain 1 (recognized by the 17B4mAb)  
30 and an intracytoplasmic proline rich motif (EP repeats) in human LAG-3  
(hLAG-3). Recently, murine lymphocyte activation gene 3 (mLAG-3) was

cloned and approximately 70 % of homology was found with hLAG-3, with the same proline rich motif in the intracytoplasmic tail.

Antigen specific stimulation of CD4<sup>+</sup> T-cell clones in the presence of anti-LAG-3 mAb leads to extended proliferation and cytokines production (5). It has been suggested a regulatory role of hLAG-3 on CD4<sup>+</sup> T lymphocyte activated, by cross-linking MHC class II molecules expressed on T-cells with LAG-3 Ig fusion proteins (6). LAG-3 MHC class II interaction inhibits signals through MHC class II molecules expressed on CD4<sup>+</sup> T-cells (decrease of proliferation and cytokines production), suggesting that both LAG-3 and MHC class II are effector molecules for the down-regulation of T helper cell mediated immune responses. The hLAG-3 Ig fusion protein was found to bind xenogenic MHC class II molecules (murine and monkey). In addition, the mLAG-3 has been proposed to transduce a positive signal in effector cells, since transgenic mice with a LAG-3 null mutation have a defect in the NK cell compartment (7).

Mouse tumor cell lines engineered to express membrane (B7.1, B7.2, CD95L, ...) or secreted molecules (IL-2, IL-12, ...) are often used to investigate immune responses or antitumor effects. This approach implies that many tumor cells are potentially antigenic (9), and become immunogenic when they express molecules. Experimental mouse tumors are classified as intrinsically immunogenic when, after a single injection into syngenic mice as nonreplicating cell vaccines, they elicit a protective immune response against a subsequent lethal challenge. Tumors that do not retain this residual immunogenicity are defined as poorly immunogenic or nonimmunogenic.

Antitumor immune responses are mediated primarily by T-cells (12). Recent studies have implicated a deficit in efficient antigen presentation and T-cell priming as being problematic for the practical implementation of an ideal tumor vaccine. Indeed, it has been demonstrated that transfecting tumor cells with genes coding for various cytokines, such as IL-2, IL-4, IL-12 or GM-CSF or genes coding for co-stimulatory molecules such as B7 not only led to primary rejection of the modified cells but often elicited protective immunity against subsequent challenge with unmodified tumor cells (13).

Professional antigen presenting cells (APCs) are capable of taking up, processing and presenting antigen to T-cells in the context of co-stimulatory signals required for T-cell activation, leading to optimal antigen presentation. In particular, it is well established that MHC class II<sup>+</sup> dendritic cells (DCs) play a crucial role in processing and presenting antigens to the immune system. The inventors hypothesized that tumor immunogenicity would be increased if tumor could be modified to directly trigger host APCs such as macrophages and dendritic cells. Indeed, it has been reported that cross-linking of MHC class II molecules specifically expressed by such cells, using mAb or superantigens, transduces signals resulting in TNF $\alpha$  and IL-12 production (14, 15). They had previously reported that Lymphocyte Activation Gene-3 (LAG-3), which is embedded in the CD4 locus (1, 16), encodes a protein that binds human and murine MHC class II molecules with higher affinities than CD4 (17, 6).

The inventors of the instant application have investigated whether hLAG-3, human CD4 (hCD4) and mLAG-3 expression on three MHC class II-mouse tumors (the poorly immunogenic sarcoma MCA 205 and the nonimmunogenic TS/A + RENCA adenocarcinoma) can mediate an immune response so as to reject mouse tumor and can induce systemic immunity.

As a result, they have discovered that human or murine LAG-3, whether expressed as membrane proteins in solid tumor cell lines or inoculated into mice as a soluble protein induced a potent immunity against highly malignant murine tumors. The immunity was T-cell dependent and antigen-specific.

They have further investigated the role of CD4 and found that human CD4 (hCD4) also induced a systemic antitumor response.



15 JUL 1988

The induced immunity has been found to be T-cell mediated, since the same antitumor response was not obtained with Nude mice lacking T- lymphocytes.

The antitumor effect was still found when using  
5 different tumor cell lines exhibiting different intrinsic immunogenicity as well as different strains of mice expressing different MHC genes.

Furthermore, the hLAG-3 and hCD4 induced effects were observed when tumor cell lines expressing hLAG-3 or  
10 hCD4 were injected at a distant site from the initial inoculation site of the wild-type tumor cell lines.

Furthermore, systemic administration of soluble hLAG-3 directly induces an inhibition of *in vivo* tumor growth.

15 All the aforementioned results demonstrate that LAG-3 and CD4 are able to elicit an antigen specific T-cell mediated immune response and may be useful as a tool in immunotherapy, in order to prevent the occurrence of a cancer among populations at risk or more generally in any  
20 immunotherapy involving an antigen-specific T-cell mediated immune response, and that LAG-3 is further useful as a tool for inhibiting *in vivo* tumor growth.

The inventors have further demonstrated that soluble LAG-3 when administered together with an antigen  
25 against which an immune response is sought, was able to work as an adjuvant for a vaccine.

This role can be explained by an improved presentation of the antigen by professional APCs (dendritic cells and macrophages) located underneath the  
30 skin and triggered via MHC class II.

Accordingly, since induction of a CD8<sup>+</sup> T-cell immunity is involved in viral (e.g. AIDS, hepatitis and herpes) and intra-cellular parasitic and bacterial (e.g.



15 JUL 2002

leprosy tuberculosis) infections and cancer, LAG-3 will be particularly useful for therapeutic vaccination against the pathogen agents involved in these diseases as well as in cancer treatment.

5 All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the  
10 applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents  
15 forms part of the common general knowledge in the art, in Australia or in any other country.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word  
20 "comprises" has a corresponding meaning. According to one of its aspects, the present invention relates to the use of a MHC class II ligand Use of a MHC class II ligand able to boost an antigen specific immune response in combination with an antigen able to induce  
25 said antigen-specific immune response, for the manufacture of a medicament for preventing or treating pathological conditions involving said antigen specific immune response, wherein the MHC class II ligand is LAG-3, or derivatives, mutants or soluble fragments of LAG-3.

30 In a first embodiment, the MHC class II binding molecule is LAG-3 as well as derivatives thereof, able to bind the MHC class ligand of LAG-3.



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By derivatives of LAG-3, in the sense of the present invention, there are meant mutants, variants and fragments of LAG-3 namely soluble fragments of LAG-3 provided that they maintain the ability of LAG-3 to bind MHC class II molecules.

5

Thus, the following forms of LAG-3 may be used :

- the whole LAG-3 protein,

- a soluble polypeptide fragment thereof consisting of at least one of the four immunoglobulin extracellular domains, namely the soluble part of LAG-3 comprised of the extracellular region stretching from the aminoacid 23 to the aminoacid 448 of the LAG-3 sequence disclosed in French Patent  
10 application FR 90 00 126,

- a fragment of LAG-3 consisting of substantially all of the first and second domains

- a fragment of LAG-3 consisting of substantially all of the first  
15 and second domains or all of the four domains, such as defined in WO 95/30750, such as

- a mutant form of soluble LAG-3 or a fragment thereof comprising the D1 and D2 extracellular domains and consisting of :

- a substitution of an aminoacid at one of the following positions :

20

- position 73 where ARG is substituted with GLU,
  - position 75 where ARG is substituted with ALA or GLU,
  - position 76 where ARG is substituted with GLU,
- or a combination of two or more of those substitutions,

- a substitution of an aminoacid at one of the following positions :

25

- position 30 where ASP is substituted with ALA ;
- position 56 where HIS is substituted with ALA ;
- position 77 where TYR is substituted with PHE ;
- position 88 where ARG is substituted with ALA ;
- position 103 where ARG is substituted with ALA ;
- 30 • position 109 where ASP is substituted with GLU ;
- position 115 where ARG is substituted with ALA ;

or a deletion of the region comprised between the position 54 and the position 66,

or a combination of two or more of those substitutions.

