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(71) Applicant(s)
SentoClone International AB

(72) Inventor(s)
Winqvist, Ola;Thorn, Magnus

(74) Agent / Attorney
FB Rice, Level 23 44 Market Street, Sydney, NSW, 2000

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(71) Applicant (for all designated States except US): SEN-
TOCLONE THERAPEUTICS AB [SE/SE]; c/o Catomi
Consulting AB, Box 5339, S-102 47 Stockholm (SE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): WINQVIST, Ola
[SE/SE]; Svankärsvägen 32F, S-756 53 Uppsala (SE).
THÖRN, Magnus [SE/SE]; Geijersgatan 21A, S-752 26
Uppsala (SE).

(74) Agent: ALBIHNS A/S; H.C. Andersens Boulevard 49,
DK-1553 Copenhagen V (DK).

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(54) Title: METHOD FOR TREATING MALIGNANT MELANOMA

(57) Abstract: The present invention discloses an immunotherapeutic method for treating patients suffering from malignant melanoma, by administering expanded tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes obtainable from one or more sentinel or metinell lymph nodes draining a malignant melanoma or a metastasis arising from malignant melanoma. The present invention provides a new effective method for treating malignant melanoma and metastatic malignant melanoma, without adverse side effects associated with the known treatments. The method comprises identification of in a patient one or more sentinel and/or metinell lymph nodes draining a malignant melanoma or a metastasis there from, resection of the one or more nodes and, optionally all or part of the tumour or metastasis, isolation of tumour-reactive T- lymphocytes from said lymph nodes, in vitro expansion of said tumour-reactive T- lymphocytes, and administration of the thus obtained tumour-reactive T-lymphocytes to the patient, wherein the T-lymphocytes are CD4+ helper and/or CD8+ T-lymphocytes.

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Method for treating malignant melanoma

This application is the Australian national phase of International Patent Application No. PCT/EP2006/012306 filed December 20, 2006, which claims priority benefit of Danish
5 application no. PA 2005 01809 filed December 21, 2005 and USSN 60/752,834 filed
December 21, 2005 and USSN 60/843,246 filed September 7, 2006, which applications
are incorporated herein by way of reference in their entirety.

Field of the invention

10 The invention relates to an immuno-therapeutically method for treating patients
suffering from malignant melanoma, by administering expanded tumour-reactive CD4+
helper and/or CD8+ T-lymphocytes obtainable from one or more sentinel or metinell
lymph nodes draining a malignant melanoma or a metastasis arising from malignant
melanoma, wherein the T-lymphocytes are not CD4+ CD25^{HI} lymphocytes, i.e. the
15 present invention does not cover regulatory T-lymphocytes.

Background of the invention

Human melanoma represents the principal cause of death in patients with skin cancer
in United States and Europe. Melanoma is estimated to be the fifth and seventh most
20 common cancers in men and women, respectively, among new cases of cancer in the
United States in 2004. An increase of more than 600% in the diagnosis of cutaneous
cancer has been seen from 1950 to 2000.

Rational treatment strategies for stage I and II patients have developed to optimize
25 survival and minimize treatment morbidities

The immune system often appears informed about tumours, as shown by an
accumulation of immune cells at tumour sites, which correlates with improved
prognosis. Immuno competent cells respond to "danger" signals, which can be
30 provided of growing tumours as a consequence of the genotoxic stress of cell
transformation and disruption of the surrounding microenvironment. Under ideal
conditions, these signals will induce inflammation, activate innate effector cells with
antitumour activity, and stimulate professional antigen-presenting cells (APCs),
particularly dendritic cells (DCs), to engulf tumour-derived antigens and migrate to
35 draining lymph nodes to trigger an adaptive response by T and B lymphocytes. Thus,
the immune system is capable of recognizing and eliminating tumour cells but

unfortunately tumours often interfere with the development and function of immune responses. However, recent advances in cellular and molecular immunology suggest strategies, which may prevent antitumour responses. Briefly, the presence of a tumour indicates that the developing cancer was able to avoid detection or to escape or to overwhelm the immune response. Progressing tumours often exhibit strategies that promote evasion from immune recognition. Examples are physical exclusion of immune cells from tumour sites, poor immunogenicity due to reduced expression of major histocompatibility complex (MHC) or costimulatory proteins, and disruption of natural killer (NK) and natural killer T (NKT) cell recognition. Further, some tumours prevent triggering of an inflammatory response by secreting proteins such as interleukin 10 (IL-10) or vascular endothelial growth factor (VEGF) that interfere with DC activation and differentiation, or by blocking the production of proinflammatory molecules by increasing expression of the STAT3 protein. Even if a response is induced, tumour cells may escape elimination by losing targeted antigens, rendering tumour-reactive T cells anergic, inducing regulatory T cells, or specifically deleting responding T cells. The tumour that finally develops reflects selection of poorly immunogenic and/or immune-resistant malignant cells.

In the adjuvant setting, tumour immunotherapy offers an appealing alternative to traditional cytostatics. One strategy has been to expand and activate NK cells *in vitro* with out specific antigen by culture with IL-2 followed by infusion of large numbers of these NK cells back into patients alone or with high doses of IL-2. This approach, or administration of high doses of IL-2 to expand and activate NK cells entirely *in vivo*, has yielded antitumour activity and remission in a subset of patients (Rosenberg SA et al., J Natl Cancer Inst 85, 622, 1993). However, life-threatening toxicity often develops, largely due to the release of tumour necrosis factor (TNF) from activated NK cells.

Other attempts to stimulate the innate specific T cell immunity have been done by different types of vaccines. Promising results from animal studies entailed a study in which autologous tumour cells and an adjuvant immunomodulating agent, bacillus Calmette-Guérin (BCG) was given in combination several times to 98 patients with colorectal cancer in a prospectively randomized study. No statistically significant differences were detected in survival but maybe a small decrease in recurrence rate in stage II colon cancer patients.

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Thus, it is obvious that there is still a need for an effective and at the same time safe treatment of malignant melanoma and metastases thereof.

Summary of the invention

5 In one example, the present invention provides a method of producing T lymphocytes for the treatment of malignant melanoma comprising:

- (i) identifying in a patient one or more sentinel and/or metinell lymph nodes draining a malignant melanoma or a metastasis there from;
- (ii) resecting the one or more nodes and, optionally all or part of the tumour or
10 metastasis;
- (iii) isolating tumour-reactive T-lymphocytes from said lymph nodes; and
- (iv) *in vitro* expanding said tumour-reactive T-lymphocytes by a process comprising:

- (a) a first phase comprising stimulating tumour-reactive CD4+ helper
15 and/or CD8+ T-lymphocytes with tumour-derived antigen together with at least one substance having agonistic activity towards the IL-2 receptor (IL-2R) to thereby promote survival of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes; and
- (b) a second phase comprising activating and promoting growth of
20 tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes, wherein said second phase is initiated when CD25 or IL-2R is down-regulated on T-lymphocytes,

thereby producing tumour-reactive T-lymphocytes comprising CD4+ helper and/or
CD8+ T-lymphocytes wherein said T lymphocytes are reactive against malignant
25 melanoma.

In another example, the present provides a method for treating a patient suffering from malignant melanoma, the method comprising:

- (i) identifying in a patient one or more sentinel and/or metinell lymph nodes
30 draining a malignant melanoma or a metastasis there from;
- (ii) resecting the one or more nodes and, optionally all or part of the tumour or metastasis;
- (iii) isolating tumour-reactive T-lymphocytes from said lymph nodes;
- (iv) *in vitro* expanding said tumour-reactive T-lymphocytes, wherein the *in vitro*
35 expansion comprises:

2b

- 5 (a) a first phase of stimulating tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes with tumour-derived antigen together with at least one substance having agonistic activity towards the IL-2 receptor (IL-2R) to promote survival of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes, and
- 10 (b) a second phase of activating and promoting growth of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes, wherein said second phase is initiated when the CD25 cell surface marker (or IL-2R marker) is down-regulated on T-lymphocytes; and
- 15 (v) administering the thus obtained tumour-reactive T-lymphocytes to the patient wherein the T-lymphocytes are CD4+ helper and/or CD8+ T-lymphocytes.

15 The lymph nodes may drain a primary tumour and/or a metastasis, such that the T-lymphocytes are derived from the primary tumour and/or metastasis and/or blood. The identification of one or more lymph nodes at (i) *supra* may comprise injecting one or more lymph node locators e.g., affinity-based or non-affinity locators, into the patient. In a further example, the one or more lymph node locators are affinity based. In yet another example, the one or more lymph node locators are non-affinity based. The

20 one or more lymph node locators may be injected into, above, around, adjacent and/or under the tumour or metastasis by a single injection or by multiple injections. The injection(s) may be by means of or form part a non-surgical procedure or a surgical procedure. Resection of lymph nodes may comprise removal of all or part of the tumour or metastasis from the patient. The T-lymphocytes are isolated and/or cultured

25 in a culture medium e.g., a serum-free medium e.g. AIMV, RPMI 1640, DMEM or MEM.

30 For expansion of tumour-reactive T-lymphocytes, the employed tumour-derived antigen may consist of or comprise a protein, polypeptide or peptide e.g., malignant melanoma-derived antigen. It is also to be understood that addition of a tumour-derived antigen encompasses addition of a denatured homogenate of a tumour e.g., a malignant melanoma.

35 A tumour-derived antigen may be added from day 2 up to and including day 5 of the first phase (a), such as, e.g., on day 2, on day 3, on day 4 or on day 5. Alternatively, or in addition, a tumour-derived antigen may be added when phase (a) is initiated or at essentially the same time. Alternatively, or in addition, a tumour-derived antigen may

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be added at the most up to 3 days following initiation of phase (a) or essentially the same time as initiating phase (a).

5 An exemplary substance having agonistic activity towards the IL-2 receptor consists of or comprises IL-2. The IL-2 may be added at a low dosage e.g., from about 100 IU/ml culture medium to about 700 IU/ml culture medium, or from about 100 IU/ml culture medium to about 600 IU/ml culture medium, or from about 100 IU/ml culture medium to about 500 IU/ml culture medium, or from about 100 IU/ml culture medium to about 400 IU/ml culture medium, or from about 100 IU/ml culture medium to about 300 IU/ml
10 culture medium or from about 100 IU/ml culture medium to about 200 IU/ml culture medium. It is to be understood that a substance having agonistic activity towards the IL-2 receptor may added regularly throughout phase (a) and optionally, regularly throughout phase (b) e.g., every 2nd, 3rd or 4th day from commencement of phase (a), and optionally, regularly throughout phase (b) e.g., every 2nd, 3rd or 4th day from
15 commencement of phase (b).

The second phase (b) may be initiated from day 17 up to and including day 23 of first phase (a) e.g. on day 17 or on day 18 or on day 19 or on day 20 or on day 21 or on day 22 or on day 23 from commencement of the first phase (a). The second phase may be
20 initiated by the addition of a tumour-derived antigen, e.g., a tumour protein, polypeptide or peptide such as comprised within a denatured homogenate of a tumour, to the T-lymphocytes for activating tumour-reactive CD25-negative T-lymphocytes. In one example, malignant melanoma-derived antigen is added to initiate the second phase.

25 In a further example, the second phase (b) comprises adding at least one substance capable of up-regulating IL-12R on the T-lymphocytes e.g., one or more substances having agonistic activity towards an interferon receptor. An exemplary substance having agonistic activity towards an interferon receptor consists of or comprises an interferon, such as interferon- α . A substance capable of up-regulating IL-12R on the T-
30 lymphocytes may be added, for example, when the level of IL-12 is at least 1-fold the level of IL-12 at day 1 of phase (b). This may include adding a substance capable of up-regulating IL-12R on the T-lymphocytes when the level of IL-12 is at least 2-fold or at least 3-fold or at least 4-fold or at least 5-fold the level of IL-12 at day 1 of phase (b). Alternatively, a substance capable of up-regulating IL-12R on the T-lymphocytes is
35 added from day 2 up to and including day 4 after the initiation of second phase (b), e.g. on day 2 or on day 3 or on day 4 from day 1 of phase (b).

2d

Alternatively, or in addition, the second phase (b) comprises adding one or more substances capable of antagonizing development of Th2 type T-lymphocytes e.g., a substance capable of neutralizing IL-4 and/or IL-5 and/or IL-10 and/or TGF-beta. For example, anti IL-4 antibody and/or anti IL-5 antibody and/or anti IL-10 antibody may be employed to antagonize development of Th2 type T-lymphocytes. The antagonist may be added on day 1 of the second phase (b). It is to be understood that an antagonist of Th2 type T-lymphocytes may be employed in conjunction with the addition of a substance capable of up-regulating IL-12R on the T-lymphocytes e.g., wherein the antagonist of Th2 type T-lymphocytes is added throughout phase (b) and advantageously commencing subsequent to addition of the IL-12R regulatory substance. For example, one or more substances capable of antagonizing development of Th2 type T-lymphocytes may be added one day following addition of the substance IL-12R regulatory substance. The substance(s) capable of antagonizing development of Th2 type T-lymphocytes may also be added every 2nd or 3rd or 4th day of phase (b).

