

COMPOSITION AND THERAPEUTIC ANTI-TUMOUR VACCINE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to US provisional patent application
5 60/954,917, filed August 9, 2007, and French patent application, FR 07
05767, filed August 8, 2007, both herein incorporated by reference.

The present invention relates to a composition which induces, in a
host, a cytotoxic response for anti-tumour purposes, and also to a
therapeutic anti-tumour vaccine containing this composition.

10 The natural immune response against tumour antigens is relatively
ineffective and various mechanisms allowing tumours to escape the anti-
tumour immune response have been identified. Conventional vaccine
approaches generate humoral responses which prove to be insufficient.

Strategies aimed at developing a cytotoxic response based on
15 antigen-presenting cells (APCs), in particular based on dendritic cells, have
been studied. The principle consists in specifically destroying the cancer
cells of a patient by stimulating said patient's own immune defences.

Dendritic cells are antigen-presenting cells (APCs) that are very
effective in generating cytotoxic effectors specific for tumour cells. They are
20 capable of phagocytizing apoptotic cells or apoptotic bodies originating from
tumour cells, and then of presenting the tumour antigens, in association with
MHC class I and II molecules, to T lymphocytes. Thus, the dendritic cell is
capable of initiating the proliferation and the generation of a clone of
specific cytolytic T lymphocytes. At the end of this reaction, the killer
25 lymphocytes thus differentiated leave the lymphoid compartment so as to
circulate in the organism and bind in the tumour. Recognition of the
antigens expressed by a tumour then induces a lytic signal and brings about
the destruction of the tumour cells.

Several anti-cancer vaccine strategies targeting dendritic cells have
30 been studied (Eymard JC, Bernard J, Bull Cancer. 2003, 90(8-9):734-43).
Some are based on manipulating the dendritic cells *in vitro*, and others are

based on stimulating the dendritic cells *in vivo*. In the first case, the dendritic cells are differentiated from blood cells taken from the patient: they are cultured and matured, "pulsed", i.e. stimulated, *ex vivo* with tumour peptides, tumour lysates, apoptotic tumour cells, or heat shock proteins extracted from autologous tumours, and finally reinjected into the patient. In the second case, the stimulation of the dendritic cells is carried out after injection, into the patient, of peptides, proteins, irradiated tumour cells or else viruses containing the antigenic peptide targeting the dendritic cells. However, the cytotoxic response obtained is rarely accompanied by clinical efficacy. The activation of the dendritic cell "conditions" its ability to effectively activate the cytolytic T lymphocyte. The level of activation of dendritic cells appears to be a delicate point in this vaccine strategy.

The use of dendritic cells *ex vivo* for obtaining an anti-tumour vaccine raises a certain number of issues on: the state of maturation of the dendritic cells, the number of cells to be injected, the route, the site and the frequency of injection for producing dendritic cells capable of migrating to the secondary lymphoid organs and of inducing an effective cytotoxic T response (Banchereau J, Schuler-Thurner B, Palucka AK, Schuler G. [Dendritic cells as vectors for therapy] *Cell*. 2001, 10, 106(3): 271-4.).

The activation of dendritic cells *in vivo* is, for its part, limited by the weak immunogenic capacity of tumour antigens and the difficulty in activating dendritic cells at a sufficient level.

The use of red blood cells as carriers for transporting antigens, encapsulated in the red blood cells or bound to their surface, and delivered to the APCs has been envisaged in several publications. The triggered immune responses have been investigated *in vitro* and *in vivo*.

Hamidi et al. have recently described the encapsulation of BSA (bovine serum albumin) as a model of an antigen in human red blood cells (Hamidi M et al., *Drug Deliv.*, 2007; 14(5):295-300 and *Int J Pharm.*, 2007, 29, 338(1-2): 70-8). The authors suggested the use of red blood cells as a vector for the presentation of antigens to APCs of the reticuloendothelial system (RES). In another review published by Hamidi et al. (*J. Control.*

Release, 2007, 118(2): 145-60), the authors indicated that a certain number of strategies have been studied to promote targeting of the RES, said targeting being promoted by ageing of the red blood cells, leading to their uptake for lysis. Other routes were mentioned, such as exposure of red blood cells to stabilizing agents, in particular crosslinking agents, coating of red blood cells with anti-RH antibodies, of IgG type to target the spleen or of IgM type to target the liver, heat shock, or exposure to oxidizing agents, enzymes or antibiotics.

A humoral immune response can be obtained *in vivo* after immunization with antigen-loaded red blood cells. The study carried out by Murray et al. made it possible to detect, in mice, IgG immunoglobulins after intravenous injection of murine red blood cells loaded with one of the following four antigens: KLH (Keyhole Limpet Haemocyanin), BSA (Bovine Serum Albumin), CTB (Cholera Toxin b Subunit) and ADA (Bovine Adenosine Deaminase). Detection of IgG1 and IgG3, which are predominant immunoglobulin isotypes during a Th2 response, and of IgG2, immunoglobulin isotype which is a marker for a Th1 response, would suggest the involvement of both types of immune responses, the humoral response and the cellular response (Murray AM et al., Vaccine., 2006 28, 24(35-36): 6129-39).

Another formulation of antigens used with red blood cells has been tested by Dominici et al. The Tat protein of the HIV-1 virus was anchored to the surface of mouse red blood cells by means of avidin/biotin coupling. The immunization of mice by intraperitoneal injection with this antigen formulation, internalized by dendritic cells, triggered a humorally-mediated immune response *in vivo*. Isotype characterization of the immunoglobulins detected indicates the induction of Th1 and Th2 responses. The anti-Tat cytotoxic activity was shown *in vitro*, by the conventional chromium-release technique, for the mice treated with red blood cells coupled to the antigens (Dominici S et al., Vaccine., 2003 16, 21(17-18): 2073-81).

A cellular response has been demonstrated *in vitro* by Corinti et al. with the same formulation. Phagocytosis of the red blood cells conjugated

with Tat protein, by dendritic cells derived from human monocytes, was shown, as was the induction of CD4+ and CD8+ responses. Moreover, maturation of dendritic cells in the presence of interferon gamma promoted the type I immune response (Corinti S. et al., Leukoc. Biol. 2002, 71(4): 652-
5 8).

Boberg et al. injected mice intraperitoneally with a vaccine constituted of peptides derived from the HIV-1 protease anchored at the surface of mouse red blood cells by means of the avidin/biotin system. They chemically modified red blood cells with the aim of promoting their
10 recognition by APCs, but a weak immune reaction was obtained. They concluded that the small amount of antigens delivered, due to the limited loading of red blood cells with antigenic peptides and blood volume injected was not compensated for by the chemical modification of the carriers supposed to promote antigen recognition by APCs (Boberg A. et al., Infect.
15 Agents Cancer. , 2007, 182: 9).

The present invention aims to provide compositions and vaccines that can be used in the treatment of cancers, according to an immunotherapy approach.

An object of the invention is therefore a composition which induces,
20 in a host, a cytotoxic cellular response directed against tumour cells, and which comprises red blood cells containing a tumour antigen.

The term "host" refers preferably to humans, but also to animals, in particular pets (especially dogs or cats) and animals for sport (especially horses).

25 According to the invention, the red blood cells contain, i.e. encapsulate, the antigen, which means that the antigen is or is essentially inside the red blood cell.

Said red blood cells are preferably designed, selected or modified so as to promote phagocytosis thereof by APCs, and most particularly by
30 dendritic cells. In particular, said red blood cells are designed, selected or modified so as to promote phagocytosis thereof in the spleen and the liver, the essential objective being to target APCs of the spleen.

In a preferred embodiment, the compositions according to the invention comprise red blood cells which contain the antigen and target the spleen. The composition promotes phagocytosis of these red blood cells by the APCs, in particular the dendritic cells, in the spleen.

5 According to a first embodiment, the red blood cells contain a tumour antigen and are in the form of an immune complex with an immunoglobulin which recognizes an epitope at the surface of said red blood cells, so as to promote the phagocytosis of said red blood cells, in particular by dendritic cells. The composition also makes it possible to promote phagocytosis by
10 macrophages. Preferably, the immunoglobulin is an immunoglobulin G.

 The formation of the immune complex involves red blood cells and at least one antibody, preferably of IgG subtype. Dendritic cells have, at their surface, receptors for the constant Fc region of immunoglobulins G (IgGs). These receptors are capable of triggering phagocytosis or internalization of
15 the antigen-IgG immune complexes thus formed and of promoting antigen presentation by MHC class I and II molecules, resulting in the generation of CD4⁺ helper lymphocytes and especially CD8⁺ cytotoxic lymphocytes (A. Regnault et al., J. Exp. Med., Janvier 1999, 189(2): 371-80).

 As antibodies which are suitable, mention may be made of anti-
20 rhesus antibodies, anti-glycophorin A antibodies and anti-CR1 antibodies (CR1 = complement receptor type 1). Anti-glycophorin A antibodies (A. Bigbee et al., Mol. Immunol., December 1983, 20(12): 1353-62) are a preferred modality.

 Preferably, the red blood cells of human origin that are used to form
25 an immune complex are heterologous red blood cells originating from a donor.

 According to a second embodiment, the red blood cells contain a tumour antigen and are heat or chemically modified, so as to promote phagocytosis of said red blood cells, in particular by dendritic cells. The
30 composition also makes it possible to promote phagocytosis by macrophages.

