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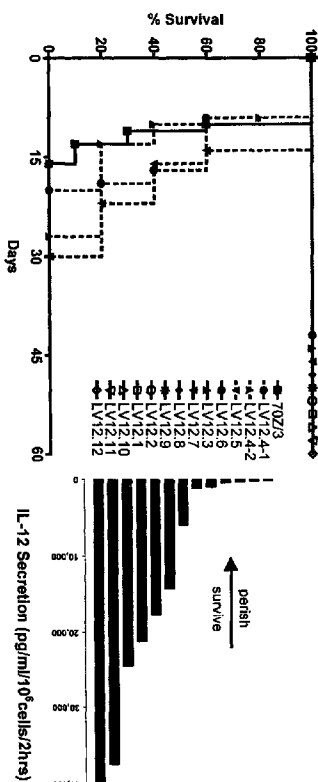
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(54) Title: IL-12 IMMUNOTHERAPY FOR CANCER

FIGURE 3





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TITLE: IL-12 Immunotherapy for Cancer**FIELD OF INVENTION**

The invention relates generally to compositions and methods for therapeutically and prophylactically treating cancer. In particular, the present invention pertains to IL-12, lentiviral vectors encoding IL-12 for transducing cells and use of the transduced cells for cancer immunotherapy.

BACKGROUND OF THE INVENTION

Cancer immunotherapy aims to overcome the inability of the immune system to efficiently protect against the establishment of tumors or reject established tumors.

Lentiviral Vectors (LVs)

Lentiviral vectors (LVs) are efficient gene transfer agents. They are stable and can be concentrated by ultracentrifugation to high titers. Compared to adenovirus, for example, they generate little immune consequences on their own reducing responses against transduced cells. Advances in LV design, safety, and long-term testing will increase their clinical adaptation. LVs have been used in cancer immunogene therapy (Metharom, P. *et al.*, 2001 ; Firat, H. *et al.*, 2002), the induction of DCs (Esslinger, C. *et al.*, 2003) and antigen presentation for CTL responses (Breckpot, K. *et al.*, 2003 ; Esslinger, C. *et al.*, 2003), and the transduction of CD34+ cells differentiated into DCs towards HIV/AIDS immunotherapy DCs (Gruber, A. *et al.*, 2003).

Interleukin-12

Cancer cells express antigens. Despite the presence of such antigens, tumors are generally not readily recognized and eliminated by the host, as evidenced by the development of disease. The inability of the immune system to protect against tumors may be due to mechanisms of evasion, active suppression, or sub-optimal activation of the response.

Cytokines are integral to both the innate and acquired immune systems. They can alter the balance of cellular and humoral responses, alter class switching of B lymphocytes and modify innate responses.

Interleukin-12 is a heterodimeric cytokine with multiple biological effects on the immune system. It is composed of two subunits, p35 and p40, both of which are required for the secretion of the active form of IL-12, p70. Interleukin-12 acts on dendritic cells (DC), leading to increased maturation and antigen presentation, which can allow for the initiation of a T cell response to tumor specific antigens. It also drives the secretion of IL-12 by DCs, creating a positive feedback mechanism to amplify the response. Once a response is initiated, IL-12 plays a fundamental role in directing the immune system towards a Th1 cytokine profile, inducing CD4⁺ T cells to secrete interferon-gamma (IFN- γ) and leading to a CD8⁺ cytotoxic T cell response.⁴ However, IL-12 is also a strong pro-inflammatory cytokine that leads to the secretion of other cytokines including tumor necrosis factor-alpha (TNF- α) which, combined with IFN- γ , is a prerequisite for the development of CD4⁺ cytotoxic T lymphocytes (CTL).⁵ Furthermore, IL-12 can promote the activation of innate immune cells such as macrophages and eosinophils through its induction of IFN- γ and other cytokines. This activation then leads to IL-12 secretion by these cells and further amplification of both the innate and acquired responses.⁴ However, high levels of IL-12, and consequently IFN- γ , have also been associated with induction of antagonistic molecules such as IL-10 and the depletion of signalling molecules downstream of IL-12, such as STAT4.⁶⁻⁸

Direct injection of recombinant IL-12 has been shown in some mouse models of leukemia.⁹⁻¹³ While initial human trials employing this approach were less promising (14-17 discussed in 4).

