Improved method for expansion of tumour-reactive T-lymphocytes for immunotherapy of patients with cancer

Field of the invention

The invention relates to an improved method for expansion and activation of tumour-reactive lymphocytes, in particular CD4+ helper and/or CD8+ T-lymphocytes. The T-lymphocytes are not CD4+ $CD25+^{Hi}$ lymphocytes, i.e. the present invention does not cover regulatory T-lymphocytes. The lymphocytes may be used for treating and/or preventing cancer.

Background of the invention

According to the immune surveillance hypothesis, the immune system is continuously sensitized against developing tumours, where experimental evidence strongly supports this notion. The identification of specific tumour antigens has created new possibilities for tumour immunotherapy and many immunotherapeutic approaches are now being translated into clinical trials. Among these, adoptive transfer of tumour antigen-specific lymphocytes seems particularly promising. These attempts have, so far, usually been based on either mononuclear cells from peripheral blood or tumour infiltrating lymphocytes (TIL) separated from fresh tumour specimens. In recent trials, treatment of patients with malignant melanoma with autologous transfer of expanded TILs, objective response rates of up to 51% has been reported. TIL cells are few, they are frequently unresponsive (anergic) due to immunosuppressive mechanisms from the tumour creating long periods for expansions to occur (several months). Furthermore, the protocols have been aiming towards the expansion of $CD8^+$ cytotoxic T cells and the cells have been reintroduced into patients preconditioned with chemotherapy and in addition the patients have been treated with high doses of interleukin-2 to provide survival of $CD8^+$ T cells.

Disclosure of the invention

The present inventors have previously shown that activation of naïve T cells may occur within the highly specialized microenvironment of secondary lymphoid organs, such as the sentinel lymph node. In other words, the sentinel node may be regarded as the primary site for the immune system to encounter tumour antigens.

The inventors have previously disclosed a general method for expansion of tumour-reactive T-lymphocytes from sentinel lymph nodes, showing that it is possible to culture

T-lymphocytes obtained from sentinel lymph nodes in order to obtain a culture of tumour-reactive T-lymphocytes. The tumour-reactive T-lymphocytes may be used for treating cancer by administering an effective amount of tumour-reactive T-lymphocytes to the patient from which the sentinel nodes were removed.

The success of a cancer treatment comprising administration of tumour-reactive T-lymphocytes are determined by factors such as, e.g., the amount of tumour-reactive T-lymphocytes obtained after the expansion step, i.e. the amount of tumour-reactive T-lymphocytes available for infusion to the patient, the time required to obtain an effective amount of tumour-reactive T-lymphocytes and the concentration and ratio of specific subpopulations of tumour-reactive T-lymphocytes obtained by the expansion method.

Accordingly, the present invention discloses an improved method for expansion of tumour-reactive CD4+ helper and/or CD8+ 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. Furthermore, the invention at the same time provides a method for directing the development of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes towards specific subpopulations. The T-lymphocytes are not CD4+ $CD25+^{Hi}$ lymphocytes, i.e. the present invention does not cover regulatory T-lymphocytes.

$CD4^{+}CD25^{Hi}$ T lymphocytes expressing the transcription factor FoxP3 are considered regulatory T cells (Treg). Tregs have the property to regulate T helper and T cytotoxic cells by inhibiting activation and proliferation and in addition Treg inhibit the production and release of useful Th1 cytokines such as IFN-gamma. Thus, the method presented here is developed in order to promote the expansion of Thelper cells and T cytotoxic T cells and to avoid expansion of Treg cells.

The tumour-reactive T-lymphocytes most often generated by the present method are CD4+ helper T-lymphocytes. One of the objects of the present expansion method is in some respect to imitate the natural pathway of the patient's own immune system, and to a certain degree let the components of the patients immune system determine whether, in the first place, CD4+ helper or CD8+ T-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+ helper T-lymphocytes. However, in some cases CD8+ T-lymphocytes are generated. If CD4+ helper T-lymphocytes are generated, they will be further expanded as described herein, however, the method may also be used for expanding CD8+ cells.