Alternatively, or in addition, the second phase (b) comprises adding one or more substances promoting the development of Th1 type T-lymphocytes e.g., substance(s) having agonistic activity towards the IL-7 and/or IL-12 and/or IL-15 and/or IL-21 receptor(s). IL-7 and/or IL-12 and/or IL-15 and/or IL-21 may be employed to promote development of Th1 type T-lymphocytes. The one or more substances promoting the development of Th1 type T-lymphocytes may be added when the level of IFN-gamma is increased compared to the level of IFN-gamma at initiation of second phase (b), for example when the level of IFN-gamma increases at least 1-fold or at least a 2 fold or at least a 3 fold or at least a 4 fold compared to the level of IFN-gamma on initiation of the second phase (b). Alternatively, or in addition, the substance(s) promoting the development of Th1 type T-lymphocytes may be added when CD25 and/or CD69 are down-regulated. Generally, one or more substances promoting the development of Th1 type T-lymphocytes is added to a concentration of from about 150 IU/ml culture medium to about 300 IU/ml culture medium, including e.g. 250 IU/ml culture medium. The one or more substances promoting the development of Th1 type T-lymphocytes are added from day 5 up to and including day 8 from commencement of second phase (b), such as, on day 5 or day 6 or day 7 or day 8.

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2e

The method of this example described according to any embodiment hereof may further comprise adding antigen presenting cells together with the tumour-derived antigen to the T-lymphocytes. The antigen presenting cells may be comprised in a preparation of irradiated peripheral blood leucocytes (PBLs) containing antigen-presenting B-cells and/or monocytes.

In a further example, the method of the present invention according to any embodiment hereof may further comprise monitoring the expression of cell surface markers e.g., CD25 and/or CD69 on the T-lymphocytes continuously during phase (a) and phase (b).

In a further example, the method of the present invention according to any embodiment hereof may further comprise harvesting the T-lymphocytes when CD25 on T-lymphocytes in the second phase (b) is down-regulated e.g., when 5% or less of the CD4 positive T-lymphocyte population express CD25. Tumour-reactive T-lymphocytes may be harvested from day 10 up to and including day 14 after initiating the second phase (b). The method of the present invention further provides for purification of the harvested T-lymphocytes and/or storing e.g., by freezing the T-lymphocytes obtained in phase (b).

In a further example, the method of the present invention according to any embodiment hereof may further comprise subjecting the T-lymphocytes to at least one additional round of phase (b) when CD25 on T-lymphocytes is down-regulated e.g., when 5% or less of the CD4 positive T-lymphocyte population expresses CD25.

Administration of the tumour-reactive T-lymphocytes may be via an intravenous, intra-arterial, intrathecal or intraperitoneal administration route. For example, at least 10 million tumour-reactive T-lymphocytes may be administered. Alternatively, at least 20 million or at least 30 million or at least 40 million or at least 50 million or at least 60 million or at least 70 million or at least 80 million tumour-reactive T-lymphocytes may be administered. It is to be understood that the administered tumour-reactive T-lymphocytes may be a combination of effector T-lymphocytes and memory T-lymphocytes e.g., wherein the percentage of effector T-lymphocytes is from about 10% to about 65%, or from about 20% to about 50% or from about 30% to about 40%. The administered tumour-reactive T-lymphocytes may be autologous T-lymphocytes, or they may be non-autologous.

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The method of the present invention described according to any embodiment hereof is useful for the preparation of CD4+ helper T lymphocytes and/or for the preparation of effector T-lymphocytes and/or for the preparation of memory T-lymphocytes and/or for the preparation of Th1 type T-lymphocytes.

5

A further example of the present invention provides a tumour-reactive T-lymphocyte e.g., a CD4+ helper T-lymphocyte or an effector T-lymphocyte or a memory T-lymphocyte or a combination thereof, including a Th1-type T lymphocyte, when prepared by the method of the present invention as described according to any

10 embodiment hereof.

A further example of the present invention provides for use of a tumour-reactive T-lymphocyte of the present invention as described according to any embodiment hereof in the preparation of a medicament for the treatment of disseminated cancer.

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A further example of the present invention provides a kit comprising a media for cultivation of T-lymphocytes e.g. AIMV, RPMI 1640, DMEM or MEM in a method of the present invention as described according to any embodiment hereof. Exemplary kits comprise one or more substances for stimulating, activating and directing tumour-

20 reactive T-lymphocytes such as one or more tumour-derived antigens and/or one or more substances having agonistic activity towards the IL-2 receptor and/or one or more substances capable of up-regulating IL-12R on T-lymphocytes and/or one or more substances capable of antagonizing development of Th2 type T-lymphocytes and/or one or more substances promoting the development of Th1 type T-lymphocytes. In

25 one example, a substance having agonistic activity towards the IL-2 receptor consists of or comprises IL-2; and/or a substance capable of up-regulating IL-12R on T-lymphocytes consists of or comprises an interferon e.g., interferon- α ; and/or a substance capable of antagonizing development of Th2 type T-lymphocytes consists of or

30 comprises a substance that neutralizes IL-4 and/or IL-5 and/or IL-10 and/or TGF-beta such as e.g., anti IL-4 antibody and/or anti IL-5 antibody and/or anti IL-10 antibody; and/or a substance for promoting the development of Th1 type T-lymphocytes consists of or comprises IL-7 and/or IL-12 and/or IL-15 and/or IL-21. A further example of the present invention provides for the kit to be formulated as a pharmaceutical composition suitable for intravenous administration.

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A further example of the present invention provides a kit comprising a syringe and a lymph node locator, such as for use in a method of the present invention as described according to any embodiment hereof.

It is within the scope of the present invention for a kit to be provided with instructions or indications for use, such as instructions in the form of computer software.

The present invention further provides for a method or product thereof, or a use of such a product, or a kit as described according to any embodiment hereof essentially as described herein with reference to the accompanying Figures and/or Examples.

As used throughout this specification and in the claims that follow, and unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Any references herein to a documentary disclosure, act, material or the like is not to be construed as an admission that such disclosure was common general knowledge in the field relevant to the present invention as it existed before the priority date of any claim.

Disclosure of the invention

Accordingly, the present invention discloses a new effective method for treating malignant melanoma and metastatic malignant melanoma, without adverse side effects associated with the known treatments.

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Adoptive immunotherapy, i.e. collection and *in vitro* expansion of autologous tumour-reactive lymphocytes, followed by transfusion back to the patient has never been done for treating malignant melanoma. The present inventors described herein a very

5 successful attempt to use sentinel or metinel node acquired lymphocytes for immunotherapy of malignant melanoma. In short, the tumour or metastasis draining lymph node(s) is identified by injection of a lymph node locator. The nodes contain T-lymphocyte clones that have been naturally activated against tumour-derived antigens *in vivo*. This specific T lymphocyte population is further expanded using an improved

10 method for expansion of tumour-reactive T-lymphocytes, wherein specific culturing conditions have been determined and optimized, and wherein specific markers on the T-lymphocytes and in the culture medium are monitored throughout the expansion phase, in order to obtain high numbers of tumour-reactive T-lymphocytes in the shortest possible time span.

15

Accordingly, the present invention relates to a method for treating patients suffering from malignant melanoma, the method comprising

- i) identifying in a patient one or more sentinel and/or metinel lymph nodes draining a malignant melanoma or a metastasis there from,
- 20 ii) resecting the one or more nodes and, optionally, all or part of the tumour or metastasis,
- iii) isolating tumour-reactive T-lymphocytes from said lymph nodes,
- iv) *in vitro* expanding said tumour-reactive T-lymphocytes,
- v) administering the thus obtained tumour-reactive T-lymphocytes to the
- 25 patient,

wherein the T-lymphocytes are CD4+ helper and/or CD8+ T-lymphocytes and not CD4+ CD25^{Hi} lymphocytes, i.e. the present invention does not cover regulatory T-lymphocytes.

30 Before going in to further details with the steps of the method of the invention, the following terms will be defined:

By the term "tumour-reactive T-lymphocytes" is intended to mean T-lymphocytes carrying a T cell receptor (TCR) specific for and recognizing a tumour antigen. Herein

35 the term tumour-reactive T-lymphocytes also tends to cover T-lymphocytes carrying a

TCR specific for and recognizing metastasis antigens. I.e. the terms tumour-reactive T-lymphocytes and metastasis-reactive T-lymphocytes may be used interchangeably.

5 By the term "tumour-reactive T-lymphocytes" is intended to mean T-lymphocytes carrying a T cell receptor specific for and recognizing a tumour antigen.

By the term "T helper cells" is intended to mean T-lymphocytes that promote adaptive immune responses when activated.

10 By the term "Th1 cells" is intended to mean T helper cells that promote cell mediated immune responses when activated, using cytokines such as IFN-gamma.

By the term "Th2 cells" is intended to mean T helper cells promoting humoral immune responses when activated, using cytokines such as IL-4.

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By the term "CD4+ helper T-lymphocytes" is intended to mean T-lymphocytes that express CD4 but not the transcription factor FoxP3.

20 By the term "CD8+ T-lymphocytes" is intended to mean T-lymphocytes that express CD8.

By the term "regulatory T-lymphocyte" is intended to mean T-lymphocytes that suppress adaptive immune responses, expressing transcription factor FoxP3.

25 By the term "specific activation" of T-lymphocytes is intended to mean antigen specific and MHC restricted T-cell receptor mediated activation. In contrast the term "unspecific activation" of T-lymphocytes is intended to mean a general activation of all T-cells, regardless of T-cell receptor specificity.

30 The term "tumour-derived antigen" intends to cover tumour cells, a homogenate of a tumour, which homogenate may be denatured, or tumour proteins, polypeptides or peptides, e.g. in the form of purified, natural, synthetic and/or recombinant protein, polypeptide or peptide. The tumour-derived antigen may be intact molecules, fragments thereof or multimers or aggregates of intact molecules and/or fragments.

35 Examples of suitable polypeptides and peptides are such that comprises from about 5 to about 30 amino acids, such as, e.g. from about 10 to 25 amino acids, from about 10

to 20 amino acids or from about 12 to 18 amino acids. If peptides are used, a final molar concentration in the culture of from about 0.1 to about 5.0 μM , such as, e.g., from about 0.1 to about 4.0 μM , from about 0.2 to about 3.0 μM , from about 0.3 to about 2.0 μM or from about 0.3 to about 1.0 μM may be used. The tumour-derived antigen may
5 be autologous or heterologous, i.e. arise from the patient to be treated or be obtained from another subject suffering from cancer. In the present Examples the inventors uses an autologous denatured tumour extract, however, as mentioned above, other sources of the tumour-derived antigen may also be feasible for use in a method according to the invention.

10

By the term "day 1 of the first phase" or e.g. "day 5 of the second phase" is to be understood the following: The day on which the lymphocytes are harvested is denoted day 0 (zero). Day 1 of the first phase is defined as the day where the expansion is initiated by addition of at least one substance having agonistic activity towards the IL-2
15 receptor, and maybe culture medium and/or tumour-derived antigen. The expansion phase i) may be initiated on day 0 (zero) or up till 2 days after harvest of the lymphocytes. The day on which the second phase is initiated by addition of tumour-derived antigen is throughout the text described as "day 1 of the second phase".

20 By the term "sentinel lymph node" is intended to mean the first lymph node(s) to receive lymphatic drainage from a tumour. Primary tumours or primary tumour areas drain to one or more so-called sentinel lymph nodes. The sentinel nodes are also the first site of metastasis and it has been shown in several solid tumour types that the risk of lymph node metastases is almost negligible if the sentinel node is free of tumour
25 cells. The term "metinel lymph node" refers to the first lymph node(s) to receive lymphatic drainage from a metastasis or a metastases area.

The term "malignant melanoma" embraces any stage including stage I, II, III and IV and substages thereof. A person skilled in the art will know that dependent on the specific
30 stage of the disease a specific treatment regimen may be necessary such as, e.g., further administration of the lymphocytes obtained as described herein and/or relevant combination treatment with drugs normally used for the treatment of malignant melanomas or metastasis thereof. Furthermore, it is contemplated that variation of the expansion method may depend on the stage of the malignant melanoma. The method
35 given herein is a general method that normally is regarded as applicable for any stage, however, some optimization may be needed for the specific stage.

The first step in the present method is the identification of one or more sentinel or metinel lymph nodes draining a malignant melanoma or a metastasis there from. The step i) of identifying one or more sentinel or metinel nodes are crucial for the method according to the invention, as the present inventors have shown that these nodes comprises a high amount of T-lymphocytes activated against tumour-derived antigens *in vivo*.