Those mutants are described in PNAS, June 1997 (4)

5 - or a physiological variant of LAG-3 comprised of a soluble 52 kD protein containing D1, D2 and D3.

According to a second embodiment, the MHC class II binding protein is CD4 or a derivative thereof able to bind the MHC class II ligand of CD4.  
10

The derivatives of CD4 are such as defined for the derivatives of LAG-3. They are namely mutants, variants and fragments of CD4 namely soluble fragments of CD4 provided that they maintain the ability of CD4 to bind MHC class II molecules.  
15

LAG-3 and CD4, namely hLAG-3 and hCD4 or the derivatives thereof such as defined above may be administered as recombinant moieties expressing said molecules, for example transfected cells or recombinant viruses.  
20

The present invention relates also to tumor cells transfected with a DNA coding for at least one MHC class II ligand, such as CD4 or LAG-3 or derivatives thereof.

25 A further object of the instant invention is also the use of cells, like tumor cells, transfected with a DNA coding for at least one MHC class II ligand, such as CD4 or LAG-3 or derivatives thereof for the manufacture of a medicament, preferably a medicament for preventing or treating pathological conditions involving an antigen specific immune response like an antigen  
30 specific T-cell mediated immune response or for treating pathological disorder like cancers.

The transfected cells are preferably mammal cells and in particular mammal tumor cells.

According to one of its aspects, the present invention relates to a process for preparing cells transfected with a DNA coding for at least one MHC class II ligand, such as CD4 or LAG-3 or derivatives thereof comprising the steps consisting of removing cells from a patient, transfecting said cells with a DNA coding for at least one MHC class II-like ligand, such as CD4 or LAG-3 or derivatives thereof and recovering the so-transfected cells.

For the preparation of tumor cells according to the invention, this process will be reproduced on tumor cells removed from a patient.

However, according to a preferred embodiment, the MHC class II binding protein, namely CD4 or LAG-3 or the derivative thereof, is administered in a free form, namely in a soluble form by inoculating them systemically, for example as an s.c, i.m or i.v injection.

The medicament according to the invention may be used as a vaccine to prevent disorders associated with an antigen specific immune response, preferably a T-cell mediated immune response.

To that end, it is administered in a suitable vehicle together with one or several antigen(s) against which an immune response is sought. The antigen may be an inactivated or attenuated infectious agent or a purified antigen, eventually obtained by protein recombinant procedures, such as an antigen of an infectious agent or a tumor antigen, which preferably are able to elicit a T-cell mediated immune response.

The vaccine may be used to prevent a subject against an infectious disease, such as a viral, bacterial or parasitic disease wherein the infectious agent elicits an antigen specific immune response, preferably a T-cell mediated immune response.

The vaccine may also be used for treating patient against an infectious disease such as mentioned hereabove, involving a T-cell mediated immune response, namely a CD8<sup>+</sup> T-cell mediated immune response.

Examples of diseases requiring a boost of an existing T-cell mediated immunity are provided in the following table.

TABLE

Pathogens	Agents	Diseases
Viruses	HIV HBV, HCV HSV, CMV, HHV HTLV1	AIDS Hepatitis Failure of transplantation, Kaposi sarcoma Cancer
Intra-cellular Bacteria	Listeria Mycobacteria	Listeriosis Leprosis, Tuberculosis
Intra-cellular Parasites	Plasmodium Etc	Malaria
	Oncogenes Etc	Most carcinomas, melanomas, leukemia

5 In such cases, the antigen is chopped in the cells and the corresponding peptides loaded into MHC class I molecules and presented at the surface of the cells where there are recognized by CD8<sup>+</sup> cells. The results of the inventors showing that LAG-3lg molecules induce efficient T-cell response in animals and stimulate immature dendritic and monocytes *in vitro* strongly suggest that LAG-3 is a natural T-cell adjuvant in situations where it can cross-link MHC class II molecules in professional APCs.

The vaccine may also be used to prevent a subject against cancer, either solid tumor cancer or leukemia.

15 The vaccine may further be used for treating a patient against cancer.

In that case, the MHC class II binding protein namely LAG-3 or CD4 is administered to a subject either subcutaneously, intradermally or as a nasal spray together with one or several antigens able to elicit an immune response, preferably a T-cell mediated immune response. The antigen may be a peptide, a lipopeptide, a recombinant protein or DNA coding for these antigens.

The anti-cancer vaccine may be inoculated to populations at risks identified by their genotype (preventive vaccine) or to patients (therapeutic vaccine) bearing a tumor or at high risk of relapse following surgery.

Whether the vaccine is used as a conventional vaccine (preventive) or a therapeutic vaccine, it may be administered as a "naked" plasmid (19) incorporating a DNA sequence encoding LAG-3 or CD4, preferably under the control of a strong promoter.

The plasmid preferably also contains DNA encoding the antigen against which an immune response is sought.

A further object of the instant invention is thus a pharmaceutical composition comprising an effective amount of a MHC class II ligand in combination with an effective amount of an antigen able to stimulate the immune system, preferably via a T-cell response.

In still another aspect, the present invention relates to the use of LAG-3 as a medicament for anti-cancer immunotherapy in patients bearing a cancerous tumor.

In that case, LAG-3 is administered preferably as a free LAG-3 protein or a derivative thereof in a pharmaceutically acceptable vehicle, preferably a soluble derivative such as defined previously.

LAG-3 may be administered as an intratumoral injection or systemic injection, for example s.c, i.v or i.m.

A further object of the present invention relates to a method for tumor gene therapy comprising the steps consisting of removing a portion of a patient tumor cells, transfecting said cells with a DNA coding for at least one MHC class II ligand, such as CD4 or LAG-3 or derivatives thereof and re-introducing the so-transfected cells into the patient.

The following examples demonstrate the activity of LAG-3 and CD4 in the prevention or treatment of pathological conditions involving a T-cell mediated immune response.

For the better understanding of the invention, it may be referred to the annexed figures wherein :

- figure 1 represents the mean tumor size of C57BL/6 mice inoculated with wild-type MCA 205 tumor cells (MCA WT), MCA 205 tumor cells transfected with hCD4 (MCA hCD4) and MCA 205 tumor cells transfected with hLAG-3 (MCA hLAG-3) ;

- figure 2 represents the results (mean tumor size) obtained after rechallenging the same mice with wild-type MCA tumors cells at a minimal tumorigenic dose ;

- figure 3 represents the results (mean tumor size) obtained after rechallenging the same mice with the irrelevant MC 38 tumor cell line ;

- figure 4 represents the results (mean tumor size) obtained with a different strain of mice (BALB/c) and a different tumor cell line (TS/A) either of wild type (TS/A wt) or transfected with hCD4 (TS/A hCD4) or hLAG-3 (TS/A hLAG-3) ;

- figure 5 represents the results (mean tumor size) obtained with existing tumors treated with different doses of MCA cells expressing hLAG-3 ;

- figure 6 represents the results (mean tumor size) obtained with soluble LAG-3 injected together with MCA cells (MCA wt, MCA wt + 25 µg LAG-3 and MCA wt + 250 µg LAG-3) ;

the figure inside the frame of figure 6 represents the percentage of mice with tumor.

- figures 7 and 8 represent results of expression of LAG-3 in the membrane of tumor infiltrating lymphocytes (TILs) in five patients (P1-P5) bearing a renal cell carcinoma (RCC).

- figure 9 illustrates rejection of hLAG-3<sup>+</sup> tumor cells mediated by CD8<sup>+</sup> lymphocytes.

a, FACS analysis of CD8 expression by TILs from control (wt MCA 205) mice compared with TILs from hLAG-3 MCA 205 mice. b, CD8<sup>+</sup> T-cells contributes to the control of hLAG-3 TS/A tumor growth. Mice received i.p. 200 µg of purified CD4 or CD8-specific mAb at day -3, -2, -1, +4 and +8. Wild type or hLAG-3 TS/A tumor cells (MTD) were inoculated s.c. at day 0.