The inventors have found that an expansion method comprising two different phases are especially useful for obtaining a high number of tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes in a relatively short time span, the two phases being

i) a first phase of stimulating tumour-reactive T-lymphocytes with tumour-derived antigen together with at least one substance having agonistic activity towards the IL-2 receptor, to promote survival of tumour-reactive T-lymphocytes, and

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

This expansion method may also be carried out using monocytes isolated from the patient as antigen specific cells. The monocytes will be administered to the patient when differentiated into dendritic cells by the use of maturating cytokines such as IL-4, GM_CSF and IL-3 followed by activation of the dendritic cells by the addition of Toll like receptor stimulating agencies such as lipopolysaccharide. The use of mature activated dendritic cells as the antigen specific population may promote and enhance the expansion of T helper cells and T cytotoxic T cells.

Definitions

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.

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.

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

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.

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.

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. 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 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.

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

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".

By the term "sentinel lymph node" is intended to mean the first lymph node(s) to receive lymphatic drainage from a tumour. The term "metinel lymph node" refers to the first lymph node(s) to receive lymphatic drainage from a metastasis.

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 according to the invention may be a mixture of lymphocytes obtained from lymph nodes draining a primary tumour and/or a metastasis, such as, e.g., a sentinel or metinel lymph node. These can be identified during surgery e.g. by injection of a lymph node locator, such as, e.g., a tracer substance, around or into the tumour or metastasis. The lymph node locator, such as, e.g., the tracer is transported in the lymph capillaries and accumulates in the sentinel/metinel node(s), thus identifying the tumour or metastasis draining lymph node(s). The inventors have recently shown that the first lymph nodes to receive drainage from a tumour are a potential rich source for naturally tumour-reactive CD4+ helper and/or CD8+ T-lymphocytes for *in vitro* expansion, as such nodes may contain a substantial amount of T-lymphocytes, that have been sensitized towards tumour-antigens and undergone in vivo expansion in the lymph nodes.

An alternative source of CD4+ helper and/or CD8+ T-lymphocytes may be the blood of a subject suffering from cancer, such as, e.g., peripheral blood. The subject may be an untreated patient that has had the disease for a long time or an already treated patient, wherefrom peripheral T-lymphocytes sensitized towards a tumour may be obtained. Other suitable sources of CD4+ helper and/or CD8+ T-lymphocytes include bone marrow, spleen tissue and tumours.

However, in a preferred embodiment of the invention, the starting material is obtained from sentinel or metinel lymph nodes.

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 immuno-tolerant of the expanded tumour-reactive T-lymphocytes).

Depending on the source of the starting material, it will 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 CD4+ helper and CD8+ 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 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 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.

Administration of unspecifically activated T-lymphocytes would have no or a very low probability of having therapeutic effect against any cancer, due to the small number of tumour relevant T lymphocytes.

In one embodiment of the invention the substances having agonistic activity towards the IL-2 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 preferred embodiment the substance is the natural ligand of the IL-2 receptor, namely IL-2.

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 helper 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 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.

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.

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

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.

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, such as, e.g., sentinel lymph nodes, or from tumours, 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. 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.

If the lymphocytes originate from blood, the tumour-derived antigen may be added already when the first phase i) is initiated, i.e. together with the substance having agonistic activity towards the IL-2 receptor, as the T-lymphocytes have not been subjected to the above-mentioned immuno-suppression by tumour cells. Accordingly, when blood is used, the tumour-derived antigen is added essentially at the same time as when phase i) is initiated or at the most up to 2 days thereafter.

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 $CD4^+$ helper 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 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 do not express CD25 at all. The 5% or less of the T-lymphocytes expressing CD25 are 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.

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.

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 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.

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% CO_2.

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.

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 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 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.

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.

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 CD4+ helper T lymphocytes secrete cytokines, proteins and/or peptides that stimulate other cells of the immune 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, 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 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+ helper 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 signalling 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. 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.

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-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, 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.

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 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 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 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 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.

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 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.

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.

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 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).

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.

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.

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.

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 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. 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 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 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 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 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). Accoridngly, 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 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-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 lest 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 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).

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, 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). 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 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.

Method of treatment

The tumour-reactive T-lymphocytes obtained by an improved expansion method as described above may be used in a method for treating a subject suffering from a disease of neoplastic origin or for effecting tumour regression in a subject having a tumour, the method comprising administering to the subject in need thereof an effective amount of tumour-reactive T-lymphocytes according to the invention.