One way of identifying the sentinel or metinel lymph node is by injecting one or more lymph node locators into the patient, i.e. any substances suitable for locating a lymph node. Such locators are preferably pharmaceutically acceptable and/or biocompatible. The locators can either be affinity based or non-affinity based. Examples of affinity based lymph node locators are antibodies in whole or fragments, nanobodies, nucleic acids such as RNA, DNA, and PNA all of which can be in turn labelled using various detection modalities. Detection of affinity based lymph node locators can be done by labeling with tracers and dyes, such as, e.g., the ones mentioned below. Visualization is then made by i) radiological methods such as x-ray, computerized tomography, scintigraphy, positron emission technique after labelling with contrast generating substances, such as, e.g., iodine containing substances or radioactive substances such as, e.g. technetium-99m, ii) magnetic resonance imaging after labelling with magnetic or paramagnetic substances, such as e.g., gadolinium, magnetodendromers or iron oxide containing particle; iii) light in the IR-visible- UV spectra by labelling with dyes, fluorescent dyes or luminescent dyes for detection by the naked eye or photon detecting devices such as CCD or CMOS sensors.

Examples of non-affinity based lymph node locators encompass tracers and dyes. These substances are transported in the lymph capillaries and accumulate through phagocytosis by macrophages in the sentinel or metinel node(s), thus identifying the tumour or metastasis draining lymph node(s).

Examples of tracers are radioactive substances such as, e.g., technetium-99 for radioactive decay based detection with photon sensitive films or sensors such as PET detectors. Further on magnetic, paramagnetic or superparamagnetic substances, such as, e.g., gadolinium containing contrast agents, iron oxide particles, magnetic oxide particles, magnetodendrimers for magnetic resonance based detection, contrast

agents, such as, e.g., iodine for radiological based detection such as, e.g., computerized tomography or regular X-ray may be used.

5 Examples of dyes encompasses e.g., azo dyes, bisazo dyes, triazo dyes, diaryl methan dye, triaryl methan dye, anthrachino dye, polycyclic aromatic carbonyl dyes, indigo dyes for visualization by luminescence, near infrared, fluorescence, UV and visible light. Further on dyes also encompass luminescent substances for luminescence based detection and fluorescent substances, such as, e.g., pico green, sybr green, red O oil, texas red for fluorescence based detection. Detection can depending on the
10 chosen wavelengths be made either by the naked eyes or photon detecting devices such as CCD or CMOS sensors.

In one embodiment the dye has an emission maximum that permits visualization by the naked eye in normal light. In another embodiment the dye has an emission maximum
15 that permits visualization by the naked eye in UV light.

Other examples of suitable dyes or tracers appear from WO 04/045650, which is hereby incorporated by reference.

20 Another, but far more time-consuming way to identify sentinel or metinel nodes is to remove and investigate a selection of lymph nodes in the presumed tumour or metastasis area. A tumour extract from the tumour or metastasis of the actual patient could then be used to identify lymph nodes containing tumour-reactive T-lymphocytes by proliferating assays.

25 The lymph node locators are injected into the patient into, above, around, adjacent and/or under the tumour or metastasis. The locator will then spread through lymph vessels leading into the metinel lymph node(s), and the one or more nodes will start to get stained within a certain period of time, such as, e.g. within 5 min to 30 min, such as,
30 e.g. within 5 min to 15 min after injection of the locator substance, where after the locator substance is imaged. As described above, imaging of the locator is of course dependent on the locator substance used.

If a dye having an emission maximum that allows visualization by the naked eye in
35 normal light is used, such as, e.g. Patent Blue, the one or more sentinel or metinel nodes are simply identified as the nodes, which are first to accumulate the coloured

dye, i.e. if Patent Blue is used, the surgeon will look for the lymph nodes first to accumulate a blue colour.

The locators may be injected by a single injection or by multiple injections, such as,
5 e.g., by two or more injections, by three or more injections, by four or more injections, by five or more injections or by six or more injections.

How to perform the injections of the lymph node locators is dependent on the location of the tumour or metastasis.

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In case of a malignant melanoma, the lymph node locators may be injected involving a surgical procedure, i.e. a procedure that includes an incision. In such cases, the surgeon will perform an incision in the area of the tumour and subsequently, a lymph node locator may be injected directly into, above, around, adjacent and/or under the
15 metastasis in order to identify the one or more sentinel lymph nodes. The lymph node locators may also be injected by a non-surgical procedure, i.e. a procedure that does not involve a surgical step, wherein a surgical step is defined as one including surgical operative procedures, i.e. involving incisions with an instrument. In the present context, an injection, i.e. the punctuation of the skin with a needle, is not considered a surgical
20 step. Accordingly, by the statement that the lymph node locator may be injected by a non-surgical procedure is intended to mean that the lymph node locator may be injected into, above, around, adjacent and/or under the metastasis directly into or through the skin.

25 Examples of situations wherein the lymph node locators may be injected into or through the skin, is e.g. cases where metastasis of malignant melanomas are located in the skin of the patient. In such situations the lymph node locator should preferentially be injected into the skin above the metastasis, or through the skin into, around, adjacent and/or under the metastasis. If the metastasis is located in the breast area of
30 the patient, the lymph node locator should preferentially be injected into the skin above the metastasis, or through the skin into, around, adjacent and/or under the metastasis.

In case of a metastasis of a malignant melanoma, the sentinel lymph nodes may not always be placed at the shortest or most logical anatomical distance from the
35 metastasis. Accordingly, as a sentinel lymph node may be placed distant from the metastasis, it may in some cases be very beneficial to inject a lymph node locator

without the need for surgery, as it may be very difficult to predict the position of such a metinel lymph node, and there by difficult to predict the place in the body to perform the surgery to remove the metinel node. In a specific embodiment of the invention, the lymph node locator is a radioactive substance, such as, e.g. technetium-99m, which
5 may be injected by a non-surgical procedure, and later imaged by performing a lymphoscintigraphy.

Some times the identification of the metinel lymph nodes may involve injection of lymph node locators by a combination of a non-surgical and a surgical step. As an example of
10 this a radioactive lymph node locator, such as, e.g., technetium-99m may be injected using a needle, i.e. without the need for surgery, and the accumulation of the locator, i.e. identification of the metinel node(s) may be performed using a gamma detector. This gives the surgeon an indication towards where the metinel nodes are located. Later on, when the patient is undergoing surgery to have the metinel lymph nodes and
15 at least part of metastasis removed, a coloured dye such as, e.g., Patent Blue Dye may be injected. Furthermore, if there is a lapse of more than about 18 to 24 hours after the first injection, it might be beneficial to add one or more extra injections with radioactive tracer dependent on the half-life of the radioactive tracer (usually about 6 hours) in order to identify the metinel nodes during surgery.

20 After having located the one or more sentinel or metinel lymph nodes by one or the other method, the surgeon will remove these in order to investigate whether the sentinel or metinel lymph nodes contain any tumour cells, and in order to obtain a culture of tumour-reactive T-lymphocytes.

25 The harvesting of lymphocytes from the one or more sentinel or metinel lymph nodes may be performed by homogenizing the sentinel or metinel lymph node material in order to obtain single cell suspensions of lymphocytes. The single cell suspensions may then be subjected to *in vitro* expansion in order to obtain tumour-reactive T-
30 lymphocytes.

In vitro expansion

The *in vitro* expansion step iv) of the method according to the invention comprises
i) a first phase of stimulating tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes
35 with tumour-derived antigen together with at least one substance having agonistic

activity towards the IL-2 receptor, to promote survival of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes, and

- ii) a second phase of activating and promoting growth of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes, wherein the second phase ii) is initiated when the CD25 cell surface marker (IL-2R marker) is down-regulated on T-lymphocytes.

Phase i)

The purpose of the first phase i) is to obtain a culture comprising a substantially high ratio of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes. The first phase is to be considered a "nursing phase" where the tumour-reactive T-lymphocytes are brought to survive and divide. Depending on the source of the T-lymphocytes (starting material for the *in vitro* expansion method), they may have phased relatively harsh conditions, such as, e.g., suppression and inhibition by factors secreted by cancer cells.

- The starting material for use in the expansion method may be a mixture of lymphocytes and antigen presenting cells obtained from lymph nodes draining a metastasis.

The T-lymphocytes to be expanded in culture can be obtained from the subject to be treated, i.e. the resulting specific tumour-reactive T-lymphocytes for administering may be autologous. However, the T-lymphocytes can also be obtained from a source other than the subject to be treated, such as, e.g. another subject suffering from a cancer. In such case the recipient and the expanded tumour-reactive T-lymphocytes are preferably immunologically compatible (or the recipient is otherwise made immunotolerant of the expanded tumour-reactive T-lymphocytes).

The starting material will most likely comprise a mixture of various lymphocytes, such as, e.g., T-lymphocytes, B-lymphocytes, antigen presenting cells, tumour-reactive T-lymphocytes and non-activated/non-reactive T-lymphocytes. In order to promote survival specifically of the tumour-reactive T-lymphocytes, tumour-derived antigen and one or more substances having agonistic activity towards the IL-2 receptor are added.

As mentioned above the first phase i) is initiated by adding at least one substance having agonistic activity towards the IL-2 receptor. The function of such substances is to stimulate T-lymphocytes via the IL-2 receptor to promote cell division of T-lymphocytes, thereby preventing cell death.

Antigen specific MHC restricted activation of T-lymphocytes promotes clonal expansion of the useful T-lymphocyte population specific for the recognition of tumour cells. On the contrary, unspecific activation of T lymphocytes will lead to the expansion of T lymphocyte clones recognizing irrelevant peptides without any relation to the
5 recognition of tumour cells, thus the majority of unspecifically expanded T lymphocytes will not recognize the tumour.

The invention aims to promote specific activation and growth of tumour-reactive CD4+ helper and CD8+ T-lymphocytes. A specific activation against a certain tumour antigen
10 enables the T-lymphocytes to have therapeutic effect when administered to a cancer patient with the same tumour type as the T-lymphocytes are activated against.

In one embodiment of the invention the substances having agonistic activity towards the IL-2 receptor are agonists. Examples of such substances include proteins,
15 polypeptides, peptides, antibodies, affibodies, and fragments thereof, fusion proteins, synthetic and/or organic molecules, such as, e.g., small molecules, and natural ligands. In a preferred embodiment the substance is the natural ligand of the IL-2 receptor, namely IL-2.

20 If IL-2 is used it is preferentially added in a low dose in order to reduce lymphocyte apoptosis and to increase the population of CD4 positive tumour-reactive T-lymphocytes. In a specific embodiment of the invention, the low dose of IL-2 is from about 100 IU/ml culture medium to about 700 IU/ml culture medium, such as, e.g., from about 100 IU/ml culture medium to about 600 IU/ml culture medium, from about 100
25 IU/ml culture medium to about 500 IU/ml culture medium, from about 100 IU/ml culture medium to about 400 IU/ml culture medium, from about 100 IU/ml culture medium to about 300 IU/ml culture medium and from about 100 IU/ml culture medium to about 200 IU/ml culture medium. In a specific embodiment, the amount of IL-2 added is 240 IU/ml.

30 In case other substances, than IL-2, having agonistic activity towards the IL-2 receptor are used the specific doses of these should be such that lead to an effect corresponding to the effect obtained by the above-mentioned doses of IL-2.

35 A further amount of the at least one substance having agonistic activity towards the IL-2 receptor may be added regularly throughout phase i), such as, e.g., every 2nd, 3rd or

4th day of phase i), in order to maintain optimal conditions for promoting cell division. By the term every 2nd, 3rd or 4th is intended to mean that at least one substance having agonistic activity towards the IL-2 receptor is added throughout phase i) every 2nd, 3rd or 4th day, starting at the 2nd, 3rd or 4th day after the first addition of the at least one substances having agonistic activity towards the IL-2 receptor, i.e. after initiating phase i).

In one embodiment the substance to be added regularly throughout phase i) is an agonist of IL-2. In a preferred embodiment the substance is IL-2.

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The further dose of substances having agonistic activity towards the IL-2 receptor, such as, e.g., IL-2, to be added regularly, such as, e.g. every 2nd, 3rd, or 4th day lies within the ranges mentioned above.

15 A further important step in the first phase i) of expansion is the addition of tumour-derived antigen in order to promote cell division of T-lymphocytes expressing T lymphocyte receptors recognizing tumour antigens, i.e. tumour-reactive T-lymphocytes.

The optimal point of time to add the tumour-antigen is depending on the source of lymphocytes. When the lymphocytes originates from lymph nodes the lymphocytes may have been subjected to close proximity and immuno-suppression by tumour cells, and need incubation with a substance having agonistic activity towards the IL-2 receptor, such as, e.g., IL-2 for some days in order to promote the ability of the T-lymphocytes to respond with proliferation upon tumour antigen presentation.

20 Accordingly, in such case the tumour-derived antigen is preferentially added from day 2 to and including day 5 of the first phase i), such as, e.g., on day 2, on day 3, on day 4 or on day 5.

The tumour-derived antigen, such as, e.g., a tumour homogenate, is likely to be endocytosed and processed by antigen presenting cells present in the starting material, such as, e.g., B-lymphocytes, dendritic cells and macrophages. In most cases the tumour-derived antigen will be presented by class II MCH molecules leading to cell division of CD4⁺ tumour-reactive T-lymphocytes. However, by cross presentation antigens taken up by endocytosis may be processed and presented in the class I pocket resulting in activation of CD8⁺ T lymphocytes. As stated above, one of the objects of the expansion method is to in some respect imitate the natural pathway of

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the patients own immune system, and to a certain degree let the components of the patients immune system determine whether CD4⁺ or CD8⁺ lymphocytes are generated, depending on whether antigen is presented by MCHI or MCHII. In most cases, the antigens will be presented by the class II MCH molecule leading to generation of CD4⁺ T-lymphocytes, however, in some cases CD8⁺ T-lymphocytes are generated.