Innovative gene therapy strategies may accelerate the development of prophylactic immunotherapy against cancer.

Summary

The inventors have demonstrated that intraperitoneal (IP) administration of low dose rIL-12 elicits a protective response against an established tumor burden and that this CD8⁺ T cell-dependent response leads to long-term immune memory. The inventors also delivered IL-12 by way of transduced tumor cells, mediated by a lentiviral delivery system to ensure that optimum concentrations of IL-12 were available at the tumor site. The method of delivering IL-12 is highly effective and is readily applied to a variety of cancers.

The application provides in one aspect, a composition comprising:

a lentiviral vector;

an IL-12 expression cassette.

In one embodiment, the IL-12 expression cassette comprises a polynucleotide optionally encoding a p35 polypeptide and a polynucleotide encoding a p40 polypeptide; or a polynucleotide encoding an IL-12 fusion polypeptide. In another embodiment the IL-12 fusion polypeptide has at least 70% sequence identity to SEQ ID NO: 4 and binds an IL-12 receptor. In a further embodiment, the lentiviral vector optionally comprises one or more of a: 5'-Long terminal repeat (LTR), HIV signal sequence, HIV Psi signal 5'-splice site (SD), delta-GAG element, Rev Responsive Element (RRE), 3'-splice site (SA), Elongation factor (EF) 1-alpha promoter and 3'-Self inactivating LTR (SIN-LTR). In yet a further embodiment, the lentiviral vector comprises a central polypurine tract optionally SEQ ID NO:2 and/or a woodchuck hepatitis virus post-transcriptional regulatory element, optionally SEQ ID NO:3; or a sequence having at least 70% sequence identity to SEQ ID NO:2 and/or SEQ ID NO:3. In another embodiment, the lentiviral vector comprises a pHR' backbone. In one embodiment, the lentiviral vector is a clinical grade vector. In one embodiment, the composition further comprises an activator polynucleotide encoding a polypeptide that converts a prodrug to a drug, optionally a modified tmpk polynucleotide. In yet a further embodiment, the

activator polynucleotide comprises a tmpk polynucleotide with at least 80% sequence identity to a modified tmpk polynucleotide described herein.

In certain embodiments, the composition further comprises a detection cassette. In one embodiment, the detection cassette comprises a CD19, truncated CD19, CD20, human CD24, murine HSA, human CD25 (huCD25), a truncated form of low affinity nerve growth factor receptor (LNGFR), truncated CD34, eGFP, eYFP, or any other fluorescent protein or erythropoietin receptor (EpoR) polynucleotide; or a polynucleotide with at least 70% sequence identity to said polynucleotide.

In another embodiment, the composition further comprises an immune modulatory cassette. In one embodiment, the immune modulatory cassette comprises a polynucleotide that encodes a polypeptide that modulates an immune cell, optionally a dendritic cell or a T cell, optionally a CD4+ T cell, optionally CD40L, IL-7, or IL-15. In another embodiment the composition is a pharmaceutical composition and further comprises a pharmaceutically acceptable carrier.

In another aspect, the application provides a vector construct comprising:
a lentiviral vector;
an IL-12 expression cassette.

In another aspect the application provides an isolated virus comprising the vector construct or composition described herein.

A further aspect provides an isolated cell secreting IL-12 at the or above the threshold level, wherein the cell is optionally transduced with the composition, the vector construct or the isolated virus described herein. In one embodiment, the cell is a cancer cell, optionally an established cell line, optionally a primary cancer cell, optionally a cancer cell derived from a subject. In another embodiment, the cancer cell is a leukemic cell, optionally an ALL cell, an AML cell, a CML cell or a CLL cell. In a further embodiment, the threshold level is at least 1500 pg/mL/10⁶ cells/2hrs of IL-12, optionally at least 1500 pg/mL/10⁶ cells/2hrs, 1500-2500 pg/mL/10⁶ cells/2hrs, 2500-5000 pg/mL/10⁶ cells/2hrs, 5000-7500 pg/mL/10⁶ cells/2hrs, 7500-10000

pg/mL/10⁶ cells/2hrs, 10000-12500 pg/mL/10⁶ cells/2hrs, 12500-15000
 pg/mL/10⁶ cells/2hrs, 15000-17500 pg/mL/10⁶ cells/2hrs, 17500-20000
 pg/mL/10⁶ cells/2hrs or at least 20000 pg/mL/10⁶ cells/2hrs of IL-12