 The heat treatment is in particular carried out under the following

conditions: heating of red blood cells for about 15 minutes to about 90 minutes, preferably from about 25 to about 50 minutes, at a temperature of between about 42 and about 55°C, preferably between about 47 and about 51°C. Typically, red blood cells are heated for about 30 minutes at
5 between about 48 and about 50°C, for example at about 48°C.

The chemical treatment is carried out using agents which modify the surface of red blood cells, and in particular bridging or crosslinking agents such as bis(sulphosuccinimidyl) suberate (BS3 or BS³), glutaraldehyde or neuraminidase.

10 In a particular embodiment, at least two methods of targeting are combined, and, for example, the composition then comprises antigen-containing red blood cells which are in the form of an immune complex and are heat or chemically treated so as to promote their uptake in the spleen and/or the liver, preferably the spleen, and phagocytosis by APCs, in
15 particular by dendritic cells.

In a third embodiment, the antigen-containing red blood cells are xenogenic. Injection of xenogenic red blood cells into humans results in the binding of the patient's natural antibodies to the injected red blood cells. The immune complex thus formed promotes phagocytosis by APCs, in
20 particular by dendritic cells. Preferably, said red blood cells are of porcine origin.

In a particular embodiment, the xenogenic red blood cells are heat or chemically modified so as to promote phagocytosis thereof.

The composition according to the invention may comprise one or
25 more tumour antigens. When there are several tumour antigens, said tumour antigens are preferably selected so as to induce an immune response against one type of tumour or of tumour cells.

The composition preferably comprises at least two tumour antigens representative of the tumour to be treated. The objective is to generate
30 several clones of cytotoxic T lymphocytes which each recognizes a specific antigenic peptide so as to develop a more effective immune response.

In a particular embodiment, the composition comprises at least two

populations of red blood cells, each encapsulating a different antigen.

The most well known antigens that can be used herein are indicated in the tables below, where they are classified by category.

5 Unique antigens:

<i>Gene/protein</i>	<i>Tumour</i>
alpha-actinin-4	Lung carcinoma
ARTC1	Melanoma
BCR-ABL fusion protein (b3a2)	Chronic myeloid leukaemia
B-RAF	Melanoma
CASP-5	Colorectal, gastric and endometrial carcinoma
CASP-8	Head and neck squamous cell carcinoma
beta-catenin	Melanoma
Cdc27	Melanoma
CDK4	Melanoma
CDKN2A	Melanoma
COA-1	Colorectal carcinoma
dek-can fusion protein	Myeloid leukaemia
EFTUD2	Melanoma
Elongation factor 2	Squamous cell carcinoma of the lung
ETV6-AML1 fusion protein	Acute lymphoblastic leukaemia
FN1	Melanoma
GNMB	Melanoma
LDLR-fucosyltransferaseAS fusion protein	Melanoma
HLA-A2d	Renal cell carcinoma
HLA-A11d	Melanoma
hsp70-2	Renal cell carcinoma
KIAA0205	Bladder tumour
MART2	Melanoma
ME1	"Non small cell" lung carcinoma
MUM-1f	Melanoma
MUM-2	Melanoma
MUM-3	Melanoma
neo-PAP	Melanoma
Myosin class I	Melanoma
NFYC	Squamous cell carcinoma of the lung
OGT	Colorectal carcinoma
OS-9	Melanoma
pml-RARalpha-fusion protein	Promyelocytic leukaemia

PRDX5	Melanoma
PTPRK	Melanoma
K-ras	Pancreatic adenocarcinoma
N-ras	Melanoma
RBAF600	Melanoma
SIRT2	Melanoma
SNRPD1	Melanoma
SYT-SSX1 or -SSX2 fusion protein	Sarcoma
Triosephosphate Isomerase	Melanoma
FLT3-ITD	Acute myeloid leukaemia
p53	Head and neck squamous carcinoma

Antigens common to several tumours:

a) Tumour-specific antigens

<i>Genes</i>
BAGE-1
GAGE-1,2,8
GAGE-3,4,5,6,7
GnTVf
HERV-K-MEL
KK-LC-1
KM-HN-1
LAGE-1
MAGE-A1
MAGE-A2
MAGE-A3
MAGE-A4
MAGE-A6
MAGE-A9
MAGE-A10
MAGE-A12
MAGE-C2
mucin k
NA-88
NY-ESO-1 / LAGE-2
SAGE
Sp17
SSX-2
SSX-4
TRAG-3
TRP2-INT2g

b) Differentiation antigens

<i>Gene / protein</i>	<i>Tumour</i>
CEA	Intestinal carcinoma
gp100 / Pmel17	Melanoma
Kallikrein 4	Prostate
mammaglobin-A	Breast cancer
Melan-A / MART-1	Melanoma
NY-BR-1	Breast cancer
OA1	Melanoma
PSA	Prostate carcinoma
RAB38 / NY-MEL-1	Melanoma
TRP-1 / gp75	Melanoma
TRP-2	Melanoma
tyrosinase	Melanoma

c) Overexpressed antigens

<i>Gene</i>	<i>Tissue expression</i>
adipophilin	adipocytes, macrophages
AIM-2	Ubiquitous (low level)
BING-4	Ubiquitous (low level)
CPSF	Ubiquitous (low level)
cyclin D1	Ubiquitous (low level)
Ep-CAM	Epithelial cells
EphA3	Numerous
FGF5	Brain, kidneys
G250 / MN / CAIX	Stomach, liver, pancreas
HER-2 / neu	Ubiquitous (low level)
IL13Ralpha2	
Intestinal carboxyl esterase	liver, intestine, kidneys
alpha-foetoprotein	Liver
M-CSF	liver, kidneys
mdm-2	ubiquitous (brain, muscle, lungs)
MMP-2	Ubiquitous
MUC1	Glandular epithelium
p53	Ubiquitous (low level)
PBF	ovaries, pancreas, spleen, liver
PRAME	Testes, ovaries, endometrium, adrenal glands
PSMA	prostate, CNS, liver
RAGE-1	Retina
RNF43	

RU2AS	testes, kidneys, bladder
secernin 1	Ubiquitous
SOX10	Ubiquitous (low level)
STEAP1	Prostate
survivin	Ubiquitous
Telomerase	testes, thymus, bone marrow, lymph nodes
WT1	testes, ovaries, bone marrow, spleen
BCLX (L)	Ubiquitous (low level)
DKK1	Testes, prostate, mesenchymatous stem cells
ENAH (hMena)	Breast, prostate, colon-rectum stroma and epithelium, pancreas, endometrium
MCSP	Endothelial cells, chondrocytes, nonstriated muscle cells
RGS5	Heart, skeletal muscle, pericytes

Van der Bruggen et al. have produced a database referencing all human tumour antigens recognized by T lymphocytes and usable in cancer immunotherapy approaches according to the invention:

- 5 <http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>.

Other antigens may be used in the invention such as: Gastrin 17, Human Chorionic Gonadotropin, EGFRvIII, HER2, HER2/neu, P501, Guanylyl Cyclase C, PAP.

The word antigen encompasses antigens of natural or synthetic or
10 artificial origin, antigens originating from the patient to be treated, antigenic fragments, derivatives or variants, so long as the antigen is able to initiate the appropriate immune response. The antigens may be for example extracted, chemically synthesized or produced via genetic engineering.

In order to generate an effective immune response, dendritic cells
15 have to be active and mature, they have to produce cytokines and chemokines and to express costimulation molecules that are necessary for the recruitment and activation of T lymphocytes.

Various types of adjuvants can be used to stimulate APCs, and in particular dendritic cells. Bacterial or viral RNA or DNA, heat shock proteins
20 (HSPs), sugars, immune complexes and cytokines are various factors which induce APC maturation, and in particular dendritic cell maturation mediated by the stimulation of specific receptors (Toll-like receptor TLR, mannose

receptor). Macrophages and dendritic cells do not all express the same receptors at their surface. The choice of the adjuvant therefore relates to a molecule capable of generating a cytotoxic immune response in humans and for which the receptor is found at the surface of the cells taking up the
5 red blood cells of the invention, and therefore in particular at the surface of dendritic cells.

According to one embodiment, the adjuvant is a molecule encapsulated inside red blood cells or attached to the surface of red blood cells. These are preferably the red blood cells containing the antigen.

10 Alternatively, the adjuvant is encapsulated in, or attached to the surface of, other red blood cells treated separately. These red blood cells may also be modified or selected so as to promote phagocytosis thereof by APCs, in particular by dendritic cells. They may thus be, as previously described, in the form of an immune complex, or thermally or chemically
15 modified, or else be xenogenic.

According to another embodiment, the adjuvant is a separate adjuvant composition, that can be administered simultaneously with or separately from the red blood cells containing the antigen.

It goes without saying that, as long as the adjuvant can be in a
20 separate composition (red blood cell composition or adjuvant composition), the adjuvant can be administered concomitantly with the red blood cells containing the antigen, as a separate administration or in the form of a mixture, or administered separately, for example after the administration of the red blood cells comprising the antigen, in particular a few hours or days
25 apart.