The method described herein may be used for treatment of any solid neoplasm of epithelial, mesenchymal or embryological origin in any anatomical location, such as e.g., for epethilal neoplasms e.g. carcinomas in the breast, colon, pancreas, bladder, small intestines, prostate, cervix, vulva, ovaries; for mesenchymal neoplasms e.g. sarcomas in the joints, bones, muscles and tendons and some haematological such as lymphomas; for embryological neoplasms, e.g. teratomas.

The definition of an effective amount of tumour-reactive T-lymphocytes 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., intravenous, intraarterial, intrathecal, or intraperitonal 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 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 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.

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.

The treatment according to the present invention may be supplemented with any other relevant treatment for cancer. 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 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 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, 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.

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 comprising a lymph node locator, such as e.g. the ones mentioned above.

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

Figure legends

Figure 1 illustrates that the sentinel node is the natural primary site for the presentation and activation of T cell reactivity towards tumour antigen.

Figure 2 shows that initially sentinel node lymphocytes are activated with tumour 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 3 illustrates that Phase I and Phase II activation results in expansion and enrichment of $CD4^+$ T helper cells.

Figure 4 illustrates that in Phase I the majority of cells are naïve 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 5 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-γ production.

Figure 6 illustrates that after expansion after phase ii) there is a dose dependent increase in antigen dependent IFN-γ production.

Figure 7 illustrates that the expansion and activation protocol promotes the expansion of antigen specific T cell clones as investigated by the selective enrichment of TCR Vβ expression.

Figure 8 A-D are CT scans of patient # 5. After transfusion of tumour-reactive lymphocytes the patient had total regress of liver metastases located in both lobes (which had been declared incurable by liver surgery), normalisation of CEA levels, disappearance of ascites and was physically well fit, exercising regularly.

Figure 9 A-F are CT scans of patient # 10. After transfusion the patient had regress of liver metastases and ascitic fluid. He was in fairly good health and further imaging showed stable disease.

Figure 10 A-H are CT scans of patient # 12. Three months after transfusion he had regress of metastases in the liver and lungs with almost a normalised CEA level at 5.9 (Normal < 4.0), disappearance of ascites and he appears clinically healthy.

Figure 11 shows T lymphocytes gated for the expression of $CD4^+$ which were stained for the expression of CD25 and the transcription factor FoxP3 at the beginning (A) and at the end (B) of an expansion. Initially (panel A), 4.8% of CD4+ T lymphocytes expressed FoxP3 and high levels of CD25, thus identified as Treg. At the end of the expansion a very small number of Tregs were present 0.3% (panel B).

Examples

Example 1

Expansion of tumour-reactive T-lymphocytes

Identification of sentinel nodes was done peroperatively using the sentinel node technique. Briefly, 1 ml of Patent blue dye was injected (Guerbet, Paris) and distributed superficially in the serosa around the tumour. Within five to ten minutes, one to three mesenteric lymph nodes were coloured blue, these sentinel nodes were marked with sutures and removed (see Figure 1). One non-sentinel mesenteric lymph node, distant from the tumour, was also identified and removed as a control.

The sentinel- and non-sentinel lymph nodes were cut in half and 1 mm thick slices were taken from the center and the periphery. The rest of the lymph nodes were sent for histopathological examination according to routine procedure. A part of the tumour, including a sample of the invasive margin, was also removed for research purposes.

Cell culture

Phase I, initial activation

The sentinel node material was kept on ice and immediately taken care of using AIM V® Media (Invitrogen) at all times. Single cell suspensions of sentinel node lymphocytes were obtained through gentle homogenisation in a loose fit glass homogenisator, and following homogenisation cells were washed twice in medium. The sentinel 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 tumour 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% CO_2 and 240 IU IL-2/mL media added every 3-4 days.

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 2) by the addition of autologus tumour 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 µg/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.

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.

Immunological evaluation

Further immunological evaluation was performed using tritium labelled thymidine incorporation proliferation assays. An aliquot of Sentinel node lymphocytes was set aside for this purpose, a single cell suspension of non-sentinel 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 $3x10^5$ cells/well in a 96-well plate and stimulated with tumour

homogenate diluted 1/100, 1/10 or Con A 10 µg/ml (Sigma) in triplicates. Proliferation was measured on day 5, 6 and 7 by adding 1 µCi of ^{3}H-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 $3x10^5$ cells/well in triplicate with tumour homogenate diluted 1/10 and 1/100, or Con A 10 µg/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 5). 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 6 A and 6B).