Another aspect provides a population of cells comprising isolated cells and/or transduced cells described herein wherein the population of cells optionally comprises at least 0.1 to 1% IL-12 producing cells, optionally leukemic cells, optionally about 0.5%, about 1%, about 1-5%, 5-10%, 10-20% or more IL-12 producing cells, optionally leukemic cells, and wherein the population of cells secretes above the threshold level optionally the threshold level necessary to induce or enhance a CD4+ T cell dependent immune response, optionally at least 1500 pg/mL/10⁶ cells/2hrs, 1500-2500 pg/mL/10⁶ cells/2hrs, 2500-5000 pg/mL/10⁶ cells/2hrs, 5000-7500 pg/mL/10⁶ cells/2hrs, 7500-10000 pg/mL/10⁶ cells/2hrs, 10000-12500 pg/mL/10⁶ cells/2hrs, 12500-15000 pg/mL/10⁶ cells/2hrs, 15000-17500 pg/mL/10⁶ cells/2hrs, 17500-20000 pg/mL/10⁶ cells/2hrs or at least 20000 pg/mL/10⁶ cells/2hrs of IL-12. In one embodiment, the population of cells is derived from a clone that secretes IL-12 above the threshold level optionally at least 1500 pg/mL/10⁶ cells/2hrs of IL-12.

A further aspect provides a composition comprising the isolated virus, cell or population of cells described herein.

Another aspect of the disclosure provides a method of expressing IL-12 in a cell, optionally a cancer cell comprising contacting the cell with the composition, the vector construct or the isolated virus under conditions that permit transduction of the cell, thereby providing a transduced cell, optionally wherein the IL-12 is secreted. In one embodiment, the method further comprises a step of isolating the transduced cell or isolating a population of cells comprising the transduced cell. In another embodiment, the method further comprises:

growth arresting the transduced cell, the population of cells or composition;
 and

introducing the transduced cell, population of cells and/or composition in a subject.

Another aspect provides a method of reducing the number of tumor cells or cancer burden in a subject in need thereof comprising administering to the subject an isolated virus, transduced cell, population of cells or composition described herein. Another aspect provides a method of treating a subject with cancer or an increased risk of cancer comprising administering to the subject an isolated virus, transduced cell, population of cells or composition described herein. In certain embodiments, the method further comprises monitoring cancer progression.

In certain embodiments, the cancer is a solid tumor. In other embodiments, the cancer is leukemia, optionally ALL, AML, CML or CLL.

A further aspect provides a method of inducing or enhancing an immune response in a subject optionally with cancer or an increased risk of cancer comprising administering to the subject an isolated virus, transduced cell, population of cells or composition described herein.

In one aspect the application provides a method of inducing or enhancing a memory immune response in a subject, optionally with cancer or an increased risk of cancer, comprising administering to the subject an isolated virus, transduced cell, population of cells or composition described herein. In certain embodiments, the immune response comprises a CD4+ T cell mediated immune response. In certain embodiments, the transduced cell is growth arrested prior to administering to the subject. In one embodiment, the transduced cell is irradiated prior to administering to the subject.

Also provided, is a method of delivering IL-12 to a subject, optionally with cancer or an increased risk of cancer, optionally, for enhancing cancer treatment comprising:

generating an IL-12 secreting cell wherein IL-12 secreted per cell is above a threshold level; and

introducing an effective number of the generated IL-12 secreting cells to the subject.

Another aspect provides a method of sustaining IFN γ levels induced by IL-12 in a host comprising:

generating an IL-12 secreting cell wherein IL-12 secreted per cell is above a threshold level; and

introducing an effective number of the generated IL-12 secreting cells to the patient.

In certain embodiments, the threshold level of IL-12 secreted is at least 1.5 fg/ml/cell/2hrs. In other embodiments, the threshold level of IL-12 secreted is at least 1.5 pg/ml cells/2hrs. In certain embodiments, the IL-12 secreting cell is generated by contacting the cell with a composition comprising a lentiviral delivery vector and an IL-12 expression cassette.

In certain embodiments, the cell is optionally a cancer cell, optionally derived from the subject with cancer. In certain embodiments, the cells are introduced by IP injection, subcutaneously or intradermally.