Data are means  $\pm$  s.e.m. of 5 mice in each group from a single experiment. These experiments were performed twice with similar results. c) Increased activity of antitumor CTLs in mice which had rejected hLAG-3/TS/A cells. Mice received transplants s.c. of  $5 \times 10^4$  hLAG-3/TS/A cells and were rechallenged at day 30 with  $2.5 \times 10^5$  parental TS/A cells. Splensens were collected at day 60 in tumor-free mice and the cells were co-cultured for 6 days with the indicated target cells that had been irradiated. Cytolytic activity against indicated target cells were examined in a standard 4 h  $^{51}\text{Cr}$  release assay with different effector-to-target ratios (E:T). Results for two mice are shown. These experiments were performed twice on 4 mice with similar results.

- figure 10 represents the results (mean tumor size) of mice (20 C57BL/6 mice) grafted with a MTD of the syngeneic MCA 205 sarcoma cells receiving a single vaccine injection of LAG-3lg. At day 6, 4 groups of 5 mice were formed and received a single s.c. vaccine injection (200  $\mu\text{l}$ ). The Ag is represented by irradiated (100 Gy) MCA 205 cells.

The experiments illustrated in the examples were carried out by using the following materials and methods.

## MATERIALS AND METHODS

### **1 Tumor cell lines**

The MHC Class I<sup>+</sup> and Class II<sup>+</sup> tumor cell lines used were : the poorly immunogenic methylcholanthrene-induced sarcoma MCA 205 cell line, (syngenic from C57BL/6 H-2<sup>b</sup> mice), the immunogenic renal carcinoma cell-line RENCA and the non-immunogenic undifferentiated spontaneous mammary adenocarcinoma TS/A cell line (both syngenic from BALB/C H-2<sup>d</sup> mice). The MC38 colon carcinoma cell line (syngenic from C57BL/6 mice) was used in rechallenge experiment as control tumor. Cells were maintained at 37°C in a humidified 10 % CO<sub>2</sub> atmosphere in air, in complete medium (RPMI 1640 culture medium supplemented with glutamine, sodium pyruvate, penicillin/streptomycin, 10 % of endotoxin free fetal calf serum and 0.05 mM 2-

$\beta$  mercapto-ethanol). For immunostaining experiments and *in vivo* experiments, cells were removed from their culture vessels with PBS containing 1mM EDTA. Before sub-cutaneous injection (s.c), cells were washed three times in cold PBS 1X and resuspended in same buffer. Cells were not cultured for longer  
5 than two weeks.

## 2. Mice

Female C57BL/6 mice, 6 or 8 weeks old, were purchased from  
10 IFFRA-CREDO Laboratories (Lyon, France). Female BALB/c mice, 4 to 8 weeks old were purchased from JANVIER Laboratoires, (France). All this mice strains were raised in specific pathogen free conditions. Female Nude were purchased from the animal facility of Institut Gustave Roussy and kept under protected microenvironments.

15

## 3. Genetic constructs

The cDNAs of hLAG-3, mLAG-3 and hCD4 were cloned into NT hygromycin plasmid vector (cloning sites are XbaI for hLAG-3 and HCD4 and  
20 XhoI for mLAG-3, under SR $\alpha$  promoter (18). The LAG-3 hcDNA cloned in a reverse orientation was used as a negative control. All tumor cell lines ( $2.5 \times 10^6$  cells) were transfected by electroporating using an Eurogentec apparatus (Belgium) : MCA 205 cells at 200V, TS/A and RENCA cells at 300V, 1500  $\mu$ F and infinite shunt resistance. Transfectants were selected in hygromycin B  
25 (Sigma) : MCA 205 in 100  $\mu$ g/ml RENCA and TS/A transfectants in 200  $\mu$ g/ml. Resistant cells expressing the transfected molecules were identified using a Elite cytofluorimeter (Coulter, Hialeah, FL) and cloned by limiting dilution. The best clone for each construction in each tumor cell lines was used in this study.

#### 4. Cytofluorimetric analysis

Resistant cells expressing the transfected molecules were  
5 stained by indirect immunofluorescence, with saturating amounts of purified or  
ascites fluids mAbs. Cells were first incubated with mAbs : 17B4 (anti-hLAG-  
3.1) (2), OKT4 (anti-hCD4), a rabbit preimmunserum (termed PIS) used as  
negative control and a rabbit immunserum anti-mLAG-3 (termed IS). The  
expression of murine MHC class I and II molecules on tumors were detected  
10 with the following mAbs : 34-1-2S for H-2 K<sup>d</sup> and D<sup>d</sup>, 28-8-6S for H-2 K<sup>b</sup> and D<sup>b</sup>,  
14-4-4S (for E<sup>d</sup>), M50114 (for IA and IE).

Cells were then washed and incubated with FITC-conjugated  
goat anti-mouse serum (GAM Coulter) or FITC-conjugated goat anti-rabbit  
serum (GAR Southern Biotechnologies Inc.). To study presence of infiltrating  
15 cells or recruitment of cells in tumor periphery, some mice were killed and  
tumor dissociated. Cells were stained by direct immunofluorescence, with  
17B4-FITC or the following mAbs (Pharmingen) : anti-mCD4-PE (L3T4), anti-  
mCD8 (Ly-2 and Ly-3.2), anti-mNK (2B4) and anti-mCD22 (Lyb-8.2). Cells  
were sorted using a Elite cytofluorimeter Coulter).

20 Positive cell lines were then cloned by limiting dilution LAG-3<sup>+</sup> or  
CD4<sup>+</sup> clones were frozen for further use.

To generate soluble LAG-3 molecules, the extracellular domains  
of hLAG-3 and mLAG-3 were fused to the hIgG1 and mIgG2a Fc portions  
respectively, as described (6). The resulting recombinant proteins, hLAG-3Ig  
25 and mLAG-3Ig, were produced in CHO cells and purified on protein-A  
columns.

#### 5. *In vivo* tumor experiments

##### 30 5.1 Tumor growth establishment and vaccination

Establishment of tumor cell lines was performed s.c using the  
minimal tumorigenic dose (MTD) at  $2 \cdot 10^5$  cells/mouse for MCA 205,  $5 \cdot 10^4$  cells

for TS/A and  $10^5$  cells for RENCA or fivefold the MTD. Mice which were tumor free 30 days after injection were rechallenged with parental tumor cell line (5 x MTD). MC38 colon carcinoma cell line was used at  $10^5$  cells as a control tumor in C57BL/6 mice that rejected TS/A tumor. Age-matched naive C57BL/6 or BALB/c mice were injected with tumor cell lines.

Tumor growth was monitored two to three times a week by measuring two perpendicular tumor diameters using calipers. At day *in vivo* tumor experiments, cells were analysed by cytofluorimeter and *in vitro* proliferation assay made.

#### 5.2 Tumor therapy models

On day 0, wild-type tumor cell lines were inoculated s.c. in the left flank (MTD). On day 0, 3 or 6, LAG-3<sup>+</sup> tumor cells were injected into the right flank (MTD or fivefold MTD) to determine the antitumor effects on the nontransfected cells at a distant site. Tumor growth was monitored as described before.

5.3 For cytometric analysis, mice were inoculated s.c. with 5 x MTD tumor cells as described above. Day 8 tumors were dissociated and analyzed with CD3-PE, CD4-PE, CD8-PE, CD80-FITC, CD86-FITC, 2B4-PE (NK-cell), CD22-PE (B-cell) or hLAG-3-FITC mAbs with an Elite cytofluorimeter (Coulter).