Among the adjuvants that can be used, mention may first and foremost be made of the preferred adjuvants below

- TLRs (Toll-like receptors) ligands, in particular imidazoquinolones, such as preferably: imidazoquinoline, e.g. imidazoquinoline CL097, imiquimod, resiquimod; CpG oligodeoxynucleotides; LPSs (lipopolysaccharides); poly(inosinic acid)-(polycytidylic acid poly(I:C));
30
- cytokines, in particular: interferon alpha, IL-2 (interleukin 2), IFN γ

(interferon gamma), GM-CSF (Granulocyte Monocyte-Colony Stimulating Factor), IL-12 (interleukin 12), TNF α (Tumour Necrosis Factor alpha).

Among the other adjuvants that can be used, mention may in particular be made of:

- 5 - bacterial constituents, in particular BCG (Bacillus Calmette Guerin), MDP (Muramyl dipeptide), TDM (Trehalose dimycolate), LPS (lipopolysaccharide), MPL (monophosphoryl lipid A);
- mineral adjuvants, in particular: aluminium hydroxide, aluminium phosphate, potassium phosphate and calcium phosphate;
- 10 - bacterial toxins, in particular: CT (cholera toxin from *Vibrio cholera*), CTB (cholera toxin from *Vibrio cholera*), PT (pertussis toxin from *Bordetella pertussis*) LT (thermolabile lymphotoxin from *Escherichia coli*);
- KLH, Keyhole limpet haemocyanin.

Techniques for encapsulating active ingredients in red blood cells are
15 known and the basic technique by lysis-resealing, which is preferred herein, is described in patents EP-A-101 341 and EP-A-679 101, to which those skilled in the art may refer. According to this technique, the primary compartment of a dialysis element (for example, dialysis tubing or a dialysis cartridge) is continuously fed with a suspension of red blood cells, while the
20 secondary compartment contains an aqueous solution which is hypotonic with respect to the suspension of red blood cells, in order to lyse the red blood cells; next, in a resealing unit, the resealing of the red blood cells is induced in the presence of the tumour antigen by increasing the osmotic and/or oncotic pressure, and then a suspension of red blood cells
25 containing the tumour antigen is collected.

Among the variants described up until now, preference is given to the method described in French Patent Application No. 0408667, which makes it possible to efficiently, reproducibly, safely and stably encapsulate the tumour antigen. This method comprises the following steps:

- 30 1 – suspension of a red blood cell pellet in an isotonic solution at a haematocrit level greater than or equal to 65%, cooling between + 1 and + 8°C,

2 – measurement of the osmotic fragility using a sample of red blood cells from said red blood cell pellet,

it being possible for steps 1 and 2 to be carried out in any order (including in parallel),

5 3 – lysis and internalization process of the tumour antigen, inside the same chamber, at a temperature constantly maintained between + 1 and + 8°C, comprising passing the suspension of red blood cells at a haematocrit level greater than or equal to 65%, and a hypotonic lysis solution cooled to between + 1 and 8°C, through a dialysis cartridge; and the lysis parameters
10 being adjusted according to the osmotic fragility previously measured; and

4 – resealing process carried out in a second chamber, inside which the temperature is between + 30 and + 40°C, and in the presence of a hypertonic solution.

The “internalization” is intended to mean penetration of the tumour
15 antigen inside the red blood cells.

In particular, for the dialysis, the red blood cell pellet is suspended in an isotonic solution at a high haematocrit level, greater than or equal to 65%, and preferably greater than or equal to 70%, and this suspension is cooled to between + 1 and + 8°C, preferably between + 2 and 6°C, typically
20 in the region of + 4°C. According to a specific embodiment, the haematocrit level is between 65% and 80%, preferably between 70% and 80%.

The osmotic fragility is advantageously measured on the red blood cells just before the lysis step. The red blood cells or the suspension containing them are (is) advantageously at a temperature close to or
25 identical to the temperature selected for the lysis. According to another advantageous feature of the invention, the osmotic fragility measurement is exploited rapidly, i.e. the lysis process is carried out shortly after the sample has been taken. Preferably, this period of time between taking the sample and beginning the lysis is less than or equal to 30 minutes, more preferably
30 still less than or equal to 25, and even less than or equal to 20 minutes.

As regards the manner in which the lysis-resealing process is carried out, with the osmotic fragility being measured and taken into account, those

skilled in the art may refer to French Patent Application No. 0408667 for further details.

According to one feature of the invention, the composition according to the invention comprises, at the end, a suspension of red blood cells at a haematocrit level of between about 40% and about 70%, preferably
5 between about 45% and about 55%, better still about 50%. It is preferably packaged in a volume of about 10 to about 250 ml. The packaging is preferably in a blood bag of the type suitable for blood transfusion. The amount of encapsulated tumour antigen corresponding to the medical
10 prescription is preferably entirely contained in the blood bag.

An object of the present invention is also a therapeutic anti-tumour vaccine comprising an effective amount of one or more compositions according to the invention. Thus, the vaccine may comprise a composition according to the invention, itself containing a population of red blood cells
15 encapsulating one or more tumour antigens, in the presence or absence of encapsulated or nonencapsulated adjuvant, or at least two compositions according to the invention with different populations of red blood cells encapsulating one or more tumour antigens, in the presence of encapsulated or nonencapsulated adjuvant.

20 An object of the invention is also a method for inducing, in a patient, a cytotoxic cellular response against tumour cells or a tumour. This method comprises the administration to this patient of an effective amount of a composition according to the invention, in particular intravenously, by injection or infusion, preferably by infusion. This method is aimed in
25 particular at inducing activation of the patient's dendritic cells and a CD8⁺ cytotoxic cellular response. As described above, a specific CD4⁺ helper and CD8⁺ cytotoxic response is obtained.

An object of the invention is also an anticancer treatment method for inducing, in a patient, a cytotoxic cellular response as described above,
30 against tumour cells or a tumour. This method comprises the administration to this patient of an effective amount of an anti-tumour vaccine according to the invention, in particular intravenously, by injection or infusion, preferably

by infusion.

According to one feature of the invention, about 10 to about 250 ml of a suspension of red blood cells at a haematocrit level of between about 40% and about 70%, preferably between about 45% and about 55%, better still
5 about 50%, are administered.

An object of the invention is also the use of a composition according to the invention, for the manufacture of a therapeutic anti-tumour, vaccine.

An object of the invention is also the use of a composition according to the invention, for inducing, in a host, a cytotoxic cellular response
10 mediated by dendritic cells and directed against tumour cells or a tumour.

Another object of the invention is a composition according to the invention, for use as a therapeutic anti-tumour vaccine.

The present invention will now be described in greater detail by means of embodiments taken by way of nonlimiting examples, and which
15 refer to the attached drawings.

Figure 1: Measurement of the phagocytosis of "antigen-loaded" red blood cells treated with the anti-TER 119 antibody, by spleen dendritic cells *in vitro*.

Figure 2: Measurement of the phagocytosis of "antigen-loaded" red
20 blood cells treated with the anti-TER 119 antibody or heat-treated, by spleen macrophages and dendritic cells *in vivo*.

Figure 3: Graph illustrating the proliferation and activation of ovalbumin-specific CD4 T cells 3 days after injection into mice.

25 (A) Cell division induces a reduction in the intensity of CFSE fluorescence. OVA-specific CD4 T cells lose half of their fluorescent material on each division. The peak observed for batch 4 represents undivided cells having a large CFSE content. All of the other peaks represent cells which have undergone 1, 2, 3, 4, 5, 6 or 7 cell divisions.
30 When the cells have divided more than 8 times, the CFSE content of the cells is practically zero.

(B) CD44 cell activation marker expression is represented as a function of the number of divisions occurring.

5 Example 1. Method for encapsulating ovalbumin in murine and human red blood cells and in porcine red blood cells

Variant 1:

Ovalbumin (protein of 45 kDa, hen egg ovalbumin) is encapsulated in murine red blood cells (OF1 mice or C57Bl/6 mice) by the method of
10 hypotonic dialysis in dialysis tubing. The red blood cell suspension is washed several times before being brought to a haematocrit of 70% for the dialysis. The dialysis is carried out in dialysis tubing in a lysis buffer of low osmolarity for about 1 hour or 30 min when the dialysis occurs after a heat treatment. The red blood cells are then resealed by means of a solution of
15 high osmolarity for 30 minutes. After a few washes, the final product is taken up in a buffer, Sag-mannitol, and haematocrit is brought to 50%.

Variant 2 for Example 1:

Ovalbumin is herein encapsulated in the murine red blood cells by the method of hypotonic dialysis in a dialysis column. The red blood cell
20 suspension is washed several times before being brought to a haematocrit of 70% for the dialysis. The dialysis is carried out in a dialysis column in a lysis buffer of low osmolarity for about 10 min. As soon as they leave the column, the red blood cells are resealed by means of a solution of high osmolarity for 30 minutes at 37°C. After a few washes, the final product is
25 taken up in a NaCl glucose buffer containing glucose SAG mannitol, or decompemented plasma, and haematocrit is brought back to 50%.

Example 2. Heat treatment on the red blood cells

When the encapsulation is realized according to variant 1 of example
30 1, heat treatment is realized before dialysis process. When encapsulation is realized according to variant 2 of example 1, heat treatment is realized after the dialysis and resealing process and before washing steps and addition of NaCl glucose buffer.

The red blood cells are washed several times before being brought to a haematocrit of 10%. They are then heated for 30 minutes at 48°C.