In certain embodiments, the immune response is initiated against a leukemia. In certain embodiments, the immune response is initiated substantially free of inducing or enhancing of a CD8⁺ T cell-dependent immune response. In certain other embodiments, the immune response leads to long-term immune memory. In certain embodiments, the immune response does not induce or enhance antagonistic cytokines.

In certain embodiments, the level of IL-12 produced is above a threshold level that enhances dendritic cell maturation and/or antigen presentation.

In another aspect, the application provides use of an isolated virus, transduced cell, population of cells or composition described herein for reducing the number of tumor cells or cancer burden in a subject in need thereof.

In another aspect the application provides use of an isolated virus, transduced cell, population of cells or composition described herein for treating a subject with cancer.

In another aspect the application provides use an isolated virus, transduced cell, population of cells or composition described herein for inducing or enhancing an immune response in a subject.

In another embodiment, the application provides use an isolated virus, transduced cell, population of cells or composition described herein for inducing or enhancing a memory immune response in a subject.

In another aspect the application provides use of an IL-12 secreting cell for delivering IL-12 to a subject, optionally with cancer or an increased risk of cancer optionally for enhancing cancer treatment:

generating an IL-12 secreting cell wherein IL-12 secreted per cell is above a threshold level; and

isolating an effective number of the generated IL-12 secreting cells for introduction to the subject.

In another aspect the application provides use of an isolated virus, transduced cell, population of cells or composition described herein, for treating a subject in need thereof, optionally a subject with cancer or an increased risk of developing cancer.

In certain embodiments, the number of cells administered ranges from 10^5 cells to 10^9 cells, optionally about 10^5 , about 10^6 cells, about 10^7 cells, about 10^8 cells, or about 10^9 cells. In other embodiments, the population of cells administered ranges from 10^5 cells to 10^9 cells, optionally about 10^5 cells, about 10^6 cells, about 10^7 , cells, about 10^8 cells, or about 10^9 cells.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Figures

The following non-limiting examples are illustrative of the present invention:

FIGURE 1a IP administered rIL-12-mediated protection of mice challenged with 70Z/3-L cells. Mice were challenged with 10^6 cells IP and received either no treatment-or injections of 0.1, 1, 10 or 20ng/mouse/day rIL-12 for 14 days ($n = 5$ mice for each group).

FIGURE 1b IP administered rIL-12 therapy leads to long-term protection against challenge with 70Z/3-L. (i) Naïve mice (A, $n = 10$) were challenged with 70Z/3-L cells on day 0 and treated for 14 days with injections of 20ng rIL-12 /mouse/day. A group of mice (B₁, $n = 7$) were included as controls for the 70Z/3-L cells (curve comparison by log rank test $p = 0.001$). (ii) After a period of 70 days, five mice from group A, having undergone rIL-12 therapy, were secondarily challenged with 10^6 70Z/3-L cells without further rIL-12 treatment. The other five animals were kept to confirm that no toxicity appeared after 70 days. Five naïve mice (B₂) were included to demonstrate the lethality of the 70Z/3-L cells (comparison of Kaplan-Meier survival curves was performed using Logrank test $p = 0.0015$).

FIGURE 1c Delayed IP administration of rIL-12 therapy leads to protection. Mice were injected with 10^4 70Z/3-L cells on day 0. A control group (70Z/3-L) did not receive treatment ($n = 4$). From days 0 through 5, groups of 4 or 5 mice (5 mice for days 0, 1 and 2, 4 mice for days 3, 4 and 5) started receiving injections of 20ng rIL-12/mouse/day for 14 days. Animals were monitored and euthanized at the appearance of symptoms. Curve comparison was performed using Logrank test. All treatment groups are significantly different from the control group ($p = 0.0029$) but are not significantly different from each other.

FIGURE 1d Requirement of T cells and IFN- γ for rIL-12-mediated protection following IP administration. Mice ($n = 5$ mice in each group) were depleted using antibodies as described in Materials and Methods. The mice were challenged with 10^6 70Z/3-L cells IP, injected with 20ng/mouse/day

rIL-12 and monitored for the appearance of symptoms. Comparison of Kaplan-Meier survival curves was performed using Logrank test ($p < 0.0018$).

FIGURE 2a Schematic representation of the LV-muIL-12 (LV-cPPT-EF1-mIL-12-WPRE) vector. LTR: long-terminal repeat; SD: splice donor; RRE; *rev* response element; SA: splice acceptor; cPPT: central polypurine tract; CMV: cytomegalovirus; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element; muIL-12: murine interleukin-12; SIN: self-inactivating LTR.