5.4 For lymphocyte depletion, mice received i.p. 200  $\mu$ g of purified (18) anti-CD4 (YTS 191.1.2) or anti-CD8 (YTS 169.4.2.1) mAb at day -3, -2, -1, +4 and +8. Wild type or hLAG-3 TS/A tumor cells were inoculated s.c. at day 0 (MTD). Cytofluorimetric analysis of control mice receiving these doses of mAb showed more than 95 % reduction of the target population in spleen (data not shown).

### 6. Studies in vitro

For cytotoxicity assays, tumor specific short term CTL were  
5 generated using mixed lymphocyte tumor cell culture. Briefly,  $3 \times 10^7$  spleen  
cells were collected at day 30 from mice that had rejected established tumors.  
These cells were stimulated with  $3 \times 10^6$  irradiated parental tumor cells in  
complete medium for 4 days and then supplemented with 50 IU/ml of  
10 recombinant hIL-2 (Cetus) for 2 days. The effector functions of splenocytes  
were tested at day 6 in a standard 4h  $^{51}\text{Cr}$  release assay (effector-to-target  
ratios from 25/1 to 200/1) against labelled target cells : autologous tumor cells,  
an H-2<sup>d</sup> irrelevant sarcoma WEHI 164 and a NK sensitive YAC cell line. The  
lysis percentage of triplicates was calculated as [(average experimental cpm -  
15 average spontaneous cpm) / (average maximum cpm - average spontaneous  
cpm)] x 100. We user-defined specific lysis as lysis of mouse splenocytes that  
rejected tumor cells minus the lysis of naive mouse splenocytes.

## RESULTS

20

### EXAMPLE I :

**Surface expression of hCD4, hLAG-3, mLAG-3 and MHC  
molecules on tumor cell lines.**

25 Transfected tumor clones were stained as detailed in section 2.2,  
and analyzed to compare level expression of hCD4, hLAG-3 and mLAG-3. The  
best clone for each construct was used in this study. The following tumor cell  
lines TS/A express high levels of MHC class I molecules and MCA 205  
express low levels of MHC class I. No significant difference was observed  
30 between MHC class I expression on parental tumor cell lines compared to  
transfected clones.

**EXAMPLE II :****Tumor establishment models and vaccination : Comparative effects of hCD4 and hLAG-3.**

5

These experiments were performed to examine the tumorigenicity of cells after gene transfection : MCA 205 and TS/A as shown in figures 1 and 4 and RENCA. The induction of antitumor immunity of LAG-3<sup>+</sup> tumor was compared to parental tumor cell lines.

10

Wild type MCA 205, TS/A and RENCA cells grew progressively when subcutaneously implanted into either syngeneic C57 BL/6 or BALB/C mice or nu/nu mice. Tumor cells stably transfected following hydromycin selection with the hLAG-3 cDNA cloned in a reverse orientation had a similar growth rate.

15

Animals receiving MCA-LAG-3 rejected their tumor. Animals receiving MCA-CD4 exhibited a lower tumor growth than animals receiving wt MCA. Two of them (over 5) completely rejected the tumor (figure 1).

Similar results were obtained with mLAG-3 (data not shown).

20

These results indicate that ectopic expression of hLAG-3, mLAG-3 and hCD4 increases the immunogenicity of MCA sarcoma cell line and prevents tumor formation of MCA transfectant, i.e. it induces a potent immunity against a highly malignant murine tumor.

Similar results are obtained with TS/A tumor cells in BALB/c mice (figure 2).

25

Similar results are further obtained with RENCA tumor cells in syngeneic BALB/C mice.

Thus, antitumor effect is obtained :

30

- in different strains of mice expressing different MHC complex genes ;

- using different tumor cell lines (exhibiting different intrinsic immunogenicity, TS/A < MCA).

Nude mice (nu/nu) were inoculated with wt MCA, MCA hLAG-3 and MCA hCD4 type tumor cells and transfectants grew similarly.

This substantiates the fact that systemic-, long-lasting-, tumor specific-hLAG-3 or hCD4- boosted immunity are T-cell mediated.

5 Mice previously inoculated with wt MCA, MCA hLAG-3 and MCA hCD4 and tumor free after 30 days injection with MTD were rechallenged (1 fold) with fivefold the MTD parental tumor cell line or with an unrelated syngeneic MC 38 colon carcinoma cell line.

The results are represented in figures 2 and 3.

10 After rechallenge, growth of wt MCA was delayed in surviving animals both for animals receiving MCA-LAG-3 cells and MCA-CD4 cells (figure 2).

No such effect was observed in animals rechallenged with irrelevant tumor MC38 (figure 3).

15 This indicates that both ectopic expression of hLAG-3 and hCD4 have an adjuvant-like effect and induces a long-lasting antigen specific immunity against the unmodified parental tumor.

20 **EXAMPLE III :**

**Therapy of wt MCA 205 tumors in C57BL/6 mice with MCA-hLAG-3**

Three groups of five mice each were used for the experiment.

25 Each group was inoculated in one flank with wt MCA and three days later, with either MCA wt (group 1), MCA-hLAG-3 at  $2 \cdot 10^5$  cells (group 2) and MCA-hLAG-3 at  $1 \cdot 10^6$  cells (group 3).

The size of the original tumor was measured in each group over 30 days. The results are represented in figure 5.

30 Injection of MCA-hLAG-3 delayed the tumor growth in a dose dependent manner.

This experiment confirms the systemic effect of LAG-3 on tumor growth and indicate that LAG-3 represents a therapeutic agent against solid tumors.

5

**EXAMPLE IV :****Therapy of wt MCA 205 tumors in C57BL/6 mice with soluble LAG-3.**

10 Three groups of five mice each were simultaneously inoculated with either wt MCA suspended in either PBS (group 1) or PBS containing soluble human LAG-3 (shLAG-3 D1D4) in amounts of 25 µg (group 2) or 250 µg (group 3).

The size of the tumors were measured for each group over a period of 30 days.

15

The results are represented in figure 6.

Co-administration of hLAG-3 D1D4 induced a dose dependent tumor growth retardation.

This demonstrates that systemic administration of soluble hLAG-3 directly induces an inhibition of *in vivo* tumor growth.

20

**EXAMPLE V :*****In vivo* LAG-3 expression on human tumor lymphocytes infiltrating (TILs) renal cell carcinoma (RCC).**

25

30 In human, LAG-3 is expressed in tissues (i.e., inflamed secondary lymphoid organs) but not on the surface of PBMCs (3), even on *in vivo*-activated CD25<sup>+</sup>, CD69<sup>+</sup> PBMCs. LAG-3 is expressed at higher levels on activated MHC class I-restricted CD8<sup>+</sup> cells than on MHC-class II-restricted CD4<sup>+</sup> cells (3) and LAG-3 expression induction by IL-12 or by the more potent combination IL-2 + IL-12 is stronger on CD8<sup>+</sup> than CD4<sup>+</sup> cells. LAG-3 is a weakly expressed activation antigen *in vitro* as well as *in vivo* and it is

sometimes difficult to assess the percentage and/or the specificity of fluorescence labelling in freshly dissociated tumors. Since LAG-3 may interfere with MHC class II<sup>+</sup> APCs in human tumors, we assessed its expression in a series of tumors known to be infiltrated by T-cells, using immunohistochemistry (the APAAP procedure). LAG-3 expression on TILs was detected in all samples tested, including 5 melanomas, 10 renal cell adenocarcinomas and 7 B-cell lymphomas.

Eight patients were investigated for LAG-3 expression on tumor infiltrating lymphocytes in renal cell carcinoma tumors.

Dissociated tumors were used for experiments by cytofluorometric assays. The expression of LAG-3 was studied among the lymphocyte population, determined by its size and granularity. Dead cells were excluded from the study by staining with propidium iodide. TILs were positively stained with 17B4, a monoclonal antibody specific of the epitope of the extraloop of LAG-3.