Flow cytometry analyses

Characterisation of cells was performed using flow cytometry initially on cells from the sentinel node, non-sentinel node, PBMC and from the tumour. From the sentinel 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 3, 4 and 5). 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.

The Vβ-repertoire was examined using the Beta mark kit (Beckman Coulter), $5x10^5$ cells/tube was stained in 10 µl of the 8 different vials containing mixtures of FITC, PE and dual-colour FITC-PE conjugated TCR Vβ antibodies and with the addition of CD8 PerCP and CD4 APC to each tube (Figure 7).

Example 2

Treatment of colon cancer by administering tumour-reactive T-lymphocytes

<u>Identification and removal of sentinel and metinel lymph nodes from colon cancer patients:</u>

Sixteen patients diagnosed with colon cancer, six woman and ten men with an average age of 62 years were studied. Patients were histopathologically classified as Duke's C or D. There were also 5 patients with Duke's B with aggressive tumour characteristics such as ulcerations, vascular or perineural invasion. Patients 7 and 14 however had earlier been surgically treated due to colon cancer and now had recurrent disease with metastases to the liver. The local ethical committee approved the study and each patient gave informed consent.

Identification of sentinel or metinel nodes was done intraoperatively. Mobilisation of the colonic tumour site was achieved by division of peritoneal adhesions in order to facilitate inspection of tumour and mesentery. Injections of Patent blue dye (Guerbet, Paris) were distributed superficially in the serosa around the tumour. Within five minutes, one to three mesenteric lymph nodes were coloured blue, these sentinel nodes were marked with sutures and removed when the resection was complete. One non-sentinel mesenteric lymph node, distant from the tumour, was handled the same way.

The sentinel- and non-sentinel lymph nodes were cut in half and 1mm thick slices were taken from the centre and the periphery. The rest of the lymph nodes were sent for histopathological examination according to routine procedure. A piece of the tumour, including a part of the invasive margin, was used for antigen preparation.

The lymphocytes obtained from the lymph nodes were then expanded as described in Example 1.

Administration of tumour-reactive T-lymphocytes:

16 patients were treated with infusion of autologous lymphocytes expanded as described in Example 1. On average 74.7 million activated and clonally expanded T cells were administered as a transfusion. No toxic side effects like fever, chills, malaise, severe fluid retention, pulmonary oedema or respiratory distress were observed.

Follow-up evaluations

Follow-up included clinical examination every third to sixth month and control of CEA levels. All stage III and IV patients were in addition investigated with computer tomography of the thorax and abdomen. The patients were followed at regular visits on

average for 13 months (range 5-20), median follow-up time was 13½ months. Out of the 16 patients who had been treated with infusion of autologous lymphocytes eight had known distant metastases at diagnosis. Four patients received their transfusions due to known recurrences and out of them three are still without signs of recurrences. One patient was operated due to a solitary liver metastases and has since then been without relaps. As it appears from Figure 8 and Figure 9, one patient with liver metastases located in both lobes (which had been declared incurable by liver surgery) had total regress of liver metastases after transfusion of tumour-reactive lymphocytes, and furthermore had normalisation of CEA levels, disappearance of ascites and is physically well fit, and exercising regularly. One further patient with liver metastases had regress of liver metastases and ascetic fluid after transfusion (see Figure 10, 11 and 12). One patient had three months after transfusion regress of metastases in the liver and lungs (see Figure 13, 14, 15, and 16) with almost a normalised CEA level at 5.9 (Normal < 4.0), disappearance of ascites and he appears clinically healthy.

Results

Sixteen patients with colon cancer or solitary colorectal liver metastases were operated on at the South Stockholm General Hospital and included in the study. The primary locations of the tumours were three in caecum, 4 in colon ascendens, 1 in colon descendens, 7 in the sigmoid colon and 1 in rectum. Seven right-sided hemicolectomies, 1 left-sided hemicolectomy, 7 sigmoid resections and 1 rectumamputation were performed. Two patients had earlier been operated on with rectumamputation and sigmoid resection; they now underwent partial liver resections due to liver metastases. One patient had recurrences at two abdominal locations and had earlier been operated due to a tumour in the caecum. At our operation two sentinel nodes draining the metastasis were identified, one in the colonic mesentery and one in the mesentery of the small intestine. An extended resection of the anastomotic ileocolonic region with mesentery was done.