Phase ii)

The purpose of the second phase ii) is to activate and expand the tumour-reactive CD4⁺ helper and/or CD8⁺ T-lymphocytes obtained by phase i) and to obtain a specific sub-population of tumour-reactive CD4⁺ helper and/or CD8⁺ T-lymphocytes by directing them into a desired pathway.

The present inventors have found, that one way of determining the optimal point in time to initiate phase ii) is by monitoring the expression of the CD25 cell surface marker on the T-lymphocytes, in order to determine specifically when the T-lymphocytes are susceptible to re-stimulation. The present inventors have found that the second phase ii) should preferably be initiated when the expression of CD25 on T-lymphocytes is down-regulated. CD25 is an activation marker, indicating that the lymphocytes have received an activating signal. If the second phase is initiated when the expression of CD25 on the T-lymphocytes is high, meaning that the lymphocytes have already received a signal, cell death would occur.

The down-regulation of CD25 is defined as that a substantial part of the T-lymphocyte population express very few or essentially none CD25 markers. In a preferred embodiment the down-regulation of CD25 is defined as that less than 5% of the T-lymphocyte population expresses CD25, i.e. 95% or more of the T-lymphocytes in the culture does not express CD25 at all. The 5% or less of the T-lymphocytes expressing CD25 is most likely regulatory CD4⁺ T-lymphocytes which have a high permanent expression of CD25. In addition the T-lymphocyte population should preferably express very few or essentially none FoxP3 markers, which are specific markers of regulatory T-lymphocytes. In a preferred embodiment the down-regulation of FoxP3 is defined as that less than 5% of the T-lymphocyte population expresses FoxP3, i.e. 95% or more of the T-lymphocytes in the culture do not express FoxP3 at all.

Besides CD25, there are also other markers, the expression of which is relevant to monitor in order to determine the optimal point in time to initiate the second phase.

Examples of such markers are the early activation marker CD69, and MCHII, which is an activation marker for T-lymphocytes. As the expression of CD69 and MCHII indicates that the "activation program" of the T-lymphocytes is already turned on, meaning that the cells are not able to respond to additional stimuli, both of these markers should preferably be down-regulated before the second phase is initiated. The term down regulation may be defined as that less than 5-10% of the T-lymphocyte population expresses CD69 and/or MCHII.

In another embodiment of the present invention, anti-CD4 antibodies are used to separate T helper cells from possible tumour cells in the culture in the expansion in phase ii) of the expansion method.

In a further or yet another embodiment of the present invention, products such as Dynabeads® with anti-CD3 and anti-CD28 antibodies are used to promote the expansion in phase ii) of the expansion method. Use of Dynabeads® CD3/CD28 will provide lymphocytes with activation signals and could also be used for separation from possible tumour cells in the culture. Dynabeads® CD3/CD28 will bind to T lymphocytes expanded antigen specifically during phase i), where these cells now can be enriched magnetically. Since the initial antigen specific activation has initiated and led to clonal T lymphocyte expansion the Dynabeads® CD3/CD28 restimulation will further promote clonal expansion since phase i) does not support activation of unspecific T lymphocyte clones.

Even though the exact starting point of phase ii) will vary depending on when the lymphocytes has acquired the preferred expression of specific markers, the second phase ii) is most often initiated from day 17 to and including day 23 of the first phase i), such as, e.g. on day 17, on day 18, on day 19, on day 20, on day 21, on day 22 or on day 23. In other words, the point in time, where the lymphocytes expresses the preferred amount and combination of markers, is most often seen as being from day 17 to day 23 of the first phase i).

The expansion of the T-lymphocytes, i.e. phase i) and ii) will most often take place in a suitable culture medium. Preferably a serum-free medium or autologous serum is used in order to avoid the risk of transmitting diseases to the patient. Examples of suitable standard media include AIMV medium, RPMI 1640, DMEM and MEM. However, other

media may also be used, comprising a suitable blend of amino acids, steroids, vitamins, growth factors, cytokines and minerals.

5 During the two phases of the expansion, the cells may be split into several culture vessels in order to maintain a suitable cell density in the cultures. The density of the T-lymphocytes in the expansion phases should preferably be from about 3 to about 6 million cells/ml of culture medium.

10 During expansion an exchange of culture medium with fresh medium, a step, which is denominated conditioning of the medium, may also be needed. The point of time to split cultures and to condition the medium may be determined based on the morphology of the cells and the cell culture density (which should not exceed about 6 million cells/ml), or the medium may contain a suitable indicator, such as, e.g., a phenol indicator. In case an indicator is included in the medium, the point of time to split
15 cultures or condition medium may be based on the color of the medium. If a phenol red indicator is used, the cells should be split or conditioned, when the medium turns yellow, indicating that the pH of the culture is turning acidic. A suitable schedule for conditioning the medium used in the present invention may be to exchange from 1/4 to 1/2, such as, e.g., 1/3 of the medium every 3-9 days, such as, e.g. once a week.

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Except for the specific conditions mentioned herein, for other parameters standard conditions for growth of lymphocyte cultures will be used, such as, e.g. a temperature of 37°C and 5% CO₂.

25 As mentioned above, the second phase ii) is initiated by the addition of tumour-derived antigen as defined above to the T-lymphocytes for activating the tumour-reactive CD25-negative T-lymphocytes, in order to promote clonal expansion of tumour-reactive T-lymphocytes.

30 In a specific embodiment of the invention antigen presenting cells (APCs) are added to the T-lymphocytes together with the tumour-derived antigen. Antigen presenting cells (APCs) include leukocytes such as, e.g., monocytes, macrophages and lymphocytes, such as, e.g., B cells. These diverse cell types have in common the ability to present antigen in a form that is recognized by specific T lymphocyte receptors. The leukocyte
35 preparation is isolated from, for example, blood, lymph fluid, bone marrow, lymphatic organ tissue or tissue culture fluid obtained from the patient to be treated. In a

- preferred embodiment the APCs cells are irradiated peripheral blood leucocytes containing antigen-presenting B-cells and/or monocytes. The amount of APCs added lies within the range of from about 0.5 million APCs/ml lymphocyte culture to about 5 million APC/ml lymphocyte culture, such as, e.g., from about 1 million APCs/ml
- 5 lymphocyte culture to about 4 million APC/ml lymphocyte culture, from about 1 million APCs/ml lymphocyte culture to about 3 million APC/ml lymphocyte culture, or from about 1 million APCs/ml lymphocyte culture to about 2 million APC/ml lymphocyte culture.
- 10 Besides the addition of tumour-derived antigen to the T-lymphocytes in order to promote clonal expansion of tumour-reactive T-lymphocytes, the second phase ii) comprises the addition of specific components the function of which are to direct the expansion of the tumour-reactive T-lymphocytes towards the desired sub-population.
- 15 As mentioned above, the present invention provides a method for the generation of tumour-reactive CD4+ helper T-lymphocytes. CD4+ helper T-lymphocytes recognizes and binds tumour antigen when the antigen is associated with a major histocompatibility complex class II molecule. Activated helper CD4+ T lymphocytes secrete cytokines, proteins and/or peptides that stimulate other cells of the immune
- 20 system, such as other lymphocytes. The most common cytokine secreted is interleukin-2 (IL-2), which is a potent T lymphocyte growth factor. Activated, proliferating CD4+ helper T-lymphocytes can differentiate into two major subtypes of cells, Th1 and Th2 cells, which are defined on the basis of specific cytokines produced. Th1 cells produce interferon-gamma and interleukin 12 (IL-12), while Th2 cells produce interleukin-4,
- 25 interleukin-5 and interleukin-13. Th1 T-lymphocytes are believed to promote activation of cytotoxic T lymphocytes (Tc), NK cells, macrophages, and monocytes, all of which can attack cancer cells and generally defend against tumours.
- T-helper (CD4+) lymphocytes of type Th1 and Th2 can differentiate into memory cells
- 30 and effector cells. Memory T-helper (CD4+) lymphocytes are specific to the antigen they first encountered and can be called upon during a secondary immune response, calling forth a more rapid and larger response to the tumour-antigens. There is evidence in humans that lymphocytes survive at least 20 years; perhaps for life. Effector CD4+ T-lymphocytes are active cells producing cytokines and INF-gamma.

For an effective treatment of cancer, administration of tumour-reactive T-lymphocytes of the Th1 type is especially beneficial, as this type is believed to promote activation of cytotoxic T lymphocytes (Tc), NK cells, macrophages, and monocytes, all of which can attack cancer cells and generally defend against tumours. I.e. in a specific embodiment the invention relates to a method for generating tumour-reactive CD4+ T-lymphocytes, and in a further embodiment, the percentage of T-lymphocytes of the Th2 type generated by the present method is 30% or less, such as, e.g., 25% or less, 20% or less, 15% or less, 10% or less, 5% or less or 0%, i.e. at least 70% of the tumour-reactive CD4+ T-lymphocytes are of the Th1 type, such as, e.g. at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or 100%.

Accordingly, the second phase may comprise the addition of a substance capable of up-regulating IL-12R on the T-lymphocytes. Up regulation of the IL-12R will increase the readiness of the T cell to receive and optimize the IL-12 cytokine activation resulting in maximal STAT-4 signaling and thus skewing the lymphocytes towards Th1 cells and IFN- γ production.

The substance(s) capable of up-regulating IL-12R on the T-lymphocytes may be substance(s) having agonistic activity towards an interferon receptor. In one embodiment of the invention the substances having agonistic activity towards the interferon receptor are agonists. Examples of such substances include proteins, polypeptides, peptides, antibodies, affibodies, and fragments thereof, fusion proteins, synthetic and/or organic molecules, such as, e.g., small molecules, and natural ligands. In a specific embodiment the substance is the natural ligand of the interferon receptor, namely an interferon, such as interferon- α .

The optimal point of time to add the substance(s) capable of up-regulating IL-12R on the T-lymphocytes, such as, e.g. a substance having agonistic activity towards an interferon receptor may be determined by measuring the level of IL-12 in the culture medium. The substance(s) should preferably be added when the level of IL-12 is at least 1 fold, such as, e.g., at least 2, at least 3 fold, at least 4 fold, or at least 5 fold increased as compared to the level of IL-12 on day 1 of phase ii). In most cases, such an increase in the level of IL-12 will be seen from day 2 to and including day 4 after initiating the second phase ii), such as, e.g. on day 2, on day 3 or on day 4.

In order to substantially avoid the generation of tumour-reactive T-lymphocytes of the Th2 type, the second phase may further comprise the addition of one or more substances capable of antagonizing development of Th2 type T-lymphocytes.

5 Examples of such substances are substances capable of neutralizing the interleukins IL-4, IL-5, IL-10, and/or TGF-beta (the latter not being an interleukin) all four of which are required for the establishment of the Th2 cytokine profile and for down regulation of Th1 cytokine production.

10 Examples of such substances include proteins, polypeptides, peptides, soluble receptors, antibodies, affibodies, and fragments thereof, fusion proteins, synthetic and/or organic molecules, such as, e.g., small molecules, and natural ligands. In a specific embodiment the substances are selected from antibodies that binds to the interleukins, thereby neutralizing them, such as, e.g. anti IL-4 antibody, anti IL-5 antibody and/or anti IL-10 antibody, together with soluble receptors (such as, e.g. TGF-
15 beta receptor I and II) and binding proteins for TGF-beta (such as, e.g. LAP and/or LTBP).

The one or more substances capable of antagonizing development of Th2 type T-lymphocytes, such as, e.g., one or more substances capable of neutralizing IL-4, IL-5,
20 IL-10 and/or TGF-beta may be added on day 1 of the second phase ii). However, as antibodies are expensive, the addition of antibodies can also be performed in a subsequent step after addition of the substance capable of up-regulating IL-12R on the T-lymphocytes, such as, e.g., one day, two days or three days after addition of the substance capable of up-regulating IL-12R on the T-lymphocytes.

25 The neutralizing substances should be added in an amount sufficient to neutralize the interleukins, such as, e.g., in a 10-100 fold (molar) excess of the amount of interleukin to be neutralized. When using antibodies, a final concentration of from about 2 to about 4 ng/ml culture medium will normally be needed. For other types of neutralizing
30 substances, a final concentration, giving the same effect as the concentration mentioned for antibodies, should be used.

In order to maintain the suppression of the development of Th2 type T-lymphocytes a further amount of the one or more substance capable of antagonizing development of
35 Th2 type T-lymphocytes, such as, e.g., one or more substance capable of neutralizing IL-4, IL-5, IL-10 and/or TGF-beta may be added regularly throughout phase ii), such as,

e.g. every 2nd, 3rd or 4th day of phase ii). It is to be understood that by the term every 2nd, 3rd or 4th is intended to mean that at least one substance capable of antagonizing development of Th2 type T-lymphocytes is added throughout phase i) every 2nd, 3rd or 4th day, starting at the 2nd, 3rd or 4th day after the first addition of the at least one
5 substance capable of antagonizing development of Th2 type T-lymphocytes.