The results are represented in figure 7 for patients P1-P3 and figure 8 for patients P4 and P5.

For all patients, a shift of the fluorescent peak was observed showing the binding of the 17B4 antibody at the surface of the lymphocytes.

Thus, in all patients, TILs actually expressed LAG-3 with a significant percentage (30 %) of RCC-TIL in unrelated patients.

In all samples CD3<sup>+</sup> T-cells were found to express LAG-3 (range 11 % - 48 %) with higher expression on CD8<sup>+</sup> T-cells.

In contrast, peripheral blood mononuclear cells were LAG-3<sup>-</sup> in these patients, showing that LAG-3 expression on lymphocytes is a phenomenon related to T-cell activation in tumors.

Furthermore by using an ELISA assay, high concentrations (about 1 ng/ml) of soluble LAG-3 were found in blood of patients with cancer.

These data show that LAG-3 is a molecule involved in the naturally occurring antitumoral response in human and support the use of LAG-3 to boost the immunosurveillance of tumor cells in human.

**EXAMPLE VI :*****CD8<sup>+</sup> T-cells mediate the primary rejection.***

5 Rejection of hLAG-3 MCA 205 and TS/A transfectants was dependent on T-lymphocytes, as no rejection was observed in T-cell-deficient *nu/nu* mice. Following the injection of 5 x MTD of these cells, 10 mm diameter tumors were excised at day 8 and both dissociated tumor cells and tumor infiltrating lymphocytes were analyzed by FACS. The wt tumors as well as the  
10 LAG-3 transfectants, initially CD80<sup>-</sup> and CD86<sup>-</sup>, had remained negative for these markers following inoculation while LAG-3 was constantly detected on hLAG-3 tumors using the anti-LAG-3 17B4 mAb (data not shown). On day 8-explanted tumors, the percentage of CD8<sup>+</sup> cells was about 31 % in hLAG-3 MCA 205 tumors versus 4 % in wt MCA 205 tumors (Fig. 9a), whilst no  
15 difference was observed when analyzing the CD4<sup>+</sup>, B or NK cell subsets. Similar results were obtained for TS/A tumors (data not shown). Finally, the respective contribution of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells to the anti-tumor response was examined by depleting mice of these cell subsets. As shown in Fig. 9b, administration of the CD8-specific mAb at the time of hLAG-3/TS/A MTD  
20 inoculation abrogates the rejection of hLAG-3/TS/A tumor cells. Participation of CD4<sup>+</sup> helper T-cells is suggested by the partial effect observed with CD4-specific mAb (Fig. 9b).

25

**EXAMPLE VII :*****Tumor-specific CTL response is enhanced by hLAG-3***

To further address the mechanism behind the anti-tumor activity of LAG-3, we evaluated its effect on the generation of cytotoxic T lymphocyte (CTL) - killing of the nonimmunogenic TS/A cells. Splenocytes were harvested  
30 from hLAG-3/TS/A tumor-implanted mice which were able to reject 5 x MTD wt tumor cells in rechallenge experiments and were restimulated *in vitro* for 6

days with irradiated TS/A cells. CTL activity was detected in splenocytes of hLAG-3 tumor cell-implanted mice (Fig. 9c), while splenocytes from naive animals displayed no cytotoxic activity (data not shown). CTL activity appears to be selective for the nonimmunogenic TS/A cells since syngeneic WEHI sarcoma cells, as well as NK-sensitive YAC cells, were not lysed (Fig. 9c).

**EXAMPLE VIII:**

**Therapy of established tumors**

10

The inventors have shown that the control of tumor growth could be achieved by using a soluble LAG- molecule as a vaccine adjuvant. A single injection of a mixture of the antigen (irradiated tumor cells) plus the booster (mLAG-3lg, 1  $\mu$ g or 0.1  $\mu$ g) was efficient (Fig. 10).

15

It is assumed that *in vivo* soluble LAG-3 molecules could signal the Langerhans cells (or any APC present at the vaccine site) via MHC class II molecules to prime efficiently CD8<sup>+</sup> T-cells.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Use of a MHC class II ligand able to boost an antigen specific immune response in combination with an antigen  
5 able to induce said antigen-specific immune response,  
for the manufacture of a medicament for preventing or  
treating pathological conditions involving said antigen  
specific immune response, wherein the MHC class II  
ligand is LAG-3, or derivatives, mutants or soluble  
10 fragments of LAG-3.
2. A use according to claim 1 wherein the antigen specific  
immune response is a T cell mediated immune response.
- 15 3. A use according to claim 2, wherein the T cell mediated  
immune response is CD8+ T cell mediated.
4. A use according to claim 3, wherein the conditions  
involving a cell immunity depending on CD8+ T cells are  
20 infectious diseases.
5. A use according to claim 4, wherein the infectious  
diseases are viral, parasitic or bacterial diseases.
- 25 6. A use according to any one of claims 1 to 5, for  
treating cancer.
7. A use according to any of claims 1 to 6, wherein the  
soluble LAG-3 fragments are selected from the group  
30 consisting of D1-D2 and D1-D4 fragments of LAG-3.
8. A use according to any of claims 1 to 7, wherein the  
medicament comprises the MHC class II ligand in the form

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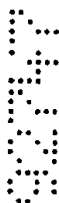
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of transfected cells expressing the ligand or in the form of a soluble molecule of that ligand.

9. A use according to any of claim 1 to 7, wherein the  
5 medicament comprises the MHC class II ligand as a naked  
plasmid incorporating a DNA sequence encoding LAG-3.
10. A use according to claim 9, wherein the plasmid  
further contains DNA encoding the antigen, against which  
10 an immune response is sought.
11. Use of LAG-3 for the manufacture of a medicament for  
anti-cancer immunotherapy.
- 15 12. A pharmaceutical composition comprising an effective  
amount of an antigen able to boost an antigen-specific  
immune response in combination with an effective amount  
of a MHC class II ligand, able to boost an antigen  
specific immune response, wherein the MHC class II  
20 ligand is LAG-3, or derivatives, mutants or soluble  
fragments of LAG-3.
13. A pharmaceutical composition according to claim 12,  
wherein the antigen-specific immune response is a T-cell  
25 mediated immune response.
14. A pharmaceutical composition according to claim 12 or  
claim 13, wherein said MHC class II ligand is hLAG-3.
- 30 15. A pharmaceutical composition according to claim 14,  
wherein the hLAG-3 is human soluble LAG-3.



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16. Use of tumor cells transfected with a DNA coding for at least one MHC class II ligand for the manufacture of a medicament according to any one of claims 1 to 8, wherein the MHC class II ligand is LAG-3, or derivatives thereof.

5

17. A process for preparing cells transfected with a DNA coding for at least one MHC class II ligand, the MHC class II ligand being LAG-3 or derivatives thereof, comprising the steps of removing cells from a patient, transfecting  
10 said cells with at least one MHC class II ligand, the MHC class II ligand being LAG-3 or derivatives thereof, and recovering the transfected cells.

18. A process according to claim 17, wherein the cells  
15 removed from the patient are tumor cells.

19. A method of preventing or treating pathological conditions involving an antigen specific immune response boosted by MHC class II ligand comprising the step of  
20 administering to a patient in need thereof an effective amount of said MHC class II ligand in combination with an antigen able to induce said antigen-specific immune response.

20. Use of a MHC class II ligand according to claim 1, substantially as herein described with reference to any of the examples or figures.

21. Use of LAG-3 according to claim 11, substantially as  
30 herein described with reference to any of the examples or figures.



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22. A process for preparing cells according to claim 17, substantially as herein described with reference to any of the examples or figures.

5

Dated this 15<sup>th</sup> day of July 2002

INSTITUT GUSTAV ROUSSY and

APPLIED RESEARCH INSTITUTE ARS HOLDING N.V.