In all patients, one to three (average 2.1) sentinel node(s) were identified intraoperatively by peritumoural patent blue injections. Among the patients with primary colonic resection on average 15.8 lymph nodes were retrieved from each specimen. After histopathological investigation of these lymph nodes five patients were classified as Duke´s C and 5 patients as Duke´s B, all of them were classified as high-risk tumours due to growth of tumour cells along nerves and in vessels at pathological anatomical investigation. Five patients had distant metastases and were at time of

metastatic resection classified as Duke's D. Two patients of them had solitary liver metastases. In addition sentinel nodes were also analysed by FACS (Fluorescence activated cell sorter) and antibodies against cytokeratin 20, which is expressed by colon cancer tumours, for the purpose to detect micrometastases. The cytokeratin 20 assessments of lymph nodes by flow cytometry were in agreement with the pathological anatomical diagnosis (not shown) except in one case where a false negative sentinel node (according to histopathological analysis) was positive in the cytokeratin 20 FACS analysis.

The sentinel node is the first lymph node draining the tumour and is therefore the first site of lymph node metastasis (Dahl et al), but the sentinel node is also the primary site for the activation of the immune system. Tumour cells, debris, necrotic cells and antigen presenting cells accumulate in the sentinel node where presentation, activation and clonal expansion of T cells directed against the tumour occur. The present inventors took advantage of this population of *in vivo* expanded T cell population of sentinel node acquired lymphocytes for *in vitro* cell culture, expansion and transfusion.

Sentinel node acquired lymphocytes is a population of T cells activated and clonally expanded against tumour antigens that can efficiently be harvested during the surgical procedure. In contrast to recent immunotherapy trials focusing on cytotoxic T cells, the aim of the present inventors was to create a protocol for *in vitro* enhancement of the *in vivo* initiated clonal expansion of T helper cells. T helper cells seem to be necessary for the effective function of cytotoxic T cells and for the creation of memory cells. Furthermore, in a T cell receptor transgenic system targeting an islet cell antigen, the transfusion of Th1 cells was found to be sufficient for the β cell destruction and development of diabetes mellitus. *In vitro* culture of sentinel node acquired lymphocytes resulted in a Th1 activation and clonal expansion of T helper cells as indicated by the dominant production of the hallmark Th1 cytokine IFN-γ and the enrichment of a restricted TCR Vβ repertoire. The tumour homogenate used to expand the T cells is likely to be endocytosed and processed by antigen presenting cells for class II presentation leading to activation of $CD4^+$ T helper cells resulting in expansion favouring T helper cells. By cross presentation antigens taken up by endocytosis may be processed and presented in the class I pocket resulting in activation of $CD8^+$ cytotoxic T cells. Interestingly, in some cases the inventors found clonal expansion of both $CD4^+$ and $CD8^+$ T cells.

The average number sentinel node acquired lymphocytes at start of expansion was 107.4 million cells (range 3.6-509 millions, median 70 millions). Cells were characterised by flow cytometry. The ratio between $CD4^+$ and $CD8^+$ cells at start was in average 4.9 (range 0.36-10, median 5.4) indicating an expansion $CD4^+$ T helper cells in sentinel nodes compared to the CD4/CD8 ratio in peripheral blood (normal range 1.0-2.5) (figure 2A). In addition B lymphocytes (CD 19) and natural killer (NK) cells (CD 56) were present in sentinel nodes (not shown). The cells were held in culture in average 36.1 days (range 23-58 days), median 33 days. Cells were monitored closely by flow cytometry at least weekly. Initially the total number of cells decreased. B cells and NK cells disappeared almost completely and the number of $CD8^+$ T killer cells was diminished. The culture procedure used promoted mainly the expansion of T helper cells since the average CD4/CD8 ratio was 92.5. Restimulation with autologous tumour antigen resulted in clonal expansion of tumour reactive T cells as assessed by investigating the T cell receptor Vβ repertoire of sentinel node acquired lymphocytes before and after *in vitro* culture.

Before transfusion expanded T cells were functionally tested against autologous tumour antigens by measuring activation and cytokine production of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4. In vitro expanded sentinel node acquired lymphocytes responded upon restimulation with tumour antigen with the production of IFN-g and no or very little IL-4 indicating that the expanded T cells were functional and Th1 responsive.