Example 3. Antibody treatment on the red blood cells containing
5 ovalbumin

The suspension of red blood cells encapsulating ovalbumin is washed several times before being brought to 10^9 cells/ml for the *in vivo* test and 10^8 cell/ml for the *in vitro* test. It is incubated with the anti-TER119 antibody (10 µg/ml for the *in vitro* test and 23 µg/ml or 5 µg/ml for the *in vivo*
10 test) for 30 minutes at 4°C. After a few washes, the final product is taken up in a buffer with injectable qualities, and haematocrit is brought to 50%.

Example 4. Chemical treatment with bis(sulphosuccinimidyl) suberate (BS3) on the red blood cells containing ovalbumin

15 The suspension of red blood cells encapsulating ovalbumin is washed several times before being brought to 1.7×10^6 cell/µl with PBS and mixed with one volume of a buffer solution of 2 mM BS3 (the BS3 solution contains glucose and phosphate buffer, pH 7.4), so as to obtain a final BS3 concentration of 1 mM. The cells are incubated for 30 minutes at room
20 temperature. The reaction is quenched by adding one volume of 20 mM Tris-HCl. After incubation at room temperature for 5 minutes, the mixture is centrifuged at 800 g for 5 min, 4°C. The cells are then washed twice with PBS containing glucose (centrifugation at 800 g) and once with SAG-mannitol (centrifugation at 1000 g) for 10 min, before constituting the final
25 products.

Example 5. Measurement of the phagocytosis of ovalbumin-containing red blood cells by dendritic cells *in vitro*

The effect of the various treatments (heat and antibody) on the
30 phagocytosis efficiency of the red blood cells obtained according to variant 1 of example 1, by dendritic cells, is measured *in vitro*. The red blood cells are labelled with a fluorescent label, CFSE (carboxyfluorescein succinimidyl

ester), for 20 min at 4°C. CFSE is a non-fluorescent dye which diffuses through the cell membrane. Once inside the cell, the molecule becomes fluorescent subsequent to its cleavage by intracellular esterases.

Dendritic cells are isolated from the spleen of C57Bl/6 mice using
5 magnetic beads. These beads carry antibodies which recognize the CD11c marker, thereby making it possible to isolate the CD11c⁺ dendritic cell fraction.

The CFSE-labelled or unlabelled red blood cells are then incubated with the dendritic cells (10×10^6 cell/ml) at a ratio of 20:1 in a final volume of
10 200 µl/well of round-bottomed 96-well culture plates for 4 hours at 37°C and 5% CO₂. After culturing for 4 hours, the red blood cells not ingested by the dendritic cells are lysed with NH₄Cl, and several washes are carried out. The capture of the CFSE fluorochrome by the dendritic cells is then measured by flow cytometry (R. Segura et al., J. Immunol, January 2006,
15 176(1): 441-50).

Three populations of red blood cells were tested:

- (A) red blood cells loaded with ovalbumin and not labelled with the CFSE fluorochrome,
- (B) red blood cells loaded with ovalbumin and labelled with CFSE,
- 20 (C) red blood cells loaded with ovalbumin, treated with the anti-TER 119 antibody and labelled with CFSE.

Results

Table 1: Percentage of dendritic cells having phagocytosed
25 fluorescent red blood cells:

<i>Red blood cell population</i>	<i>% of dendritic cells</i>
(A)	4%
(B)	27%
(C)	36%

The murine red blood cells loaded with ovalbumin and treated with the anti-TER 119 antibody were more efficiently phagocytosed by the dendritic cells isolated from the spleen than the untreated red blood cells *in*

vitro, after 4 hours of coculture (Figure 1, respectively C and B). 36% of the dendritic cells phagocytosed the red blood cells carrying the antibody, against only 27% in the absence of antibody. Phagocytosis of the antibody-treated red blood cells by a population not expressing the CD11c dendritic cell marker was also observed (10.9%; Figure 1, C).

Example 6. Measurement of the phagocytosis of red blood cells containing ovalbumin, by macrophages and dendritic cells of the spleen and liver *in vivo* on mice

This study is an allogenic study since OF1 mice red blood cells containing ovalbumin are injected to not consanguineous C57Bl/6 mice.

Three batches of 74×10^7 red blood cells, from OF1 mice, loaded with ovalbumin (variant 1 of example 1) heat treated, treated with the anti-TER 119 antibody (as described in examples 2 and 3, respectively) or not treated are prepared. These batches are divided up in the following way:

Batch 1: no heat or antibody treatment (Figure 2, A and D)

Batch 2: heat treated (Figure 2, B and E)

Batch 3: treated with the anti-TER 119 antibody (Figure 2, C and F).

Each batch is labelled with CFSE and injected intravenously into C57Bl/6 mice. Three hours after the injection, the blood, the spleen and the liver of the mice are taken. The percentage of fluorescent red blood cells circulating in the blood of the mice is measured by flow cytometry. The fluorescence incorporated into the spleen macrophages expressing the F4/80 marker (Figure 2, A, B, C), into the liver macrophages expressing the F4/80 marker and into the spleen dendritic cells expressing the CD11c marker (Figure 2, D, E, F) is measured by flow cytometry.

Results

Table 2: Percentage of macrophages or dendritic cells from the spleen, having phagocytosed fluorescent red blood cells 3 hours after injection into the mouse:

Batches	Macrophages	Dendritic cells
1	28%	5%

2	68%	19%
3	81%	22%

3 hours after injection, the murine red blood cells loaded with ovalbumin and heat-treated or treated with the anti-TER 119 antibody are almost no longer present in the blood of the mouse (1.6% and 1%), whereas
 5 there are still untreated, ovalbumin-loaded red blood cells in the blood of the mouse (4.6%).

The red blood cells that have been heat-treated or treated with the anti-TER 119 antibody are phagocytosed by the F4/80 macrophages and CD11c dendritic cells of the spleen (Figure 2).

10 The red blood cells treated with the anti-TER 119 antibody were more efficiently phagocytosed by the F4/80 macrophages of the spleen than the heat-treated red blood cells or the untreated red blood cells (Figure 2, A, B, C). 81% of the spleen macrophages phagocytosed the antibody-treated red blood cells, against 68% of macrophages having phagocytosed the heat-
 15 treated red blood cells and only 28% in the untreated batch (Table 2).

The heat-treated or antibody-treated red blood cells were also more efficiently phagocytosed by the CD11c dendritic cells from the spleen than the untreated red blood cells (Figure 2). Respectively 22% and 19% of dendritic cells phagocytosed the antibody-treated red blood cells and the
 20 heat-treated red blood cells, against only 5% in the case of the untreated red blood cells (Table 2).

Phagocytosis of the antibody-treated red blood cells by a population from the spleen not expressing the CD11c dendritic cell marker or the F4/80 macrophage marker was also observed (11.9% and 12.8%, Figure 2).

25 Table 3: Percentage of liver macrophages having phagocytosed fluorescent red blood cells 3 hours after injection into the mouse.

<i>Batches</i>	<i>Macrophages</i>
1	24%
2	40%
3	50%

The heat-treated red blood cells or the red blood cells treated with the anti-TER 119 antibody are phagocytosed by the F4/80 macrophages of the liver.

5 The red blood cells treated with the anti-TER 119 antibody were more efficiently phagocytosed by the F4/80 macrophages of the liver than the heat-treated red blood cells or than the untreated red blood cells. 50% of the liver macrophages phagocytosed the antibody-treated red blood cells, against 40% of macrophages having phagocytosed the heat-treated red blood cells and only 24% in the untreated batch (table 3).

10 In conclusion, the binding of the antibody to the red blood cells and the heat treatment allowed efficient targeting of the red blood cells in the spleen and the liver, and a significant increase in the percentage of dendritic cells and of macrophages capable of phagocytizing these red blood cells.

15

Example 7. Measurement of the phagocytosis of mouse red blood cells containing fluorescent ovalbumin by bone marrow macrophages and dendritic cells *in vivo*

20 This study is an autologous study, since OF1 mouse red blood cells are injected into OF1 mice.

Four batches of 132×10^7 OF1 mouse red blood cells loaded with fluorescent ovalbumin (Serlabo Technologies, ref WO-LS003054) prepared using variant 2 of example 1 and treated with the anti-TER 119 antibody, 25 with heat or with BS3 (as described in Examples 3, 2 and 4, respectively) or not treated are prepared. Each batch of red blood cells is injected intravenously into two mice. One and a half hours after the injection, the mice are sacrificed and the femurs of the mice are removed. The fluorescence incorporated into the macrophages expressing the F4/80 30 marker or the CD11b marker, the granulocytes expressing the Gr1 marker, the myeloid dendritic cells expressing the CD11c and CD11b markers and into the plasmacytoid dendritic cells expressing the CD11c and CD8

markers is measured by flow cytometry.