By their Patent Attorneys

10 GRIFFITH HACK

Fellows Institute of Patent and

Trade Mark Attorneys of Australia

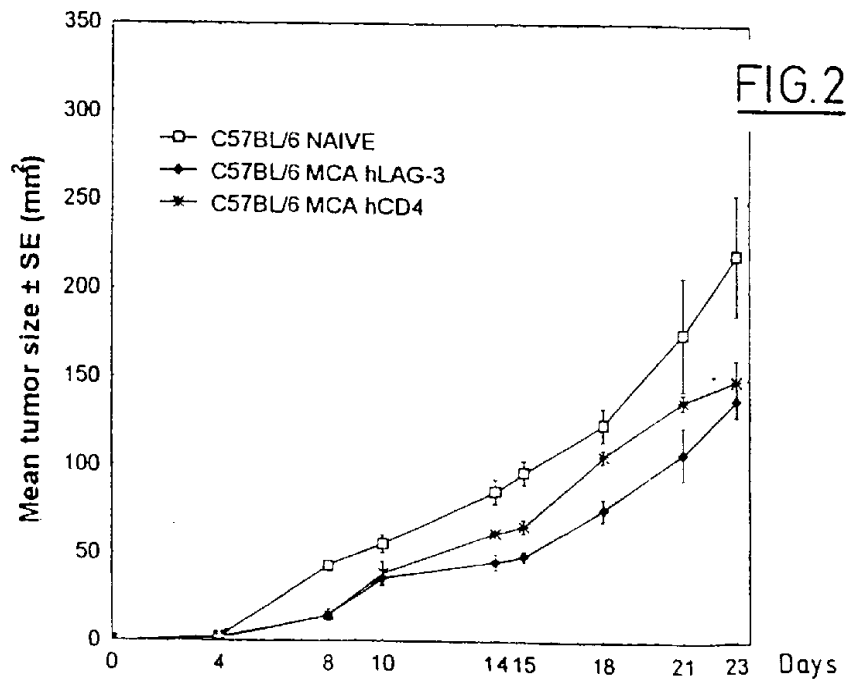
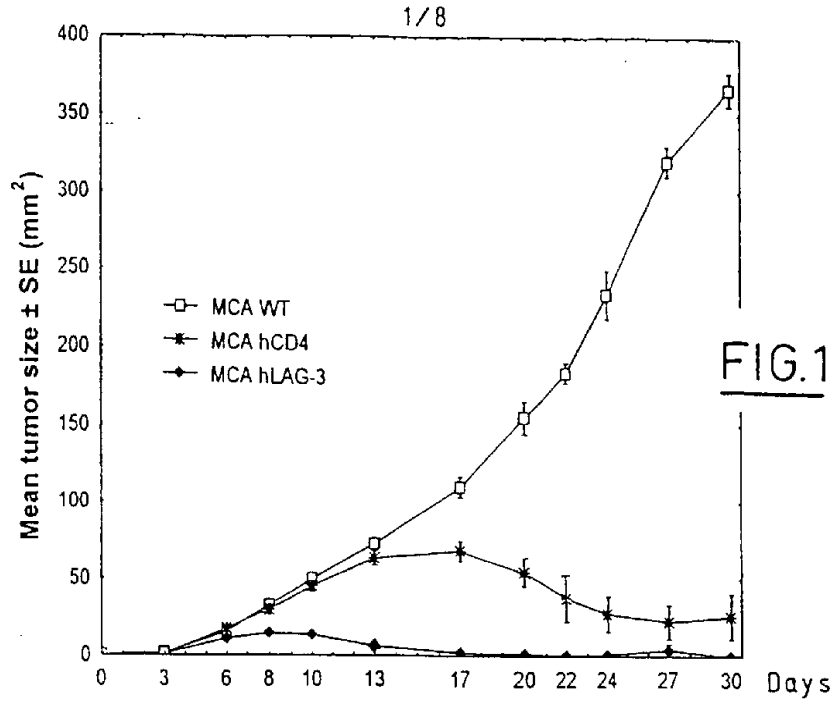
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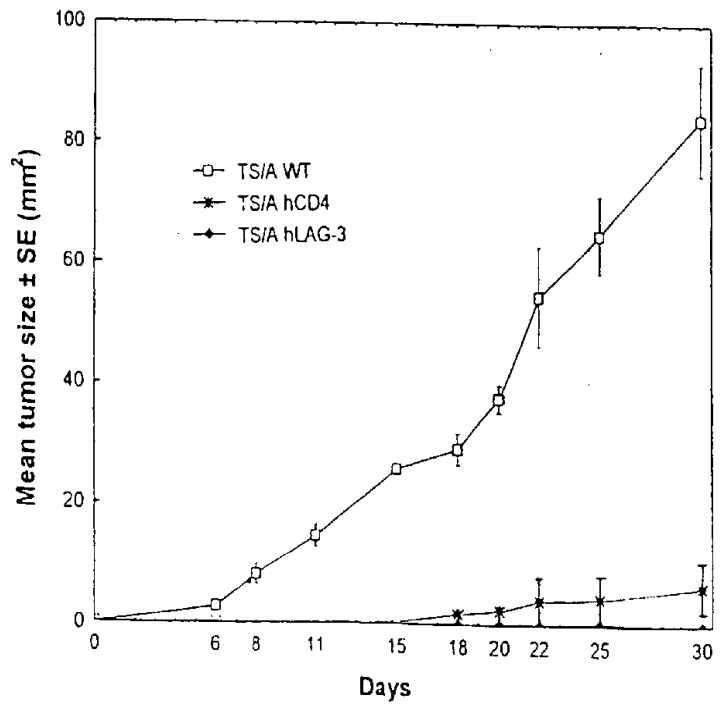