Furthermore, as for phase i) a further amount of a substance having agonistic activity towards the IL-2 receptor, such as, e.g., an agonist may be added regularly throughout phase ii) such as, e.g., every 2nd to 4th day of phase ii), i.e. on the 2nd, 3rd or 4th day in
10 order to maintain optimal conditions promoting cell division. The dose of the substance to be added regularly lies within the optimal ranges mentioned under phase i) for addition of substances having agonistic activity towards the IL-2 receptor, such as, e.g., IL-2.

15 In order to favor the generation of Th1 type tumour-reactive T-lymphocytes, the second phase ii) may comprise adding one or more substances promoting the development of Th1 type T-lymphocytes. Examples of such substances are substances having agonistic activity towards the IL-7, IL-12, IL-15 and/or IL-21 receptor. More specific, the substances may be agonists for the IL-7, IL-12, IL-15 and/or IL-21 receptor. Examples
20 of such agonists include proteins, polypeptides, peptides, antibodies, affibodies, and fragments thereof, fusion proteins, synthetic and/or organic molecules, such as, e.g., small molecules, and natural ligands. In a specific embodiment the substances are the natural ligands of the IL-7, IL-12, IL-15 and/or IL-21 receptor, respectively, such as IL-7, IL-12, IL-15 and/or IL-21.

25 The effect of IL-12 is activating the IFN-gamma inducing STAT pathway by stimulating the IL-12R thereby promoting activation of Th1 lymphocytes. The function of IL-21 is to enhance proliferation, activation and development towards a Th1 type of T-lymphocytes.

30 Both IL-7 and IL-15 work by promoting homeostatic expansion of the T-lymphocytes, enhancing the enumeration of activated Th1 programmed T-lymphocytes.

The optimal point of time to add one or more substances promoting development of
35 Th1 type T-lymphocytes is when the T-lymphocytes are susceptible to modification. If the substances are added when the T-lymphocytes are not susceptible to modification,

the addition will have no effect, i.e. the development of Th1 type T-lymphocytes will not be favoured. In order to determine the optimal point in time for adding substances promoting development of Th1 type T-lymphocytes, such as, e.g., substances having agonistic activity towards the IL-7, IL-12, IL-15 and/or IL-21 receptor, the production of INF- γ by the T-lymphocytes, may be monitored. In a preferred embodiment, the one or more substances promoting the development of Th1 type T-lymphocytes, such as, e.g., substances having agonistic activity towards the IL-7, IL-12, IL-15 and/or IL-21 receptor should be added when the level of IFN-gamma is increased as compared to the level of IFN-gamma on initiation of second phase ii).

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In a specific embodiment, the increase in IFN-gamma level may be determined as at least a 1 fold increase in IFN-gamma level, such as, e.g., at least a 2 fold, at least a 3 fold, at least a 4 fold increase as compared to the level of IFN-gamma on initiation of the second phase ii). Often will such an increase can be correlated to that the content IFN-gamma in the culture medium should be at least 100 picogram/ml culture medium, such as, e.g. at least 150 picogram/ml culture medium, at least 200 picogram/ml culture medium or at least 250 picogram/ml culture medium.

15

When determining the optimal point in time to add substances promoting development of Th1 type T-lymphocytes, such as, e.g., substances having agonistic activity towards the IL-7, IL-12, IL-15 and/or IL-21 receptor, one may further look at the expression of the activation markers CD25 and CD69 on CD4+ T-lymphocytes, which markers should preferentially be up-regulated. By up-regulation is understood that at least about 40% to about 60% or more of the CD4+ T-lymphocytes should express CD25 and CD69 as compared to the expression of CD25 and CD69 on T-lymphocytes on day 1 of phase ii), showing that the T-lymphocytes have received an activating signal.

20

25

Normally, the optimal point of time for adding the substances promoting development of Th1 type T-lymphocytes will fall subsequent to the steps of adding the substances capable of up-regulating IL-12R on the T-lymphocytes and the substances capable of antagonizing development of Th2 type T-lymphocytes. More specific the optimal point in time to add the substances promoting development of Th1 type T-lymphocytes will fall between day 5 to day 8 after initiating the second phase ii), such as, on day 5, day 6, day 7 or day 8.

30

35

In case IL-7, IL-12, IL-15 and/or IL-21 are added the concentration of each of these substances in the culture medium should lie within the range from about 150 IU/ml culture medium to about 300 IU/ml culture medium, such as, e.g. 250 IU/ml culture medium. When other substances than the specific ones mentioned is used, they should
5 be added to the culture in final concentration, which leads to the same effect as the addition of IL-7, IL-12, IL-15 and/or IL-21 within the specific ranges mentioned will give.

As mentioned above, the present method is preferentially used for the expansion of T-lymphocytes in order to achieve CD4+ tumour-reactive T-lymphocytes of the Th1 type.
10 One further aspect of the invention is that by using the method described herein for expanding tumour-reactive T-lymphocytes, a relatively high amount of T-lymphocytes of the memory type will be obtained. In treating cancer it is of course important that the patient to be treated receive a high amount of effector tumour-reactive CD4+ T-lymphocytes, as these – as mentioned above - promote activation of cytotoxic T
15 lymphocytes (Tc), NK cells, macrophages, and monocytes, all of which can attack cancer cells and generally defend against tumours.

However, by at the same time administering a substantial amount of memory tumour-reactive CD4+ T-lymphocytes, the patient achieve up to life long protection towards
20 recurrence of the tumour or metastasis of the primary tumour.

Accordingly, the present invention relates to a method for the preparation of memory T-lymphocytes. Normally, when a culture of tumour-reactive T-lymphocytes are expanded according to the present invention from about 35% to about 90% of tumour-reactive T-lymphocytes of the memory type, such as, e.g. from about 40% to about 90%, from
25 about 50% to about 80% or from about 60% to about 70%, will be obtained. The present inventors speculates that the fact that the lymphocytes in phase i) are allowed to regenerated before tumour antigen is added, together with the relatively slow expansion phase leads to formation of a high ratio of memory lymphocytes to effector
30 lymphocytes.

As mentioned above the expression of the cell surface activation markers CD25 and CD69 on the T-lymphocytes may be used for determining when to initiate important steps of the present method, such as, e.g., when to initiate the second phase ii).
35 Accordingly, it may be beneficial to continuously monitor the expression of CD25 and

CD69 throughout phase i) and phase ii), such as, e.g., every 2nd, every 3rd or every 4th day.

As one of the purposes of the present method is to obtain a high number of specific
5 CD4+ tumour-reactive T-lymphocytes, which may be used for administering to a
patient, the tumour-reactive T-lymphocytes may be harvested at some point, leading to
the termination of the expansion step. The optimal point of time to harvest the tumour-
reactive T-lymphocytes is when the expression of CD25 on the T-lymphocytes is down-
regulated, where the down-regulation is defined as that 5% or less of the CD4+ T-
10 lymphocyte population expresses CD25. The optimal point in time to harvest may also
be determined based on measurement of the amount of IFN-gamma produced. The
IFN-gamma production should be at least 2 fold increased, such as, e.g., at least 3
fold, at least 4 fold or at least 5 fold increased as compared to initial IFN-gamma
production, which normally correspond to a level of IFN-gamma of at least 100 pg/ml of
15 culture medium.

Normally, this event will occur from day 10 to and including day 14 after initiating the
second phase ii), i.e. normally the cells will be harvested from day 10 to and including
day 14 after initiating the second phase ii).

20

Accordingly, the entire process for expansion of tumour-reactive T-lymphocytes
according to the invention may in general take from about 25 days to and including
about 45 days, such as, e.g. from about 26 days to and including about 44 days, from
about 27 days to and including 43 days, from about 27 days, to and including 42 days,
25 from about 27 days to and including 41 days, and from about 27 days to and including
about 40 days.

Instead of harvesting the tumour-reactive T-lymphocytes when the CD25 marker is
down regulated, they may be subjected to one or more additional rounds of phase ii).

30 This could be beneficial to do if the amount of tumour-reactive T-lymphocytes obtained
by the expression method is not considered an effective amount to be administered to
a patient suffering from cancer, or if the patient is in a chemo-therapy treatment
regimen, where it may be considered beneficial to postpone the administration of T-
lymphocytes until the chemo-therapy treatment is finished. In order to determine
35 whether the tumour-reactive T-lymphocytes should be subjected to one or more
additional rounds of phase ii) one may look at the level of IFN-gamma produced, and/or

the total number of tumour-reactive T-lymphocytes obtained and/or the expression of CD25. In the case the IFN- γ levels is 30 pg/ml culture medium or less, such as, e.g. 20 pg/ml culture medium or less, and/or the total number of T cells are unsatisfactory, additional rounds of phase ii) may be initiated beginning when the majority of T cells are CD25 negative (i.e. less than 5% of the T-lymphocytes population express CD25) and thereby susceptible to restimulation.

After harvest the tumour-reactive T-lymphocytes may be purified by any conventional means, such as, e.g. by using density gradient, such as, e.g., a Ficoll medium. A portion of the tumour-reactive T-lymphocytes may be stored by freezing in a suitable freezing medium after harvesting and purifying the tumour-reactive T-lymphocytes.

Administration

The tumour-reactive T-lymphocytes obtained by an improved expansion method are used herein for treating patients suffering from malignant melanoma.

The definition of an effective amount of tumour-reactive T-lymphocytes to be administered is depending on the specific type of lymphocytes, the ratio of memory to effector T-lymphocytes and on the severity of the disease. However, in average a minimum of at least 10 million, such as, e.g. at least 20 million, at least 30 million, at least 40 million, at least 50 million, at least 60 million, at least 70 million or at least 80 million tumour-reactive T-lymphocytes may be administered. The present inventors have not identified any upper limit with respect to the amount of tumour-reactive T-lymphocytes to be administered in a single dose.

In a preferred embodiment the tumour-reactive T-lymphocytes for administration comprises a combination of effector T-lymphocytes and memory T-lymphocytes. More specific the amount of tumour-reactive T-lymphocytes of the memory type may be from about 35% to about 90%, such as, e.g. from about 40% to about 90%, from about 50% to about 80% or from about 60% to about 70%, and a percentage of effector T-lymphocytes from about 10% to about 65%, such as, e.g., from about 20% to about 50% or from about 30% to about 40%.

The tumour-reactive T-lymphocytes may be formulated as a pharmaceutical composition suitable for parenteral administration to the patient such as, e.g., intravenously, intraarterially, intrathecally or intraperitoneally administration.

When the tumour-reactive T-lymphocytes are administered parenterally, they may be formulated in an isotonic medium, i.e. in a medium having the same tonicity as blood, and comprising one or more substances preventing aggregation of the cells. A specific
5 example of a suitable medium is a 0.9% NaCl solution comprising up to 3% human serum albumin such as, e.g. up to 2% human serum albumin or up to 1% human serum albumin. For intravenously administration the concentration of tumour-reactive T-lymphocytes in the composition to be administered normally lies within the range
10 from about 0.5 million lymphocytes/ml medium to about 4 million lymphocytes/ml medium, such as, e.g., from about 0.5 million lymphocytes/ml medium to about 3 million lymphocytes/ml medium, from about 0.5 million lymphocytes/ml medium to about 2 million lymphocytes/ml medium or from about 1 million lymphocytes/ml medium to about 2 million lymphocytes/ml medium.

15 The composition comprising tumour-reactive T-lymphocytes may be administered as a single dose or multiple doses. It may be infused over 1 to 2 hours.

The treatment method may be performed once or repeated depending on the severity of the disease. Furthermore, the treatment may be reiterated upon recurrence of the disease.

20

The treatment according to the present invention may be supplemented with any other relevant treatment for malignant melanoma. Such supplemental treatment may be given before, at the same time or after the administration of the lymphocytes and it may be given at frequencies normally used for such treatments. A suitable example of
25 supplemental treatment is chemotherapy and the like.

Kits

The invention further relates to kits for use in a method according to the invention, the kit comprising a medium for cultivation of T-lymphocytes. The medium may be any
30 suitable serum-free medium, such as, e.g., AIMV, RPMI 1640, DMEM or MEM.

The kit may further comprise one or more substances for stimulating, activating and directing tumour-reactive T-lymphocytes. Examples of such substances may be tumour-derived antigen, substances having agonistic activity towards the IL-2 receptor,
35 substances capable of up-regulating IL-12R on the T-lymphocytes, substances capable of antagonizing development of Th2 type T-lymphocytes and/or substances promoting the development of Th1 type T-lymphocytes.

More specific, such substances may be IL-2, interferon-alpha, anti-IL-4 antibody, anti-IL-5 antibody, anti-IL-10 antibody, IL-7, IL-12, IL-15 and/or IL-21.

- 5 The kit may also comprise a pharmaceutical composition suitable for intravenous administration. The pharmaceutical composition may be mixed with the population of tumour-reactive T-lymphocytes before administration.

The kit may also comprise one or more syringes and a lymph node locator. In one
10 embodiment, the syringes are prefilled with a lymph node locator.