Batch 1: ovalbumin-loaded red blood cells, treated with BS3

Batch 2: ovalbumin-loaded red blood cells, heat-treated

Batch 3: ovalbumin-loaded red blood cells, treated with the anti-TER 119
5 antibody

Batch 4: ovalbumin-loaded red blood cells

Batch 5: NaCl glucose

Results

10 Table 4: Percentage of bone marrow macrophages, granulocytes or dendritic cells having phagocytosed fluorescent ovalbumin 1 hour 30 min after the injection:

Batches	F4/80 macrophages	CD11b macrophages	Granulocytes	Myeloid dendritic	Plasmacytoid dendritic
1	2.5	0.05	12.5	2.7	2.4
2	3.3	0	15.5	2.2	3.4
3	17.7	1.75	21	15.3	6
4	2.4	0	13	1.6	2.4
5	0.5	0.1	1.3	0.8	1.5

15

One and a half hours after injection, the ovalbumin-loaded red blood cells treated with the anti-TER 119 antibody were efficiently phagocytosed by the F4/80 macrophages, the granulocytes and the dendritic cells of the bone marrow. The myeloid dendritic cells of the bone
20 marrow are the cells most involved in the phagocytosis of the ovalbumin-loaded red blood cells treated with the anti-TER 119 antibody.

In conclusion, the use of red blood cells treated with the anti-TER 119 antibody allows the best targeting and phagocytosis of the red blood cells by immune cells in the bone marrow.

25

Example 8. Measurement of the ovalbumin-specific CD4 T cell response after a single injection of ovalbumin-loaded red blood cells and of Poly(I:C) adjuvant

The evaluation of the OVA (ovalbumin)-specific CD4 T cell response consists in measuring the percentage of OVA-specific CD4 T cells, the proliferation of these cells, the level of activation of these cells and the
5 production of IFN γ (γ = gamma).

The evaluation of the CD4 T cell response is realized using an adaptation of the methods described in Russo V. et al. The Journal of Clinical Investigation, 2007, 117 : 3087-3096 ; Stoitzner P. et al., The Journal of Immunology 2008, 180 : 1991-1998. The CD4 T cells of OT-II
10 transgenic mice (Charles River, ref C57BL/6-Tg(TcraTcrb425Cbn/Crl)) are used to measure the OVA-specific CD4 T cell response. The OT-II mice are mice expressing only CD4 T cells which recognize the ovalbumin peptide 323-339 associated with major histocompatibility complex class II molecules.

15 The OT-II transgenic CD4 T cells are isolated from the spleen of OT-II mice and labelled with CFSE, before being injected intravenously into Ly5.1 mice. OT-II mice and Ly5.1 mice have the same genetic background, but the cells of both types of mice can be identified by means of the CD45 marker. This is because the cells of OT-II mice express the CD45.2 marker,
20 whereas the Ly5.1 mice express the CD45.1 marker.

20 hours after the injection of the transgenic mouse CD4 T cells, the Ly5.1 mice receive an intravenous injection of the following batches. Three mice are injected with the batches containing red blood cells and two mice are injected with the batches containing free OVA or the control.

25 Two batches of 183×10^7 red blood cells of C57Bl/6 mice loaded with ovalbumin (Serlabo Technologies, ref WO-LS003054) prepared using variant 2 of example 1 and treated or not with the anti-TER 119 antibody (as described in example 3) are prepared. The adjuvant, Poly (I:C) (Invivogen, ref tlrl-pic), is added to these batches, as well as to the batch
30 containing free ovalbumin. The amount of Poly (I:C) injected per mouse is 25 μ g. The amount of OVA injected into the mice is indicated in Table 5.

This is an autogenic study since ovalbumine-loaded C57Bl/6 mice

red blood cells are injected to Ly5.1 mice. C57Bl/6, Ly5.1 and OT-II mice have the same genetic background.

Batch 1: Poly (I:C) and ovalbumin-loaded red blood cells treated with the anti-TER 119 antibody

5 Batch 2: Poly (I:C) and ovalbumin-loaded red blood cells

Batch 3: Poly (I:C) and free ovalbumin

Batch 4: Plasma

Three days after the injection of the batches, the mice are sacrificed and the spleens of the mice are removed. The percentage of OVA-specific
10 CD4 T cells in the spleen of the mice is measured by flow cytometry using the CD4 and CD45.2 markers (Table 5). The number of OVA-specific CD4 T cells is calculated from the percentage of OVA-specific CD4 T cells and the number of total lymphocytes counted with trypan blue (Invitrogen, ref 15250061) (Table 5).

15 The proliferation and the activation of the OVA-specific CD4 T cells in the spleen of the mice is measured by flow cytometry using the CD4, CD44 and CD45.2 markers and using CFSE (Tables 6 and 7). At each cell division, the amount of CFSE contained in the OVA-specific CD4 T cells is divided by a factor of 2, which makes it possible to determine the number of
20 cell divisions by flow cytometry (Figure 3).

The production of IFN γ by the mice splenocytes is measured in the culture supernatants after 3 days of in vitro stimulation in the presence of 10 μ g/ml of ovalbumin peptide 323-339 (Neomps, ref) by means of an ELISA assay.

25 Results

Table 5: Percentage and number of OVA-specific CD4 T cells three days after injection of the batches (mean $\pm$ standard deviation):

Batches	Amount of OVA injected into the mice (μ g)	% of OVA-specific CD4 T cells	Number of OVA-specific CD4 T cells (millions of cells)
1	160	$6.6 \pm 1.4^*$	$8 \pm 2^*$
2	129	$3.6 \pm 0.9^{**}$	$4.1 \pm 2^*$
3	150	0.8 ± 0.3	1.3 ± 0.7
4	0	0.1 ± 0.1	0.2 ± 0.1

Three days after injection of the batches, the mice injected with Poly (I:C) and ovalbumin-loaded red blood cells treated or not with the anti-TER 119 antibody have a significantly higher percentage and number of OVA-specific CD4 T cells than the mice injected with free OVA and Poly (I:C) (Student test $*p=0.01$, $**p=0.02$).

Furthermore, the batch containing ovalbumin-loaded red blood cells treated with the anti-TER 119 antibody is more effective in inducing an increase in the number of OVA-specific CD4 T cells than the batch containing untreated, ovalbumin-loaded red blood cells (Student test $p=0.04$).

Table 6: Percentage of OVA-specific CD4 T cells which have divided 0, 1, 2, 3, 4, 5, 6 or 7 times three days after injection of the batches:

Batches	% of OVA-specific CD4 T cells at each division cycle (mean $\pm$ standard deviation)							
	0	1	2	3	4	5	6	7
1	0 ± 0	0 ± 0	0.1 ± 0	0.3 ± 0	1.7 ± 0	9.4 ± 0.1	35.9 ± 0.3	52.5 ± 0.2
2	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.8 ± 0.1	4.2 ± 0.8	19.2 ± 3.2	43.7 ± 2.2	31.5 ± 6.3
3	1.9 ± 1.4	6.9 ± 5.2	15.6 ± 9.5	18.7 ± 5.6	19.7 ± 0.4	16.4 ± 5.3	13.9 ± 10.1	6.8 ± 5.9
4	69.7 ± 4.9	1.3 ± 1	0.1 ± 0.2	0.3 ± 0.4	5.6 ± 3.7	3.7 ± 1.1	5.3 ± 2.8	13.6 ± 2.5

These observations are confirmed by the in vivo cell proliferation results (Table 6 and Figure 3). Cell division induces a decrease of the CFSE fluorescence intensity: disparition of half of the fluorescent material at each division (Figure 3A). The OVA-specific CD4 T cells divide more rapidly

(Table 6 and Figure 3A) for the mice injected with Poly (I:C) and ovalbumin-loaded red blood cells treated with the anti-TER 119 antibody (6 to 7 divisions) in comparison with the mice injected with free OVA and Poly (I:C) (3 to 4 divisions). Furthermore, the batch containing ovalbumin-loaded red blood cells treated with the anti-TER 119 antibody appears to be more effective in inducing cell proliferation than the batch containing untreated, ovalbumin-loaded red blood cells.

Table 7: Level of activation of OVA-specific CD4 T cells three days after injection of the batches:

Batche	Mean of OVA-specific CD4 T cells activation fluorescence (mean $\pm$ standard deviation)
1	32.6 $\pm$ 2.3
2	37.6 $\pm$ 3.5
3	34.6 $\pm$ 3.9
4	11.4 $\pm$ 0.5

The results of the cell activation level (characterized by the level of expression of the CD44 marker) show that all the dividing OVA-specific CD4 T cells have a high cell activation level (Table 7 and Figure 3). In addition, the activation level of the OVA specific CD4 T cells can be correlated to the number of divisions of these cells: the more the cells have divided and have lost their CFSE marker content, the more they express a high level of activation marker (Figure 3B).

Table 8: Production of IFN γ by splenocytes stimulated in vitro with 10 μ g/ml of ovalbumin peptide 323-339 for 3 days:

Batches	Production of IFN γ by splenocytes in ng/ml (mean $\pm$ standard deviation)
1	56.3

2	60 ± 26.8
3	9.4 ± 1.6
4	6.7 ± 1

The results of IFN γ production by splenocytes show a strong production of IFN γ by the splenocytes of mice injected with Poly (I:C) and ovalbumin-loaded red blood cells treated or not treated with the
5 anti-TER119 antibody.

In conclusion, these results demonstrate the superiority of the ovalbumin-loaded red blood cells treated or not with the anti-TER 119 antibody, on the activation and proliferation of OVA-specific CD4 T cells capable of producing IFN γ .