Six patients with Duke's D were treated in the study. Two patients staged as Duke´s D at surgery with metastases to the liver and to the lungs and liver, respectively displayed marked regression of disease (pat 5 and 12). After transfusion of lymphocytes the first patient had total regress of liver metastases located in both lobes (which had been declared incurable by liver surgery) (figure 3) normalisation of CEA levels, disappearance of ascites and appear healthy. Patient 12 shows regress of metastases in the liver and lungs with almost a normalised CEA level at 5.9 (Normal < 4.0), disappearance of ascites and he appears clinically healthy. Patient 1 displayed a regression of the size of liver metastasis, and initially a decrease in CEA levels, disappearance of ascites and she was in excellent shape when she suddenly died (day 191), what appears to have been a lung embolus. Two Duke's D patients display stable disease without progression of metastasis or increase in CEA levels. The oldest patient no 7 in the study displayed stable disease for five months, but thereafter CEA levels

started to increase and she died at age 83. No autopsy was performed. One patient was staged as Duke´s C at surgery but soon developed metastases to the liver and lungs (Duke´s D), but following transfusion and chemotherapy a regress of the lung and liver metastases were seen with only slightly elevated CEA levels. The patients classified as Duke's C all have normal CEA levels and appear without any signs of radiological or clinical recurrence of disease. Four of the Duke's B patients are healthy with normal CEA levels and have no signs of recurrent disease. Patient no 9 classified as Duke's B, but with an aggressive growing tumour shows signs of recurrent disease with elevated CEA levels (67) and signs of liver metastases.

To investigate the fate of transfused T cells the present inventors analysed T cell proliferation against tumour extract in peripheral blood. As mentioned before, they could not demonstrate any T cell reactivity in peripheral blood against autologous tumour antigens in any of the patients prior to transfusion. However, we were able to detect T cell proliferation against autologous tumour antigens in peripheral blood in all investigated patients up to 10 months after transfusion indicating the presence of clonally expanded circulating tumour-reactive T cells.

Summary of patient characteristics

Below is a table of all participants in the study, sorted after Duke's classification at surgery:

Participant characteristics

Age/ Sex	Duke's Classification	Infused cells ($x10^6$)	CD4/ CD8 [a]	IFN-γ (pg/ml)	Overall survival (months)	Response
67/M	B	4	92 / 0.2	ND	31	SD
67/F	B	8	15 / 51	ND	30	SD
71/M	B	50	74 / 15	2091	29	SD
74/M	B	63	64 / 22	ND	29	SD
66/M	B	152	82 / 1.5	1411	27	SD
64/F	C	110	64 / 25	ND	34	SD
58/F	C	16	77 / 18	417	23	SD
61/F	D	1	3.7 / 35	ND	6	SD
47/M	D	80	24 / 16	ND	36	CR
54/M	D	40	37 / 24	ND	36	SD

65/M	D	270	82 / 15	ND	36	CR
42/F	D	80	66 / 11	ND	33	CR
82/F	D	40	98 / 0.1	ND	6	SD
74/M	D	130	73 / 22	142	30	CR
33/M	D	72	72 / 1.5	908	12	PR
66/M	D	25	37 / 27	764	26	PR

[a]) The numbers represent the percentage of CD4 and CD8 positive cells detected with FACS.

Discussion

To the knowledge of the present inventors, sentinel or metinel node-based immunotherapy in patients with colon cancer has never been presented before. Thus, this is the first attempt to use lymphocytes acquired from sentinel or metinel nodes for therapy. There are some major differences between the present study and e.g. treatment with high-dose IL-2 (Rosenberg). Firstly the use of sentinel node acquired lymphocytes that have been *in vitro* stimulated by autologous tumour homogenate and APCs, causes a highly specific cellular immune response towards the tumour. Only T cells with high affinity to the primary tumour will survive until transfusion. In a systemic generalized treatment with high-dose IL-2 intravenously to patients all lymphocytes will be equally stimulated and reasonably only a very small fraction of them are tumour specific. The present inventors believe that since the sentinel node(s) are the first draining lymph nodes to a tumour there will be an excessive accumulation of tumour specific lymphocytes. The proliferation and transfusion of true tumour recognising T cells should create a massive tumour specific reaction. Secondly the high-dose IL-2 regimen causes high-toxicity and severe complications, long treatment periods and high costs. The transfusions according to the present method have been given without complications during about one hour and the patients are often discharged the same day. Thirdly, the present protocol aim towards expansion of T helper cells from sentinel nodes, in contrast to expansion of cytotoxic T cells harvested as tumour infiltrating lymphocytes.