The kits may also comprise instructions for use, such as, e.g. instructions in the form of computer software.

15 **Figure legends**

Figure 1 shows the injection of isotope or patent blue around the malignant melanoma or around the scar after initial melanoma removal where after the lymphatic drainage of the tumour will stain and reveal the sentinel node.

20

Figure 2A and 2B shows plantar malignant melanoma injected with radioactive tracer and patent blue. Sentinel node identified by scintigraphy (Figure 2C) and by open surgery and dissection in area inguinalis (Figure 2D).

25 Figure 3 shows a time course study of sentinel node acquired lymphocytes. '0' represents no addition of autologous tumour extract, 'MM 1/100' represents addition of a 1/100 dilution of tumour extract, and 'MM 1/50' represents addition of a 1/50 dilution of tumour extract. Proliferation, determined as cpm by scintillation counting, is seen at the highest concentration of autologous tumour extract.

30

Figure 4 illustrates that sentinel node acquired lymphocytes (S_{lgn}) are proliferating dose dependently against autologous tumour extract, whereas no proliferation was seen among lymphocytes from a negative node (N_{lgn}) or from tumour infiltrating lymphocytes (TIL). The different bars illustrate proliferation in cpm, determined by
35 scintillation counting, from 0, 1/100, and 1/10 dilution of tumour extract.

Figure 5 shows that sentinel node acquired lymphocytes can be activated and expanded in the presence of autologous tumour extract and interleukin-2 as described above. Results are based on expansion of three different sentinel nodes from a patient with malignant melanoma. All experiments were carried out using the same expansion protocol, but generated various successes. An expansion of 1×10^9 cells was achieved in expansion 3.

Figure 6 shows the cellular expression of CD8 and CD4 T helper cell markers as investigated by flow cytometry. At the end of the expansion after approximately 30 days the majority of cells express the CD4 T helper cell marker.

Figure 7 shows the V β repertoire before and after expansion as investigated by flow cytometry. The majority of cells at the end of the expansion belong to the V β 2 family indicating clonal expansion of tumour reactive T cells.

Figure 8 shows the presence of metastatic malignant melanoma cells in sentinel lymph nodes with metastasis as investigated by flow cytometry using antibodies against HMB-45. SSC indicates side scatter, which is an indication of the granularity of cells.

Figure 9 shows that initially sentinel node lymphocytes are activated with tumor antigen and low dose IL-2 resulting in activation and expression of the activation marker CD25 (Top panel). The end of phase I activation phase is defined by the decreased number of CD4⁺ T cells expressing CD25 (Bottom panel). When less than 5% of the CD4⁺ T cells express CD25 phase II is initiated with restimulation with antigen.

Figure 10 illustrates that Phase I and Phase II activation results in expansion and enrichment of CD4⁺ T helper cells.

Figure 11 illustrates that in Phase I the majority of cells are naive CD62L⁺ cells or activated CD69⁺CD62L⁺ cells. After Phase II the majority of the cells are CD62L⁻ and are composed of memory and effector CD4⁺ T helper cells. CD62L⁻ T cells are not expressing the preferred lymph node homing molecule, thus they are seeking inflammatory areas in non-lymphatic organs.

Figure 12 shows primary cells stimulated in Phase I from the tumour (Tumour infiltrating lymphocytes), sentinel nodes (SN) and an irrelevant lymph node (LN) results in no little IFN-gamma production.

- 5 Figure 13 illustrates that after expansion after phase ii) there is a dose dependent increase in antigen dependent IFN-gamma production.

Figure 14 illustrates that the expansion and activation protocol promotes the expansion of antigen specific T cell clones as investigated by the selective enrichment of TCR V
10 beta expression.

The following examples tend to illustrate the invention without limiting it in any way.

15 **Examples**

Example 1

Acquirement and expansion of tumour-reactive T-lymphocytes from patient with malignant melanoma

20

Materials and methods

Patients

Patients with malignant melanoma were included in the study. The study was approved
25 by the local ethical committee and informed consent was given by the patients.

Identification of sentinel nodes

One ml Patent blue dye (Guerbet, Paris) in addition to 0.4 ml of the isotope $^{40}\text{MBq}^{99\text{mTc}}$ -Albu-Res was injected superficially around the primary tumour. Sentinel
30 lymph nodes were identified by hand held γ -counter.

Preparation of specimens

The sentinel and non-sentinel nodes were cut in half. Slices were cut from the central and the peripheral part of the nodes for flow cytometry, proliferation analysis and
35 expansion. The rest of the node underwent routine histopathological examination. One

piece of the primary tumour (including part of the invasive margin) was removed for flow cytometry analyses and as an antigen source.

Flow cytometry analyses

5 PBL, lymph node cells and tumour cell suspensions at 1×10^6 cells/sample were subjected to investigation using flow cytometry (FACS). Cells were washed in PBS containing 2% FCS and 0.05% NaN_3 and stained with fluorophore conjugated antibodies against the cell surface molecules CD4 PE, CD8 PerCp (Becton Dickinson) and the very early activation marker CD69 FITC (Pharmingen). The $\text{V}\beta$ -TCR repertoire
10 was examined using the Beta mark kit (Beckman Coulter), 5×10^5 cells/tube being stained in 10 μl of the 8 different vials containing mixtures of FITC, PE and dual-colour FITC-PE conjugated TCR $\text{V}\beta$ antibodies. In addition, direct conjugated antibodies CD8 PerCP and CD4 APC were added to each tube. After staining, cells were investigated
15 using a FACSCalibur (Becton Dickinson) and data were analyzed using the Cellquest computer software (Becton Dickinson).

Immunological evaluation

Single cell suspensions from lymph nodes and tumours were obtained by gentle pressure using a loose fit glass homogenizer. Peripheral blood leukocytes (PBL) were
20 purified by ficoll-paque (Pharmacia, Amersham). Cells were resuspended and washed twice in RPMI 1640 (Life technologies) containing 2.5% fetal calf serum (FCS) (Life technologies). Finally, cells were resuspended in RPMI 1640 proliferation media containing 10% human AB serum (Sigma), 1% penicillin-streptomycin (Sigma) and 1% glutamine (Sigma). Tumour samples were homogenized using a Ultra-turrax in 5
25 volumes (w/v) of 2 x PBS followed by 5 minutes denaturation at 97°C. Tumour homogenates were diluted to 1:10 and 1:100 in complete proliferation media. Purified PBL and lymph node cells were used at 3×10^5 cells/well in time course proliferation assays against diluted tumour homogenate, Con A 10 $\mu\text{g}/\text{ml}$ (Sigma) or tyrosinase peptide 100 $\mu\text{g}/\text{ml}$ (Sigma) in triplicate. Proliferation was measured by adding 1 μCi of
30 ^3H -Thymidine/well (Amersham) 18 hours prior to harvesting. Samples were subjected to scintillation counting.

Example 2

General method for expansion of tumour-reactive T-lymphocytes

35

Cell culture

Phase I, initial activation

The node material was kept on ice and immediately taken care of using AIM V® Media (Invitrogen) at all times. Single cell suspensions of sentinel or metinel node lymphocytes was obtained through gentle homogenisation in a loose fit glass homogenisator, and following homogenisation cells were washed twice in medium. The metinel node lymphocytes were put in cell culture flasks at 4 million cells/ ml and interleukin-2 (IL-2) (Proleukin®, Chiron) was added to a concentration of 240 IU/ml medium.

Autologous metastasis extract was prepared by homogenisation with an Ultra Turrax in 5 volumes (w/v) 2 x PBS followed by denaturation for 5 minutes at 97°C. Three to four days after initiation of the cell culture autologous tumour extract was added at a concentration of 1/100. For long-term culture the cells were kept in a cell incubator at 37°C and 5% CO₂ and 240 IU IL-2/ml media added every 3-4 days.

15

Phase II, activation and expansion

After 18-22 days the cell cultures were monitored for the expression of CD25. When the number of CD25 expressing cells was diminished below 5% the cells were restimulated in Phase II (Figure 9) by the addition of autologus metastasis extract at a concentration of 1/100. For efficient antigen presentation autologous PBMC were collected using Ficoll-Paque PLUS (Amersham Biosciences, GE Healthcare), radiated with 2500 rad and added to the cell cultures. Three days after restimulation interferon- α (Introna) in conc. 100-500 IU/ml and anti IL-4 antibody was added to a concentration of 2 μ g/ml. After 5 to 8 days IL-12 (4 ng/ml) was added to the expansion in order to promote induction of IFN- γ producing Th1 cells.

25

The day before transfusion to the patient the cell cultures were subject to purification using a Ficoll-Paque PLUS (Amersham Biosciences, GE Healthcare) in order to retrieve the viable cells in the culture. On the day of transfusion the cells were washed twice in Saline solution (Natriumklorid Baxter Viaflo 9 mg/ml, Baxter) and then transferred to a transfer bag containing 100-200 ml of saline solution and 1% Human Serum Albumin (Baxter). Investigations for microbial presence were performed prior to transfusion. Infusions of the cells were performed during 1-2 hours under professional medical supervision.

30

Immunological evaluation

35

Further immunological evaluation was performed using tritium labeled thymidine incorporation proliferation assays. An aliquot of metinel node lymphocytes was set aside for this purpose, a single cell suspension of non-metinel node lymphocytes was obtained by gentle pressure in a loose fit glass homogenisator and peripheral blood leukocytes were purified by Ficoll-Paque PLUS (Amersham Biosciences, GE Healthcare).

Cells were resuspended and washed twice in RPMI 1640 (Life technologies) containing 2.5% fetal calf serum (FCS) (Life technologies). Finally, cells were resuspended in RPMI 1640 proliferation media containing 10% human AB serum (Sigma), 1% penicillin-streptomycin (Sigma) and 1% glutamine (Sigma). Lymph node cells and purified PBL were used at 3×10^5 cells/well in a 96-well plate and stimulated with metastasis homogenate diluted 1/100, 1/10 or Con A 10 $\mu\text{g/ml}$ (Sigma) in triplicates. Proliferation was measured on day 5, 6 and 7 by adding 1 μCi of ^3H -Thymidine/well (Amersham) 18 hours prior to harvesting. Samples were subjected to scintillation counting.

At the start of cell culture, stimulations of lymph node cells and PBL, for the measurement of IFN- γ secretion, were performed in 96-well plates with 3×10^5 cells/well in triplicate with tumour homogenate diluted 1/10 and 1/100, or Con A 10 $\mu\text{g/ml}$ (Sigma). The amount of secreted IFN- γ was measured with ELISA (Human IFN- γ DuoSet, R&D Systems) on culture supernatants in pooled samples of the triplicates (Figure 12). At the end of cell cultures samples of the supernatant was removed and IFN- γ and IL-4 secretion measured in triplicates with ELISA (Human IFN- DuoSet and Human IL-4 DuoSet, R&D Systems) (Figure 13 A and 13 B).

Flow cytometry analyses

Characterization of cells was performed using flow cytometry initially on cells from the metinel node, non-metinel node, PBMC and from the metastasis. From the metinel node acquired lymphocytes in culture samples were taken every two to three weeks for flow cytometry analyses. Cells were incubated for 30 minutes in PBS supplemented with 2% FCS and 0.05% NaN_3 (FACS buffer) with antibodies against markers for immune cell subpopulations and for lymphocyte activation (Figure 9, 10 and 11). Antibodies conjugated with Fluorescein isothiocyanate (FITC) against the following markers were used: CD69, HLA-DR, CD45RA, CD25, conjugated with phycoerythrin

(PE): CD62L, CD19, CD45RO, CD56, conjugated with Peridinin-Chlorophyll-Protein
(PerCP): CD8, CD3, conjugated with allophycocyanin (APC): CD4, CD14, CD8.

5 The V β -repertoire was examined using the Beta mark kit (Beckman Coulter), 5×10^5 cells/tube was stained in 10 μ l of the 8 different vials containing mixtures of FITC, PE and dual-colour FITC-PE conjugated TCR V β antibodies and with the addition of CD8 PerCP and CD4 APC to each tube (Figure 14).

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of producing T lymphocytes for the treatment of malignant melanoma comprising:

5 (i) identifying in a patient one or more sentinel and/or metinell lymph nodes draining a malignant melanoma or a metastasis there from;

(ii) resecting the one or more nodes and, optionally all or part of the tumour or metastasis;

(iii) isolating tumour-reactive T-lymphocytes from said lymph nodes; and

10 (iv) *in vitro* expanding said tumour-reactive T-lymphocytes by a process comprising:

(a) a first phase comprising stimulating tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes with tumour-derived antigen together with at least one substance having agonistic activity towards the IL-2 receptor (IL-2R) to thereby promote survival of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes; and

15 (b) a second phase comprising activating and promoting growth of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes, wherein said second phase is initiated when CD25 or IL-2R is down-regulated on T-lymphocytes,

20 thereby producing tumour-reactive T-lymphocytes comprising CD4+ helper and/or CD8+ T-lymphocytes wherein said T lymphocytes are reactive against malignant melanoma.