10

Example 9. Measurement of the ovalbumin-specific CD8 T cell response after a single injection of ovalbumin-loaded red blood cells and of Poly (I:C) adjuvant

15 The evaluation of the OVA-specific CD8 T cell response consists in measuring the percentage of OVA-specific CD8 T cells, the production of IFN γ and the cell lysis, *in vivo*, of the cells presenting the ovalbumin peptide 257-264 associated with major histocompatibility complex class I molecules. This study is an allogenic study, since ovalbumin-loaded red blood cells
20 from OF1 mice are injected into not consanguineous C57Bl/6 mice.

Two batches of 167×10^7 ovalbumin-loaded OF1 mouse red blood cells prepared using variant 2 of example 1 and treated or not with the anti-TER 119 antibody (as described in example 3) are prepared. The adjuvant, Poly (I:C), is added to these batches, and also to the batch containing free
25 ovalbumin. The amount of Poly (I:C) injected per mouse is 25 μ g. The amount of OVA injected into the mice is indicated in Table 9.

The batches are injected intravenously into C57Bl/6 mice. A minimum of 4 mice are injected with a given batch. *In vivo* cell lysis is measured using an adaptation of the method described by Hervas-Stubbs S. et al., Blood

2007, 109 : 5318-5326. Cells presenting the ovalbumin peptide 257-264 associated with major histocompatibility complex class I molecules are injected into the immunized mice. Briefly, six days after the injection, the mice receive an injection of 0.5×10^6 splenocytes presenting the ovalbumin
5 257-264 peptide (Neomps, ref SC1302) and labelled with a moderate concentration of CFSE, and of 0.5×10^6 splenocytes not presenting the peptide and labelled with high concentrations of CFSE (cell lysis control).

Latex beads coupled to ovalbumine peptide 257-264 (BOVAp) and poly(I:C) are used as positive control to induce a CD8 T immune response
10 against ovalbumine. It has been shown that the injection of BOVAp and of poly(I:C) induces an increase of the percentage of ovalbumine-specific CD8 T cells and the destruction of cells presenting the OVA257-264 peptide *in vivo* (Herva-Stubbs).

Batch 1: Poly (I:C) and ovalbumin-loaded red blood cells treated with the
15 anti-TER 119 antibody

Batch 2: Poly (I:C) and ovalbumin-loaded red blood cells

Batch 3: Latex beads coupled to the ovalbumin peptide 257-264

Batch 4: Poly (I:C) and latex beads coupled to the ovalbumin peptide 257-
264

20 Batch 5: Poly (I:C) and free ovalbumin

Batch 6: NaCl glucose + plasma

16 hours after injection of the splenocytes, the mice are sacrificed by euthanasia and the spleens of the mice are removed. The percentage of OVA-specific CD8 T cells in the spleen of the mice is measured by flow
25 cytometry using tetramer and the CD8 marker (Table 9). IFN γ production and expression of the marker associated with degranulation (CD107) by CD8 T cells was measured by flow cytometry after *in vitro* stimulation for 4 hours in the presence of 0.1 μ g/ml of ovalbumin peptide 257-264 (Table 10). *In vivo* cell lysis was measured by flow cytometry using CFSE (Table 11).

Results

Table 9: Percentage of OVA-specific CD8 T cells seven days after injection of batches (mean $\pm$ standard deviation):

5

Batch	Quantity of OVA injected into mice (μ g)	% of OVA-specific CD8 T cells (mean $\pm$ standard deviation)	% of OVA-non-specific T cells (mean $\pm$ standard deviation)
1	95	14 $\pm$ 3.2*	2.4 $\pm$ 0.2
2	91	12.5 $\pm$ 5.2**	1.2 $\pm$ 0.5
3	0	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1
4	0	2.5 $\pm$ 0.4*	0.9 $\pm$ 0.1
5	130	2.4 $\pm$ 1.4	1 $\pm$ 0.2
6	0	0.7 $\pm$ 0.4	1 $\pm$ 0.1

Mice injected with Poly(I:C) and ovalbumin-charged red blood cells which had either been treated or had not been treated with anti-TER119 antibody have a significantly higher percentage of OVA-specific CD8 T cells than mice injected with free OVA and Poly(I:C) or mice injected with Poly(I:C) and BOVAp (Student's test: *p = 0.001 and **p = 0.01).

10

Table 10: Production of IFN γ by CD8 T cells stimulated in vitro with 0.1 μ g/ml of ovalbumin peptide 257-264 for 4 hours:

15

Batch	% CD8 T cells producing IFN γ	% of CD8 T cells producing IFN γ and expressing CD107 marker
1	8.7 $\pm$ 1.8*	71 $\pm$ 4*
2	6.6 $\pm$ 1.7*	71 $\pm$ 4*
3	1.4 $\pm$ 0.1	33 $\pm$ 4.5
4	2.8 $\pm$ 0.5	57 $\pm$ 5.5
5	1.8 $\pm$ 0.5	46 $\pm$ 7
6	0.7 $\pm$ 0.1	18 $\pm$ 2

The CD8 T cells generated are effective and cytotoxic since they produce IFN γ and express the CD107 marker in response to stimulation with the ovalbumin peptide (Table 10). The percentage of CD8 T cells

producing IFN γ and expressing CD107 is significantly higher for mice injected with Poly(I:C) and ovalbumin-charged red blood cells which had either been treated or had not been treated with anti-TER119 antibody than for mice injected with free OVA and Poly(I:C) or Poly(I:C) and BOVAp
 5 (Student's test, $p < 0.008$).

Table 11: Percentage of in vivo anti-OVA-specific cell lysis (mean $\pm$ standard deviation):

Batch	% cell lysis, in vivo (mean $\pm$ standard deviation)
1	82 $\pm$ 7
2	83 $\pm$ 4
5	53 $\pm$ 8
6	0

10

The cell lysis results correlate with expression of the marker associated with degranulation (Tables 10 and 11). Injection of Poly(I:C) and ovalbumin-charged red blood cells which had either been treated or had not been treated with anti-TER119 antibody induced lysis of cells displaying
 15 ovalbumin peptide 257-264 in a manner which was more effective than injection of Poly(I:C) and free ovalbumin (Table 10).

In conclusion, these results demonstrate the superiority of ovalbumin-charged red blood cells which have either been treated or have not been treated with anti-TER119 antibody in the activation and proliferation of
 20 cytotoxic CD8 T cells capable of producing IFN γ , degranulating and lysing cells displaying ovalbumin peptide 257-264.

Example 10. Measuring the maintenance of the ovalbumin-specific CD8 T cell response 30 days after a single injection of ovalbumin-charged
 25 red blood cells and adjuvant Poly(I:C)

Evaluating the maintenance of the OVA-specific CD8 T cell response consisted of measuring the percentage of OVA-specific CD8 T cells and cell lysis in vivo 34 days after a single injection.

This study was an allogenic study, since red blood cells charged with
 5 OF1 mouse ovalbumin were injected into non-consanguineous C57Bl/6 mice.

Two batches were prepared of 150×10^7 of ovalbumin-charged OF1 mouse red blood cells obtained in accordance with variant 2 of Example 1 which had or had not been treated with anti-TER119 antibody (as described
 10 in Example 3). The adjuvant, Poly(I:C), was added to these batches, and also to the batch containing free ovalbumin or BOVAp. The quantity of Poly(I:C) injected per mouse was 25 µg. The quantity of OVA injected into the mice is shown in Table 12.

The batches were injected intravenously into C57Bl/6 mice. Three
 15 mice were injected with a given batch. To measure cell lysis in vivo, cells displaying ovalbumin peptide 257-264 associated with molecules of the major histocompatibility complex of class I were injected into immunized mice. 33 days after injection, the mice received an injection of 0.5×10^6 splenocytes displaying ovalbumin peptide 257-264 (NeoMPS, reference
 20 SC1302) and labelled with a moderate concentration of CFSE and of 0.5×10^6 splenocytes not displaying the peptide and labelled with a high concentration of CFSE (cell lysis assay).

Batch 1: Poly(I:C) and ovalbumin-charged red blood cells, treated with anti-TER119 antibody

25 Batch 2: Poly(I:C) and ovalbumin-charged red blood cells

Batch 3: Latex beads coupled with ovalbumin peptide 257-264:
 BOVAp

Batch 4: Poly(I:C) and BOVAp

Batch 5: Poly(I:C) and free ovalbumin

30 Batch 6: NaCl glucosated + plasma

16 hours after injecting the splenocytes, the mice were euthanized and their spleens were removed. The percentage of OVA-specific CD8 T

cells in the spleens of the mice was measured by flow cytometry using tetramer and CD8 marker (Table 12). In vivo cell lysis was measured by flow cytometry using CFSE (Table 13).

- 5 Table 12: Percentage of OVA-specific CD8 T cells 34 days after injection of batches (mean $\pm$ standard deviation)

Batch	Quantity of OVA injected into mice (μ g)	% of OVA-specific CD8 T cells (mean $\pm$ standard deviation)
1	170	1.5 $\pm$ 0.2
2	138	1.7 $\pm$ 0.3
3	0	1.6 $\pm$ 0.1
4	0	1.4 $\pm$ 0.1
5	150	2.2 $\pm$ 0.2
6	0	1.8 $\pm$ 0.06

- 34 days after injection, the percentage of OVA-specific CD8 T cells was equivalent for each group (Table 12). This result is not surprising given that the peak CD8 T cell proliferation was at about day 7 and it was followed by a contraction phase (Hervas-Stubbs S et al, Blood, 2007, 109: 5318-5326).