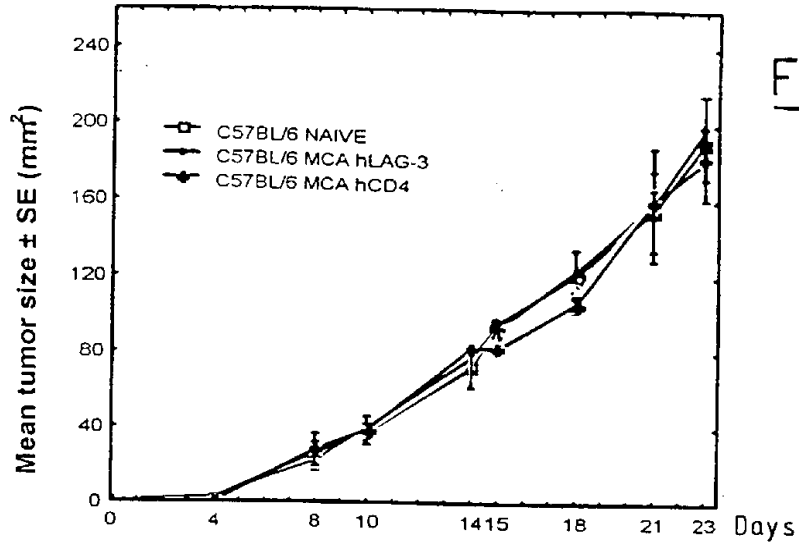
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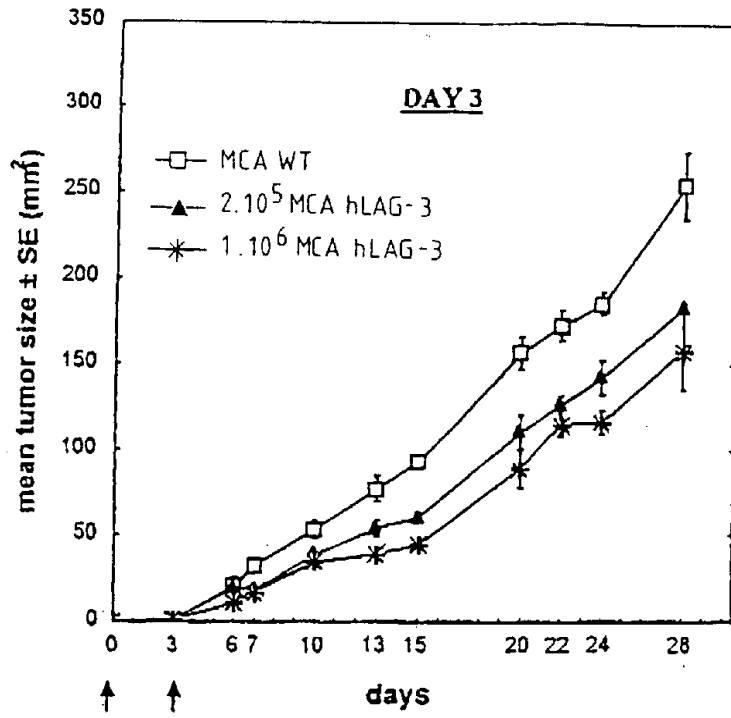
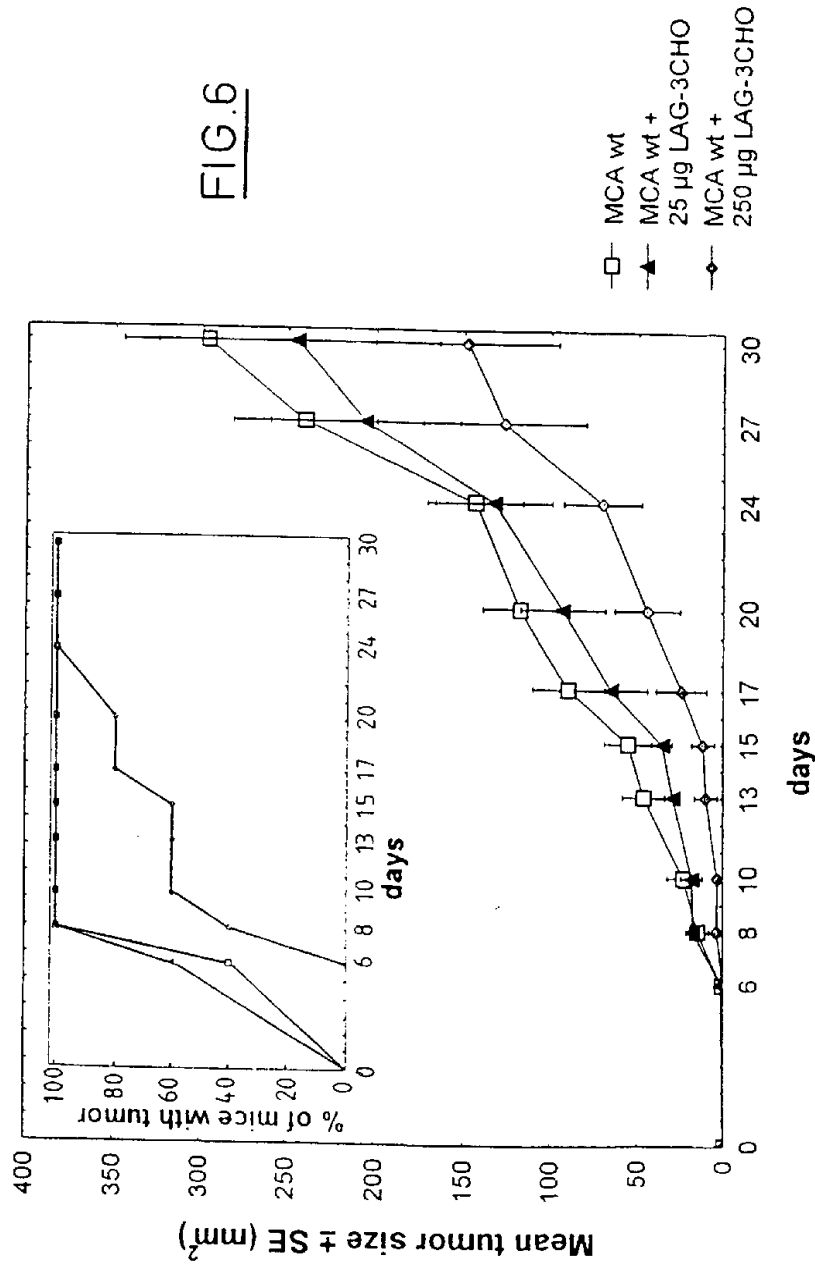
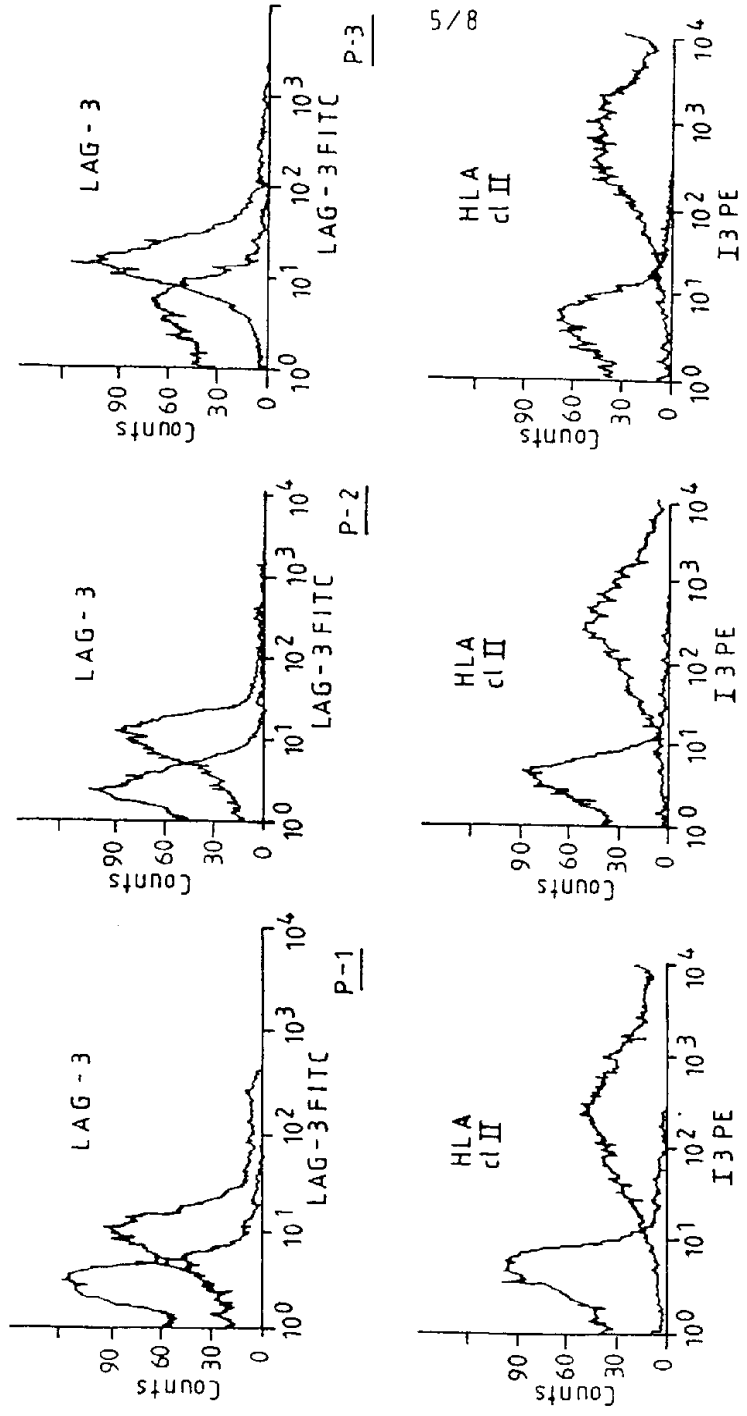