25 2. The method according to claim 1, wherein the down-regulation of CD25 on T-lymphocytes comprises 5% or less of the T- lymphocyte population expressing CD25 on their surface.

30 3. The method according to claim 1 or 2, wherein the first phase is initiated by adding the at least one substance having agonistic activity towards IL-2R.

4. The method according to any one of claims 1 to 3, wherein the second phase is initiated by adding malignant melanoma-derived antigen to the lymphocytes to thereby activate tumour-reactive CD25-negative T-lymphocytes.

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5. The method according to any one of claims 1 to 4, wherein the tumour-derived antigen comprises or is comprised within a denatured homogenate of a tumour.
6. The method according to claim 4 or 5, comprising adding antigen presenting cells together with a malignant melanoma-derived antigen to the T-lymphocytes.
7. The method according to claim 6, wherein the antigen presenting cells are in a preparation of irradiated peripheral blood leucocytes comprising antigen-presenting B-cells and/or monocytes.
8. The method according to any one of claims 1 to 7, comprising adding at least one substance capable of up-regulating interleukin-12 receptor (IL-12R) on the T-lymphocytes in the second phase.
9. The method according to any one of claims 1 to 8, comprising adding one or more substances capable of antagonizing development of Th2 type T-lymphocytes in the second phase.
10. The method according to any one of claims 1 to 8, comprising adding an amount of one or more substances capable of antagonizing development of Th2 type T-lymphocytes regularly throughout the second phase.
11. The method according to claim 9 or 10, wherein at least one substance capable of antagonizing development of Th2 type T-lymphocytes comprises at least one substance capable of neutralizing IL-4 and/or IL-5 and/or IL-10 and/or TGF-beta.
12. The method according to claim 11, wherein at least one substance capable of neutralizing IL-4 and/or IL-5 and/or IL-10 and/or TGF-beta comprises anti IL-4 antibody and/or anti IL-5 antibody and/or anti IL-10 antibody.
13. The method according to any one of claims 1 to 12, comprising adding one or more substances capable of promoting the development of Th1 type T-lymphocytes in the second phase.
14. The method according to claim 13, wherein the one or more substances promoting the development of Th1 type T-lymphocytes comprise one or more

substances having agonistic activity towards the IL-7 receptor and/or IL-12 receptor and/or IL-15 receptor and/or IL-21 receptor.

15. The method according to claim 14, wherein the one or more substances having agonistic activity towards the IL-7 receptor and/or IL-12 receptor and/or IL-15 receptor and/or IL-21 receptor comprises IL-7 and/or IL-12 and/or IL-15 and/or IL-21.

16. The method according to any one of claims 1 to 15 further comprising continuously monitoring the expression of one or more cell surface markers comprising CD25 and/or CD69 during the first phase and second phase, and harvesting the T-lymphocytes when CD25 on T-lymphocytes in the second phase is down-regulated.

17. The method according to any one of claims 1 to 16, further comprising subjecting the T-lymphocytes to at least one additional round of the second phase when CD25 on T-lymphocytes is down-regulated.

18. The method according to any one of claims 1 to 17, wherein the T-lymphocytes are derived from lymph nodes draining a malignant melanoma tumour and/or a metastasis or blood.

19. The method according to any one of claims 1 to 18, wherein the T-lymphocytes produced at (iv) are Th1 type T-lymphocytes of the memory and/or effector type.

20. A preparation of T-lymphocytes reactive against malignant melanoma when prepared by the method according to any one of claims 1 to 19.

21. A pharmaceutical composition comprising tumour-reactive T-lymphocytes produced by the method according to any one of claims 1 to 19 in an isotonic medium

22. The pharmaceutical composition according to claim 21, wherein the isotonic medium is 0.9% sodium chloride, and the medium further contains up to 3% human serum albumin.

23. A method of treating a patient suffering from malignant melanoma comprising administering tumour-reactive T-lymphocytes produced by the method according to any

one of claims 1 to 19 or the pharmaceutical composition according to claim 21 or claim 22 to the patient.

24. Use of the preparation of T-lymphocytes according to claim 20 or the
5 pharmaceutical composition according to claim 21 or claim 22 in medicine.

25. Use of the preparation of T-lymphocytes according to claim 20 in the
manufacture of a medicament for treating malignant melanoma.

10 26. The method according to any one of claims 1 to 19 or the preparation of T-
lymphocytes according to claim 20 or the pharmaceutical according to claim 21 or 22 or
the method of claim 23 or the use according to claim 24 or 25 substantially as
hereinbefore described with reference to any one of the Examples and/or Figures.

DATED this NINTH day of JUNE 2011

SentoClone International AB
Patent Attorneys for the Applicant:
F.B. RICE .

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Fig. 1

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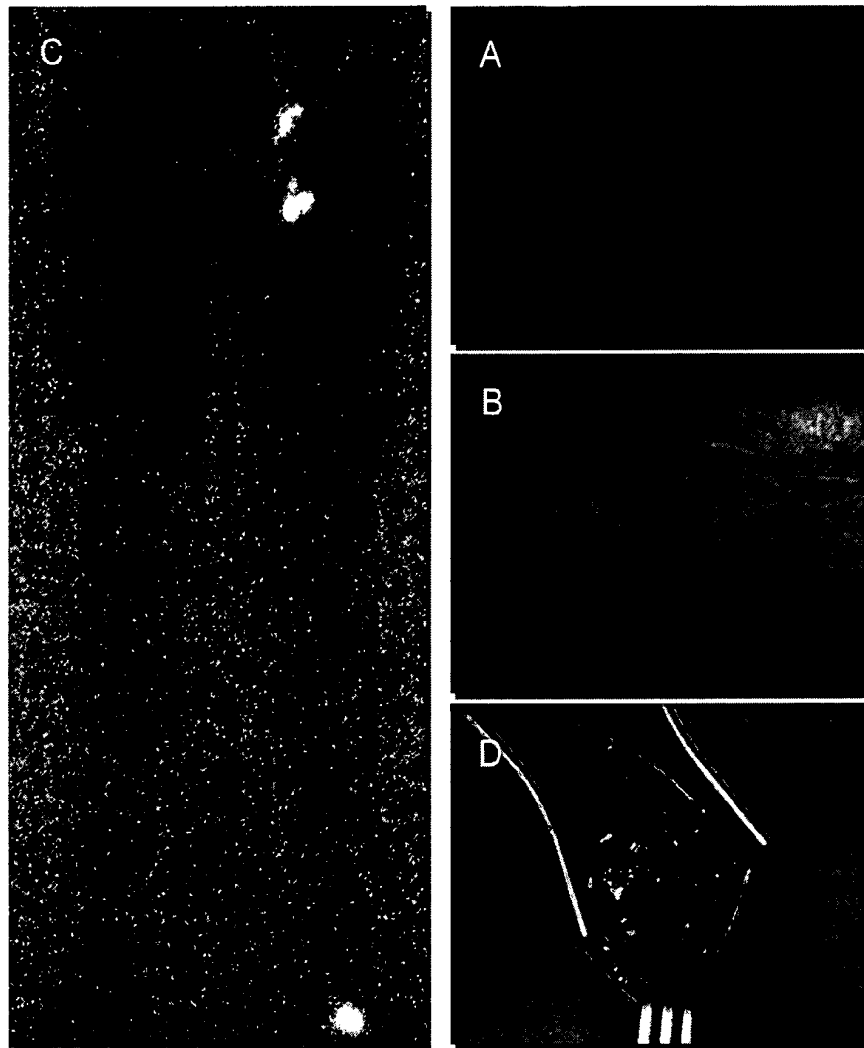


Fig. 2

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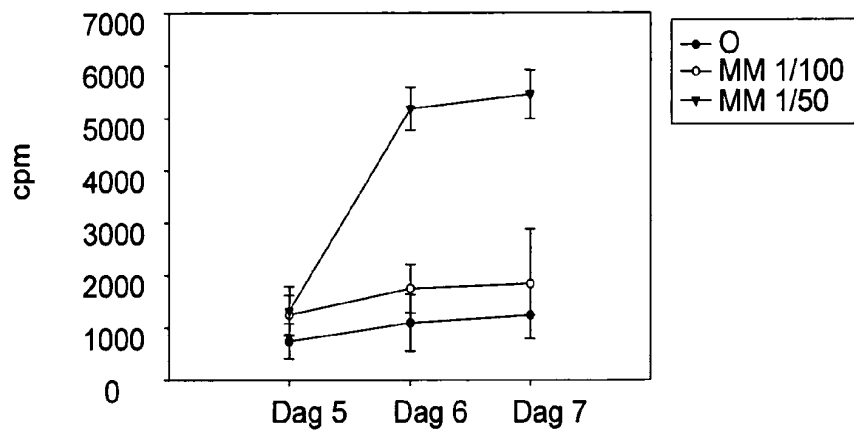


Fig. 3

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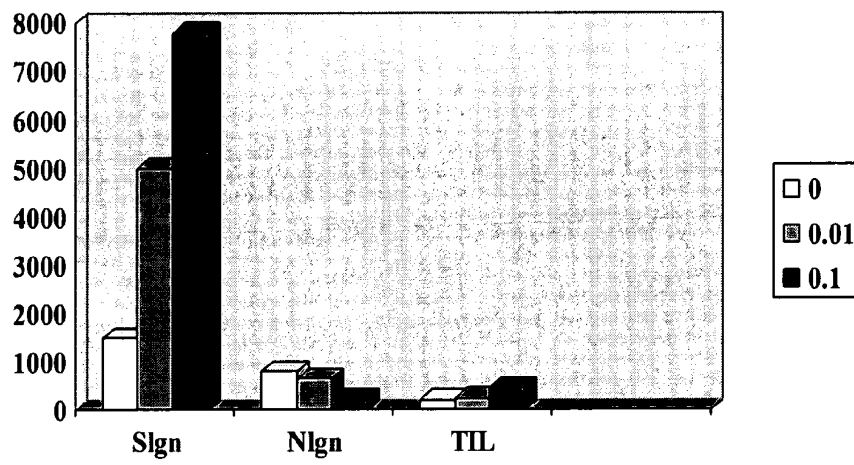


Fig. 4

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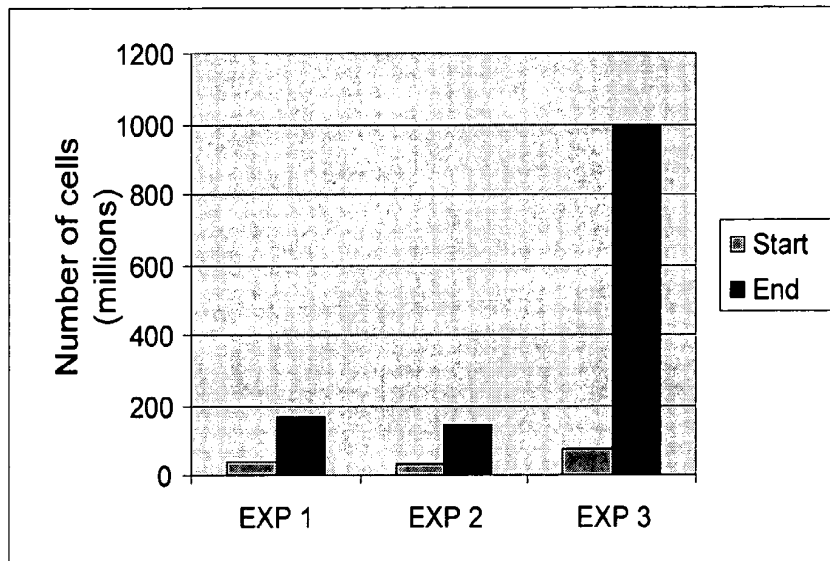
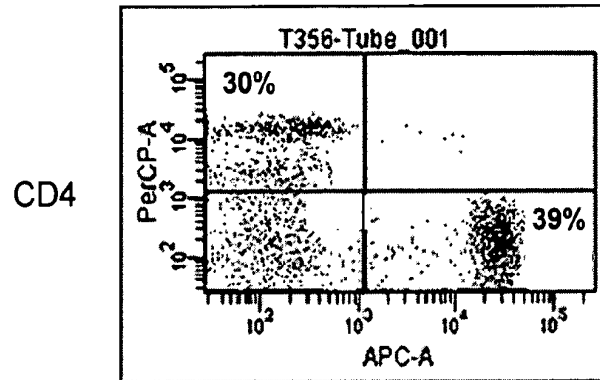