- 15 Table 13: Percentage of anti-OVA-specific cell lysis in vivo (mean $\pm$ standard deviation):

Batch	% cell lysis in vivo (mean $\pm$ standard deviation)
1	49 $\pm$ 9*
2	54 $\pm$ 28
3	12 $\pm$ 5.4
4	21.6 $\pm$ 12
5	20.4 $\pm$ 19
6	0.9 $\pm$ 0.9

- 34 days after injection, the OVA-specific CD8 T cells were still capable of lysing cells displaying ovalbumin peptide 257-264 (Table 13). Injection of Poly(I:C) and ovalbumin-charged red blood cells treated with

anti-TER119 antibody induced cell lysis in a manner which was more effective than injection of Poly(I:C) and BOVAp (*Student's test, $p < 0.04$).

In conclusion, these results demonstrate that ovalbumin-charged red blood cells are superior not only as regards activation and proliferation of cytotoxic CD8 T cells, but also as regards maintenance/survival of these T cells which are capable of lysing cells displaying ovalbumin peptide 257-264.

Example 11. Measurement of the ovalbumin-specific CD8 T cell response after a single injection of chemically treated, ovalbumin-loaded red blood cells and of Poly (I:C) adjuvant

This study is an allogenic study, since ovalbumin-loaded red blood cells from OF1 mice are injected into not consanguineous C57Bl/6 mice.

A batch of 132×10^7 ovalbumin-loaded OF1 mouse red blood cells prepared using variant 2 of example 1 and chemically treated with 1mM BS3 (as described in example 4) is prepared. The adjuvant Poly (I:C), is added to this batch, and to the batch containing free ovalbumin. The amount of Poly (I:C) injected per mouse is 25 μ g. The amount of OVA injected into the mice is indicated in Table 14.

Batch 1: Poly (I:C) and ovalbumin-loaded red blood cells treated with 1mM BS3

Batch 2: Poly (I:C) and free ovalbumin

Batch 3: Poly (I:C)

Batch 4: NaCl containing glucose

The batches are injected intravenously into C57Bl/6 mice. A minimum of 3 mice are injected with a given batch. Seven days after the injection, the mice are sacrificed and the spleens of the mice are removed. The percentage of OVA-specific CD8 T cells in the spleen of the mice is measured by flow cytometry using tetramer and the CD8 marker (Table 14).

Results

Table 14: Percentage and number of OVA-specific CD8 T cells seven days after injection of the batches (mean $\pm$ standard deviation):

Batche	Amount of OVA injected into the mice	% of OVA-specific CD8 T cells (mean $\pm$ standard deviation)
1	38	1.8 $\pm$ 0.6
2	150	1.2 $\pm$ 0.3
3	0	1 $\pm$
4	0	0.8 $\pm$ 0.1

5

The mice injected with Poly (I:C) and ovalbumin-loaded red blood cells treated with BS3 have a higher percentage of OVA-specific CD8 T cells than the mice injected with free OVA and Poly (I:C) or than the mice injected with Poly (I:C). These results are not statistically different, but it should be noted that the amount of free OVA injected is more than three times greater than the amount of OVA injected in the batch containing the OVA-loaded red blood cells.

In conclusion, these results show that the ovalbumin-loaded red blood cells treated with BS3 are also capable of generating OVA-specific CD8 T cells.

Example 12. Measurement of the ovalbumin-specific CD8 T cell response after a single injection of ovalbumin-loaded red blood cells and of CL-097 adjuvant

20

This study is an allogenic study, since ovalbumin-loaded red blood cells from OF1 mice are injected into not consanguineous C57Bl/6 mice.

Batches of 119×10^7 ovalbumin-loaded OF1 mouse red blood cells prepared using variant 1 of example 1 and treated or not with the anti-TER 119 antibody (as described in example 3) are prepared. The adjuvant, CL097 (Invivogen, ref tlrl-c97), is or is not added to these batches. The amount of CL097 injected per mouse is 0.15 μ g. The amount of OVA

injected into the mice is indicated in Table 15.

Batch 1: CL097 and ovalbumin-loaded red blood cells treated with the anti-TER 119 antibody

Batch 2: ovalbumin-loaded red blood cells treated with the anti-TER 119 antibody

Batch 3: CL097 and ovalbumin-loaded red blood cells

Batch 4: ovalbumin-loaded red blood cells

Batch 5: NaCl containing glucose

The batches are injected intravenously into C57Bl/6 mice. Four days after the injection, the mice are sacrificed and the spleens of the mice are removed. The IFN γ production by the splenocytes of mice injected with the various batches is measured in the culture supernatants after three days of *in vitro* stimulation in the presence of 0.1 μ g/ml of ovalbumin peptide 257-264 by means of ELISA assay.

Results

Table 15: IFN γ production by splenocytes stimulated *in vitro* with 0.1 μ g/ml of ovalbumin peptide 257-264 for 3 days:

Batche	Amount of OVA injected into the mice (μ g)	IFN γ production by splenocytes in ng/ml
1	83	15.
2	83	5.7
3	103	11.
4	103	7.4
5	0	0.4

The results of IFN γ production by the splenocytes show a greater IFN γ production when the mice received an injection of both the CL097 adjuvant and ovalbumin-loaded red blood cells treated or not with the anti-TER 119 antibody.

In conclusion the CL097 adjuvant also potentiates the IFN γ response

induced by the injection of the ovalbumin-loaded red blood cells.

Example 13. Measure of tumoral growth after one injection of adjuvant Poly (I :C) and treated or untreated ovalbumin-loaded erythrocytes in mice

5

The purpose of this study is to measure the tumor growth of EG.7 cell line in C57Bl/6 mice after injection of a single dose of Poly (I:C) and treated or untreated ovalbumin-loaded erythrocytes. EG.7 tumor cells are obtained from ATCC (ATCC-CRL-2113). EG.7 cells originate from the EL.4 cell line, which synthesize and secrete OVA constitutively.

This is an allogenic study since ovalbumin-loaded erythrocytes from OF1 mice are injected into C57Bl/6 mice. The model in this experiment is a prophylactic model.

Two batches of 165×10^7 antibody-treated or untreated ovalbumin-loaded erythrocytes from OF1 mice are prepared according to variant 2 of example 1. The adjuvant, Poly (I: C), is added to those batches. 25 μg of Poly (I: C) is injected per mice. The negative control is a suspension of unloaded erythrocytes. OVA amounts injected to mice are indicated in table 16.

20 Batch 1: Poly (I: C) and antibody-treated ovalbumin-loaded erythrocytes

Batch 2: Poly (I: C) and ovalbumin-loaded erythrocytes

Batch 3: unloaded erythrocytes

The batches are injected intravenously to C57Bl/6 mice (10 mice per group). Seven days after batch injection, 2.2×10^6 EG.7 cells per mice are injected subcutaneously. Mice are followed for tumor growth. Tumor growth is assessed by measuring the diameter of the tumor in centimeters (recorded as the average of two perpendicular diameter measurements). Tumor size is measured 3 times per week at 2 days interval during 14 days. Mice with tumors of more than 2.0 cm of diameter are killed.

30

Table 16: OVA amount injected per mice

Batches	OVA quantity injected to mice (µg)
1	134
2	134
3	0

Table 17: Tumor growth

Batch es	Tumor growth (mm ³) the days following tumor cell injection						
	0	3	5	7	10	12	14
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	0	363 ± 200	756 ± 462	1009 ± 445	1198 ± 541

5

7 days after EG.7 injection, the size of the tumors is measurable on the flank of the mice previously injected with unloaded erythrocytes and the tumors continue to growth until the end of the study. Whereas no tumors are observed on the flank of the mice injected with Poly (I:C) and ovalbumin-loaded red blood cells treated or not with the anti-TER 119 antibody. Observation of the mice is still ongoing.

10

In conclusion, these results demonstrate that ovalbumin-loaded red blood cells treated or not with the anti-TER 119 antibody are efficient to prevent the growth of OVA expressing tumor cells.

15

Example 14. Measurement of the phagocytosis of pig red blood cells by macrophages and dendritic cells in vivo

This study is a xenogenic study, since pig red blood cells are injected into C57Bl/6 mice.

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The pig red blood cells are washed three times in NaCl containing glucose, before being labelled with CFSE as described above. The red blood cells are washed again three times in NaCl containing glucose, and

then brought back to a haematocrit of 50%. A batch of 142×10^7 pig red blood cells is injected intravenously into three C57Bl/6 mice. One hour after the injection, the mice are sacrificed and the spleens, the livers and the femurs of the mice are removed. The fluorescence incorporated into the macrophages expressing the F4/80 marker or the CD11b marker, into the myeloid dendritic cells expressing the CD11c and CD11b markers and into the plasmacytoid dendritic cells expressing the CD11c and CD8 markers is measured by flow cytometry.