FIG. 5

FIG. 6





**FIG.7**

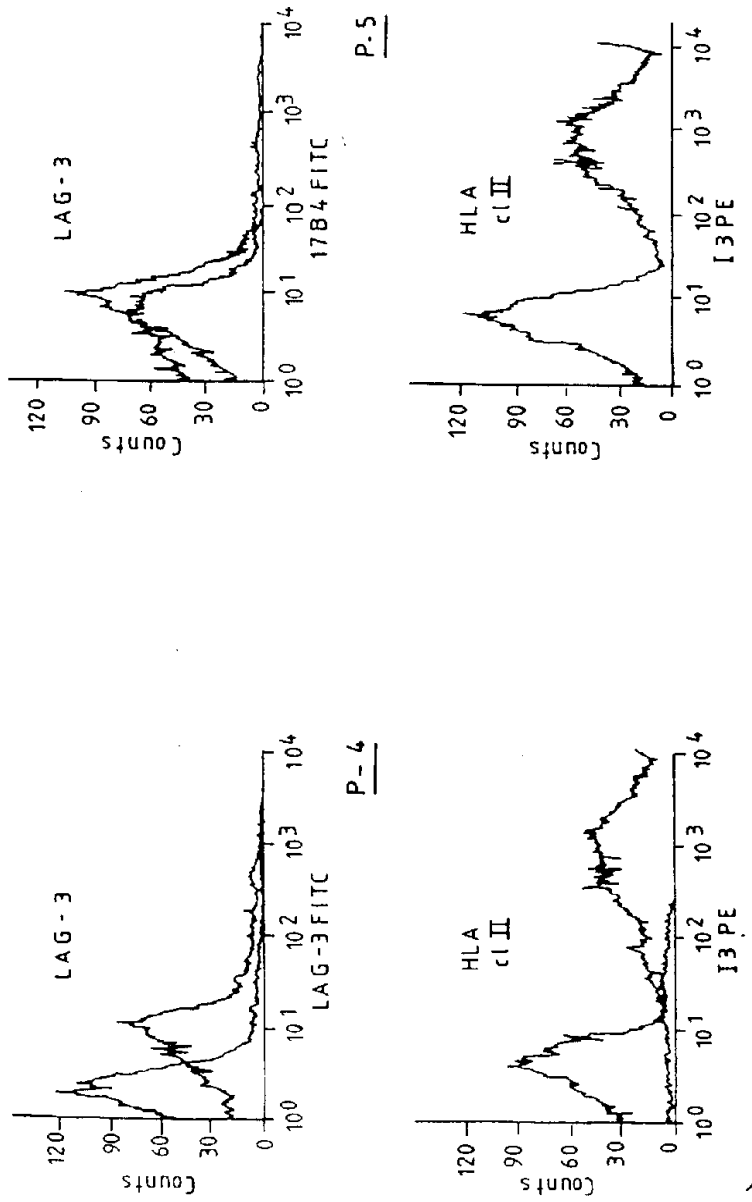
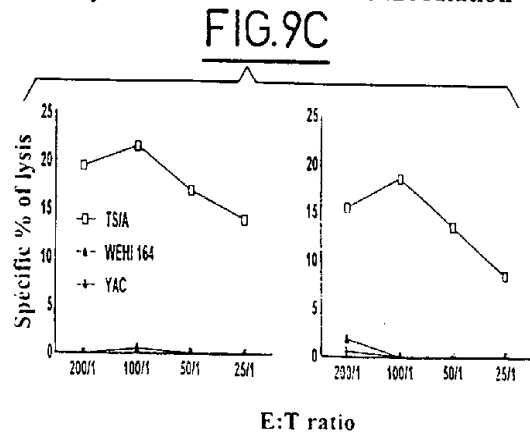
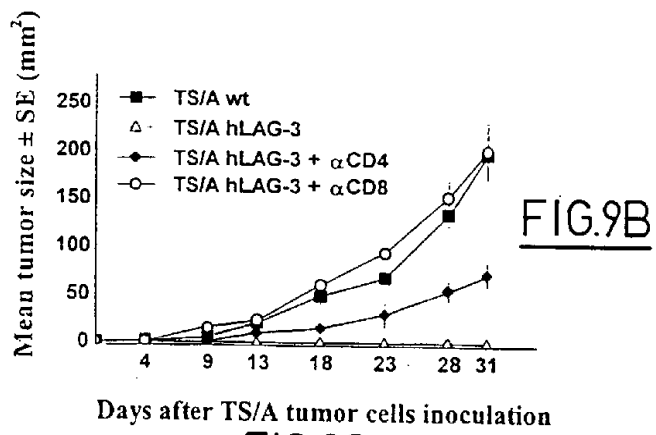
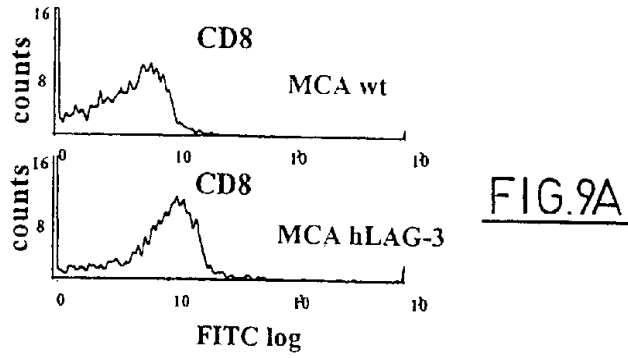
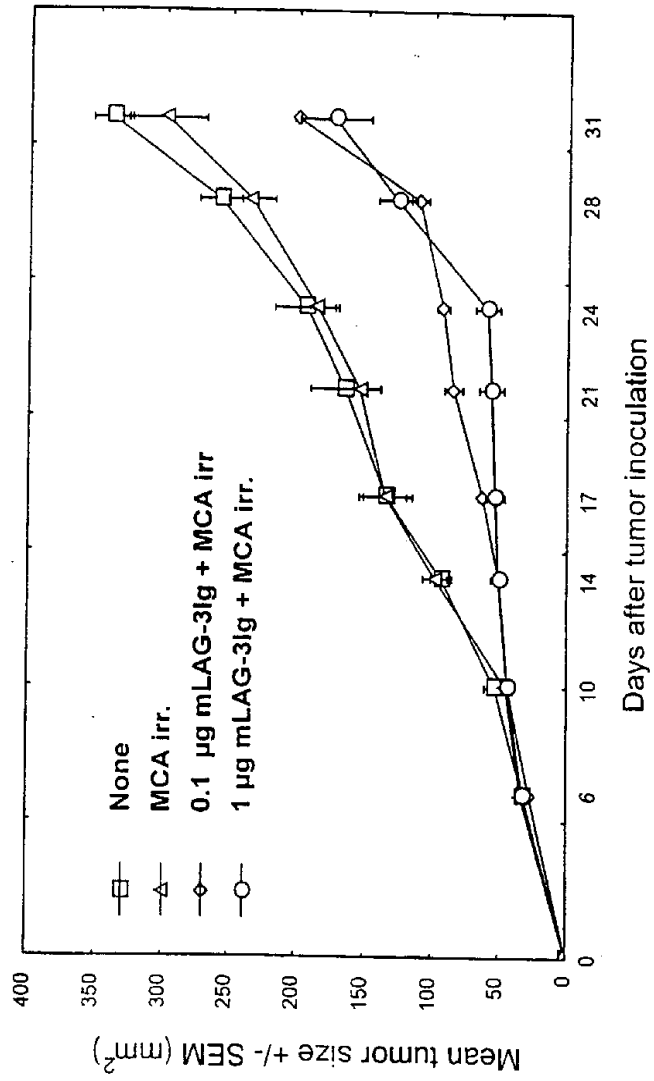


FIG. 8





**FIG.10**