This study shows that freshly isolated sentinel node acquired lymphocytes possesses a proliferative ability in vitro against autologous tumour homogenate and can without complications be transfused to the patient as adoptive immunotherapy. There is a strong indication to that treatment with expanded sentinel node acquired lymphocytes may improve the outcome of patients with high-risk or disseminated colon cancer, as well as patients suffering from types of solid cancer.

Claims

1. A method for in vitro expansion of tumour-reactive CD4+ T helper and/or CD8+ T-lymphocytes, the method comprising

i) a first phase of stimulating tumour-reactive CD4+ T helper and/or CD8+ T-lymphocytes 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+ T helper and/or CD8+ T-lymphocytes; and

ii) a second phase of activating and promoting growth of tumour-reactive CD4+ T helper and/or CD8+ T-lymphocytes, wherein the second phase ii) is initiated when the CD25 cell surface marker is down-regulated on CD4+ T helper and/or CD8+ T-lymphocytes, wherein down-regulation is defined as that 5% or less of the T-lymphocyte population expresses CD25 and wherein phase ii) is initiated by the addition of tumour-derived antigen to the T-lymphocytes for activating tumour-reactive CD25-negative T-lymphocytes.

2. A method according to claim 1, wherein the first phase i) is initiated by adding the at least one substance having agonistic activity towards the IL-2 receptor.

3. A method according to claim 2, wherein the substance having agonistic activity towards the IL-2 receptor is IL-2.

4. A method according to any of the preceding claims, wherein the tumour-derived antigen is a denatured homogenate of a tumour.

5. A method according to claim 4, wherein the tumour-derived antigen is autologous.

6. A method according to any of the preceding claims , which further comprises addition to the T-lymphocytes of antigen presenting cells together with the tumour-derived antigen.

7. A method according to claim 6, wherein the antigen presenting cells are irradiated peripheral blood leucocytes containing antigen-presenting B-cells and/or monocytes.

8. A method according to any of the preceding claims, wherein the second phase ii) comprises adding at least one substance capable of up-regulating IL-12R on the T-lymphocytes.

9. A method according to any of the preceding claims, wherein the second phase ii) comprises adding one or more substances capable of antagonizing development of Th2 type T-lymphocytes.

10. A method according to claim 9, wherein the one or more substances capable of antagonizing development of Th2 type T-lymphocytes are one or more substances capable of neutralizing IL-4, IL-5, IL-10, and/or TGF-beta.

11. A method according to claim 10, wherein the one or more substances capable of neutralizing IL-4, IL-5, IL-10, and/or TGF-beta are anti IL-4 antibody, anti IL-5 antibody and/or anti IL-10 antibody.

12. A method according to any of the preceding claims, wherein a further amount of the one or more substance capable of antagonizing development of 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 is added regularly throughout phase ii).

13. A method according to any of the preceding claims, wherein the second phase ii) comprises adding one or more substances promoting the development of Th1 type T-lymphocytes.

14. A method according to claim 13, wherein the one or more substances promoting the development of Th1 type T-lymphocytes is substances having agonistic activity towards the IL-7, IL-12, IL-15 and/or IL-21 receptor.

15. A method according to claim 14, wherein the one or more substances is selected from IL-7, IL-12, IL-15 and IL-21.

16. A method according to any of the preceding claims for the preparation of Th1-lymphocytes of the memory and/or effector type.

17. A method according to any of the preceding claims, which further comprises monitoring the expression of cell surface markers, such as, e.g., CD25 and/or CD69 on the T-lymphocytes continuously during the first phase i) and second phase ii) and wherein the T-lymphocytes are harvested when CD25 on T-lymphocytes in the second phase ii) is down-regulated.

18. A method according to claim 17, wherein the T-lymphocytes are subjected to at least one additional round of phase ii), when CD25 on T-lymphocytes is down-regulated.

19. A method according to any of the preceding claims, wherein the T-lymphocytes are derived from lymph nodes draining a primary tumour and/or a metastasis, or they are derived from blood.