Batch 1: pig red blood cells

10 Batch 2: NaCl containing glucose

Results

Table 18: Percentage of spleen macrophages or dendritic cells having phagocytosed fluorescent red blood cells 1 hour after injection:

15

Batches	F4/80 macrophages	CD11b macrophages	Myeloid dendritic cells	Plasmacytoid dendritic cells
1	27 ± 6	4 ± 2	21 ± 5.5	45 ± 6
2	0	1	4	3

Table 19: Percentage of bone marrow macrophages or dendritic cells having phagocytosed fluorescent red blood cells 1 hour after injection:

20

Batches	F4/80 macrophages	CD11b macrophages	Myeloid dendritic cells	Plasmacytoid dendritic cells
1	5.3 ± 7.5	0.8 ± 1	5 ± 3	5 ± 7
2	6.3	0	6	12.9

Table 20: Percentage of liver macrophages or dendritic cells having phagocytosed fluorescent red blood cells 1 hour after injection:

25

Batche	CD11b macrophages	Myeloid dendritic cells	Plasmacytoid dendritic cells
1	1.1 ± 1.1	3 ±	1.2 ±
2	1.7	3.4	0.7

One hour after injection, the pig red blood cells were efficiently phagocytosed by the F4/80 macrophages and the dendritic cells of the spleen (Table 18). The plasmacytoid dendritic cells of the spleen are the cells most involved in the phagocytosis of the pig red blood cells. In the bone marrow and the liver, the macrophages and the dendritic cells did not phagocytize fluorescent red blood cells (Tables 19 and 20).

In conclusion, the use of pig red blood cells allows targeting and phagocytosis of the red blood cells by the spleen cells involved in the generation of immune responses (Lou Y., 2007, J of Immunol, 178: 1534–1541).

Example 15. Characteristics of pig red blood cells comprising ovalbumin.

Two batches were prepared of pig red blood cells which had either been charged with ovalbumin (obtained in accordance with variant 2 of Example 1) or had not been charged therewith.

Batch 1: Pig red blood cells charged with OVA

Batch 2: Pig red blood cells not charged with OVA.

Batches from the starting material were characterized at the end of production and 18 hours after production. The mean globular volume, the mean corpuscular haemoglobin and the concentration of red blood cells was measured using an ABX cell counter. The osmotic fragility, which corresponds to the concentration of NaCl inducing 50% haemolysis of red blood cells, was measured using an Osmocells instrument (SD Medical). The extracellular haemoglobin was measured by spectrophotometry. The corpuscular concentration of ovalbumin, the extracellular concentration of ovalbumin and the mean globular quantity of ovalbumin were determined using an ELISA test.

Table 21: Characteristics of pig red blood cells comprising ovalbumin:

Characteristic s	Day of production			18 hours after production		
	Starting material	Batch 1	Batch 2	Starting material	Batch 1	Batch 2
Mean globular volume (μm^3)	65	55	57	65	56.0	58
Mean corpuscular haemoglobin (g/dl)	31.9	27.7	25.9	31.4	28.9	25.8
Osmotic fragility (salinity inducing 50% haemolysis in g/l of NaCl)				3.98	2.51	3.33
Haematocrit of suspension (%)	36.8	48.2	50	36.5	46.3	48
Concentration of red blood cells ($10^6/\text{mm}^3$)	5.64	9.1	8.1	5.9	9	8.4
Extracellular haemoglobin (g/dl)	0.04	0.27	0.196	0.05	0.79	0.56
Corpuscular ovalbumin concentration (mg/ml of red blood cells)		0.66	0		0.71	0
Extracellular ovalbumin concentration (mg/ml of end product)		0.02	0		0.02	0
Mean globular quantity (mg/ 10^9 GR)		0.03	0		0.04	0

5 As expected, the dialysis procedure caused a reduction in the globular volume and the corpuscular haemoglobin. Furthermore, the osmotic fragility of the batches was lower than that of the red blood cells of

the initial blood (Table 21). No major differences were observed between the batch comprising the ovalbumin (batch 1) and that which did not comprise it (batch 2).

For batch 1, the quantity of extracellular ovalbumin was relatively low
5 compared with the quantity of encapsulated ovalbumin and had not increased 18 hours after production, thereby demonstrating the stability of the red blood cells encapsulating the ovalbumin.

Thus, it is possible to encapsulate ovalbumin in pig red blood cells.

CLAIMS

1. Composition which induces, in a host, a cytotoxic cellular response against tumour cells, and which comprises red blood cells containing a
5 tumour antigen.

2. Composition according to Claim 1, wherein the red blood cells (1) contain an antigen and (2) are in the form of an immune complex with an immunoglobulin which recognizes an epitope at the surface of the red blood cells, so as to promote phagocytosis of said red blood cells by dendritic
10 cells.

3. Composition according to Claim 2, wherein the red blood cells form an immune complex with an anti-rhesus or anti-glycophorin A or anti-CR1 antibody.

4. Composition according to Claim 2 or 3, wherein the immunoglobulin
15 is an IgG.

5. Composition according to Claim 1, wherein the red blood cells (1) contain an antigen and (2) are heat-treated or chemically treated so as to promote phagocytosis of said red blood cells by dendritic cells.

6. Composition according to Claim 2 wherein the red blood cells in the
20 form of an immune complex are heat-treated or chemically treated.

7. Composition according to any one of Claims 1, 2, 5 or 6, wherein the red blood cells are xenogenic red blood cells.

8. Composition according to any one of Claims 1 to 7, which further comprises an adjuvant for activating dendritic cell maturation.

25 9. Composition according to Claim 8, wherein the adjuvant is present in the red blood cells, at their surface and/or outside the red blood cells.

10. Composition according to Claim 9, wherein the adjuvant is a TLR (Toll-like receptor) ligand or a cytokine.

11. Composition according to Claim 10, wherein the TLR ligand is
30 selected from imidazoquinolines belonging to the group consisting of imidazoquinoline, imiquimod, resiquimod, CpG oligodeoxynucleotides LPSs (lipopolysaccharides) or poly(inosinic acid)-poly(cytidylic acid).

12. Composition according to Claim 10, wherein the cytokine is selected from the group consisting of interferon alpha, IL-2 (interleukin 2), IFN γ (interferon gamma), GM-CSF (Granulocyte Monocyte-Colony Stimulating Factor), IL-12 (interleukin 12) or TNF α (Tumor Necrosis Factor alpha).

5 13. Composition according to any one of Claims 1 to 12, comprising at least two tumour antigens representative of the tumour to be treated.

14. Composition according to any one of Claims 1 to 13, wherein the tumour antigen is selected from the group consisting of the following antigens: alpha-actinin-4; ARTC1; BCR-ABL fusion protein (b3a2); B-RAF; CASP-5; CASP-8; beta-catenin; Cdc27; CDK4; CDKN2A; COA-1; dek-can fusion protein; EFTUD2; Elongation factor 2; ETV6-AML1 fusion protein; FN1; GPNMB; LDLR-fucosyltransferaseAS fusion protein; HLA-A2d; HLA-A11d; hsp70-2; KIAA0205; MART2; ME1; MUM-1f; MUM-2; MUM-3; neo-PAP; Myosin class I; NFYC; OGT; OS-9; pml-RARalpha fusion protein; PRDX5; PTPRK; K-ras; N-ras; RBAF600; SIRT2; SNRPD1; SYT-SSX1 or -SSX2 fusion protein; Triosephosphate Isomerase; BAGE-1; GAGE-1,2,8; GAGE-3,4,5,6,7; GnTVf; HERV-K-MEL; KK-LC-1; KM-HN-1; LAGE-1; MAGE-A1; MAGE-A2; MAGE-A3; MAGE-A4; MAGE-A6; MAGE-A9; MAGE-A10; MAGE-A12; MAGE-C2; mucin k; NA-88; NY-ESO-1 / LAGE-2; SAGE; Sp17; SSX-2; SSX-4; TRAG-3; TRP2-INT2g; CEA ; gp100 / Pmel17 ; Kallikrein 4 ; mammaglobin-A ; Melan-A / MART-1; NY-BR-1 ; OA1 ; PSA ; RAB38 / NY-MEL-1 ; TRP-1 / gp75 ; TRP-2; tyrosinase ; adipophilin; AIM-2 ; BING-4 ; CPSF; cyclin D1 ; Ep-CAM ; EphA3 ; FGF5; G250 / MN / CAIX; HER-2 / neu; IL13Ralpha2; Intestinal carboxyl esterase; alpha-foetoprotein; M-CSF ; mdm-2 ; MMP-2 ; MUC1 ; p53; PBF ; PRAME ; PSMA ; RAGE-1 ; RNF43 ; RU2AS ; secernin 1; SOX10 ; STEAP1; survivin ; Telomerase ; WT1 ; FLT3-ITD ; BCLX(L) ; DKK1 ; ENAH(hMena) ; MCSP ; RGS5 ; Gastrin-17 ; Human Chorionic Gonadotropin, EGFRvIII, HER2, HER2/neu, P501, Guanylyl Cyclase C, PAP.

30 15. Therapeutic anti-tumour vaccine, comprising an effective amount of a composition according to any one of Claims 1 to 14.

16. Use of a composition according to any one of Claims 1 to 14, for the

manufacture of a therapeutic anti-tumour vaccine.

17. Composition according to any one of claims 1 to 14, for use as a therapeutic anti-tumour vaccine.

18. Method for inducing, in a patient, a cytotoxic cellular response
5 against tumour cells or a tumour, comprising the administration to this patient of an effective amount of a composition according to any one of claims 1 to 14.

19. An anticancer treatment method for inducing, in a patient, a cytotoxic cellular immune response against tumour cells or a tumour, comprising the
10 administration to this patient of an effective amount of an anti-tumour vaccine according to claim 15.