Fig. 5

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Start



CD8

End

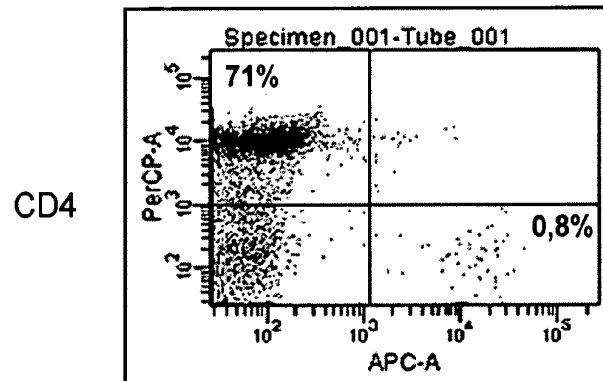


Fig. 6

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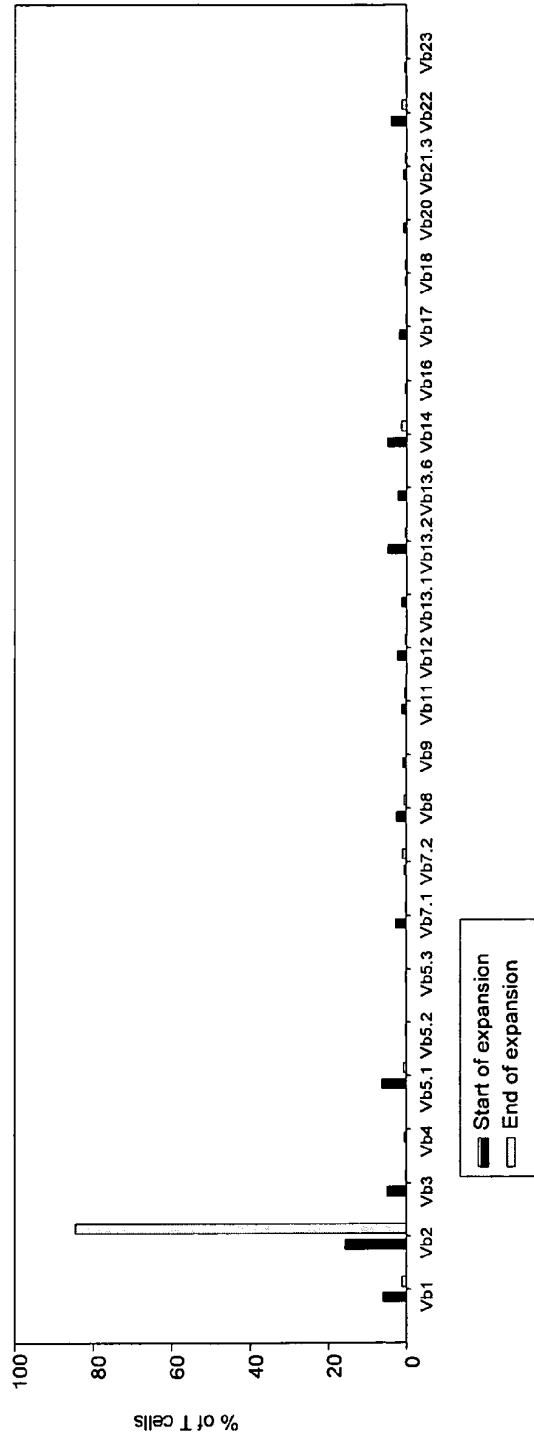
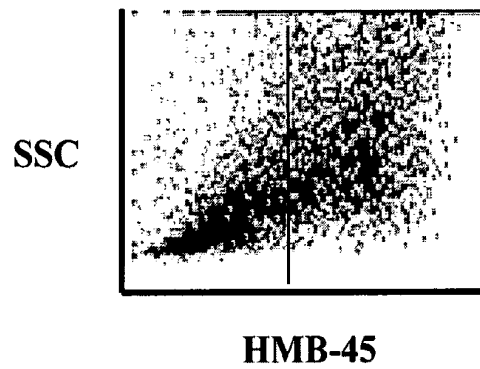


Fig. 7

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**Sentinel lymph node
with metastasis**



Negative lymph node

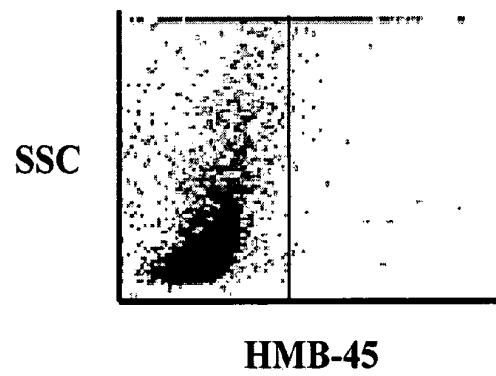


Fig. 6

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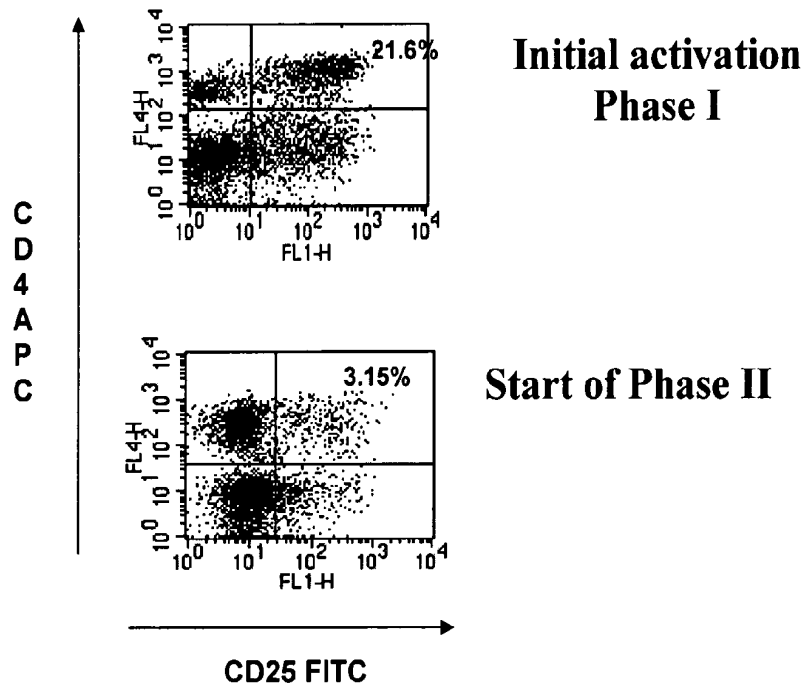


Fig. 9

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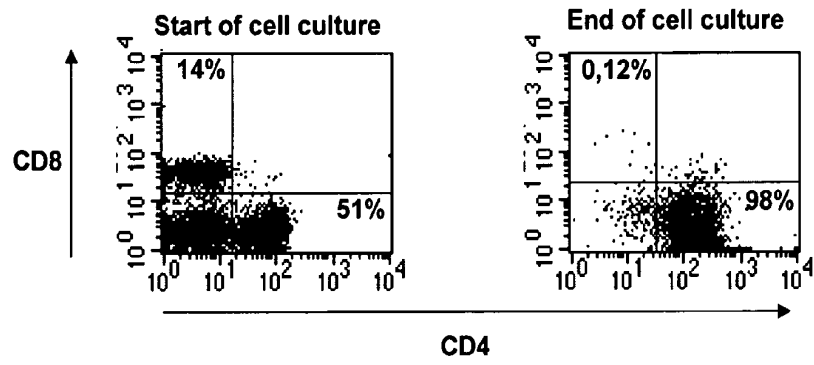


Fig. 10

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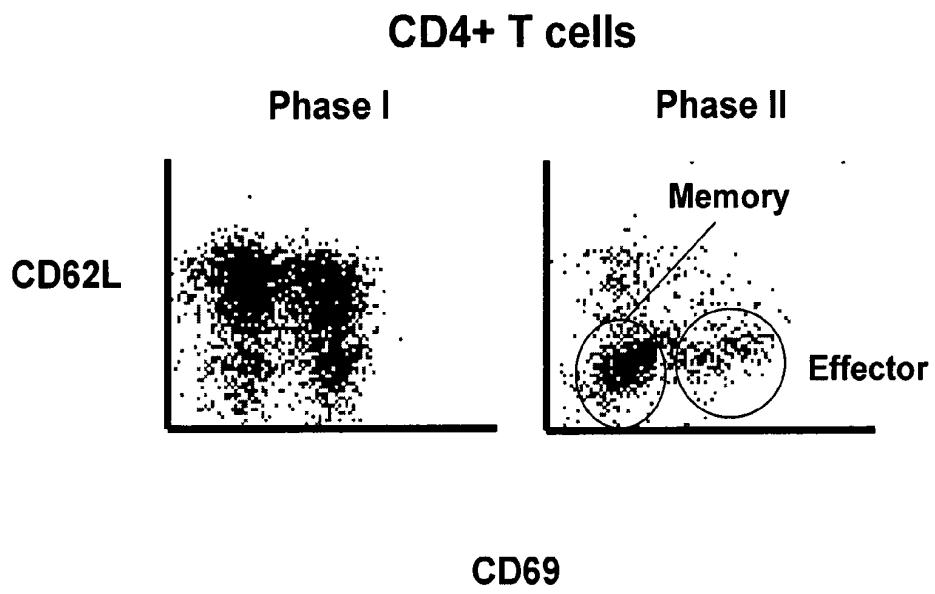


Fig. 11

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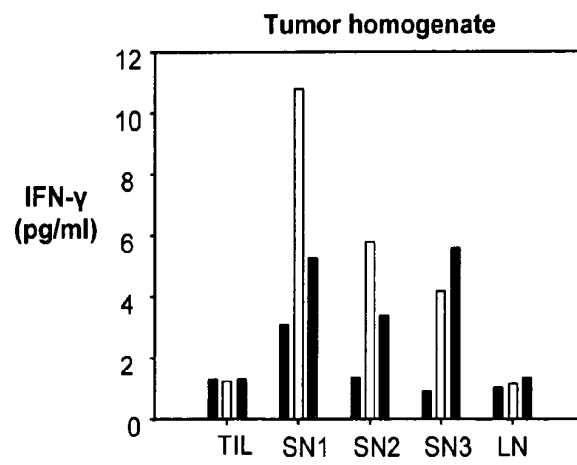


Fig. 12

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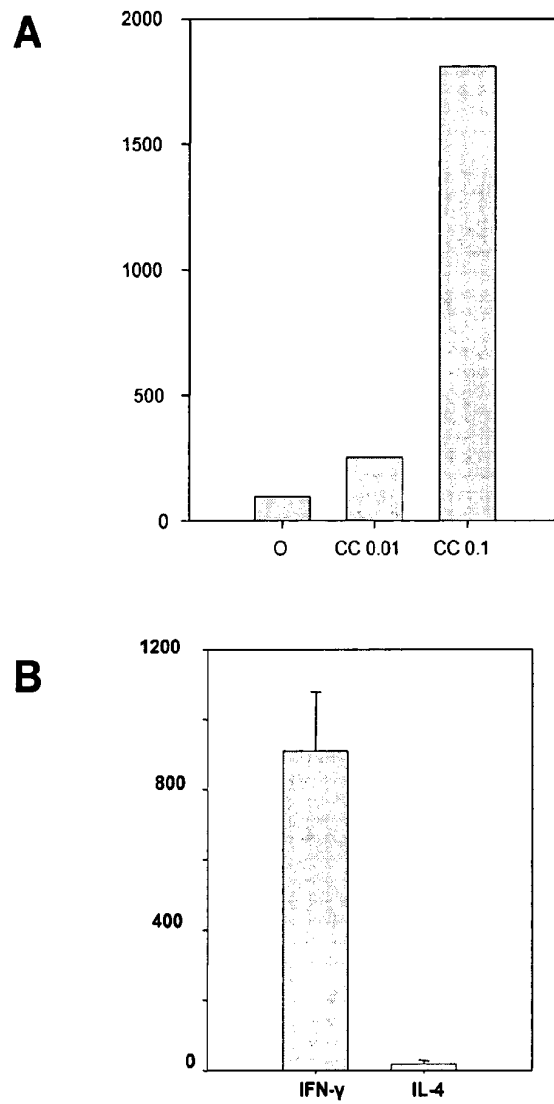


Fig. 13

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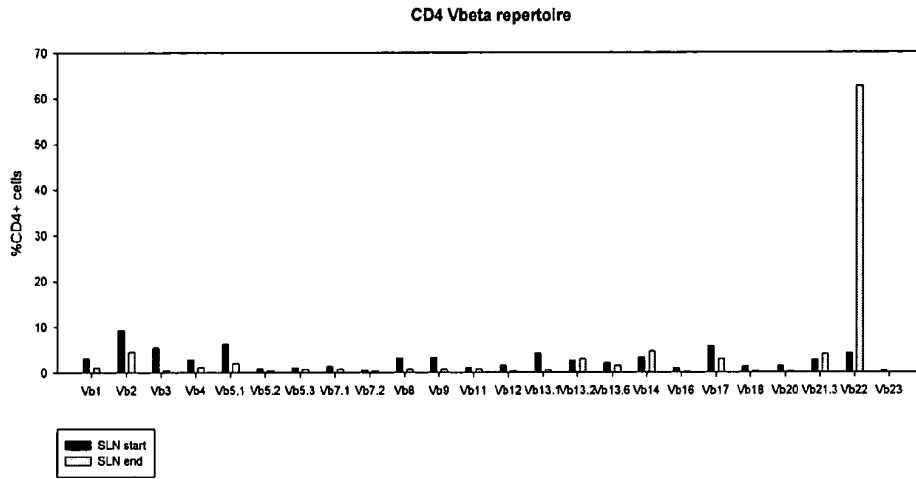


Fig. 14