FIGURE 2b Interleukin-12 secretion by vector-transduced clones is a stable trait. Levels of IL-12 secretion were measured by ELISA on 2-5 independent occasions and seen to remain fairly constant; differences are not statistically significant.

FIGURE 3 Leukemia cell mediated IL-12 therapy leads to protection of challenged mice. Mice were injected IP with PBS or 10^6 cells of either the parent line, 70Z/3-L, or one of the vector-transduced clones and monitored for the appearance of symptoms. Clones secrete varying levels of IL-12 and a theoretical threshold was established, below which protection is not conferred.

FIGURE 4 Leukemia cell mediated IL-12 therapy leads to protection of challenged mice when only a portion of the cells are vector-transduced. Mice were injected IP with 10^6 cells of the parent line, 70Z/3-L, and varying proportions a.) 2%, 10% and 50% of the LV12.1 secreting clone or b.) 0.1%, 0.5%, 1% and 10% of LV12.2 and LV12.3, and monitored for the appearance of symptoms.

FIGURE 5 Leukemia cell mediated IL-12 therapy leads to long-term and specific protection against challenge with 70Z/3-L. Mice were initially challenged with either 10^6 LV12.2 cells or injected with PBS. More than 110 days following the primary challenge, primed mice ($n = 4$ in each group) were secondarily challenged with either 10^6 70Z/3-L or 10^6 L1210 cells. The PBS injected mice ($n = 5$ in each group) also received either 10^6 70Z/3-L or 10^6 L1210 cells to control for their efficiency to lead to morbidity, or another

injection of PBS and monitored for appearance of symptoms. Kaplan-Meier survival curve comparison was performed using Logrank test, $p < 0.0001$.

FIGURE 6 Requirement of the CD4⁺ T cell subset for leukemia cell-mediated protection of challenged mice. Mice ($n = 5$ in each group) were depleted using antibodies as described in Materials and Methods. The mice were challenged with 10^6 LV12.2 cells IP and monitored for the appearance of symptoms. Kaplan-Meier curve comparison was performed using Logrank test, $p = 0.0084$.

FIGURE 7 Cytokine expression profiles of mice receiving IP administration and leukemia cell-mediated IL-12 therapies. The mice ($n = 4$ in each group) receiving IP administered rIL-12 therapy were challenged with 10^6 70Z/3-L cells and received either no treatment or injections of 10 or 20ng/mouse/day rIL-12 for 14 days. Mice ($n=4$ in each group) receiving leukemia cell-mediated IL-12 therapy were challenged with 10^6 70Z/3-L cells IP and received either no treatment or treatment with various proportions (0.5%, 1% or 10%) of the vector-transduced clone LV12.2. Serum samples were collected and analyzed on days 7, 10 and 20 as described in Materials and Methods. (*- all mice from group 2 in the leukemia cell-mediated model were dead by day 20 such that serum was not collected from this group).

DETAILED DESCRIPTION

The inventors have shown that administration of low dose recombinant IL-12 (rIL-12) elicits a protective response against an established leukemia burden and that rejection is mediated by a CD4⁺ and CD8⁺ T cell-dependent immune response which leads to long-term immune memory without the induction of antagonistic cytokines. The inventors have compared this protocol to a cell therapy approach in which leukemic cells were transduced with a lentivirus vector (LV) engineering expression of murine IL-12 (both subunits) cDNA. Clones of the leukemic cells producing a wide range of IL-12 were established. Injection of IL-12 producing leukemic cells provoked long term and specific immunity without the induction of antagonistic mechanisms. Leukemia clearance in this instance, however, was mediated by a CD4⁺ cellular subset alone, suggesting a qualitatively different route to immunity than that seen in systemic therapy. The inventors found that injection of as few as 1% IL-12 producing leukemic cells along with 99% untransduced leukemic cells, was sufficient to elicit protective immunity as long as each of these cells produced IL-12 above a necessary threshold. This finding may explain the failure of many human cell therapy based protocols because in these cases IL-12 production is measured on bulk populations making it impossible to know if sufficient IL-12 is being produced in the local environment influenced by the IL-12 producing cell. The average production reported in these studies is well below the threshold reported in the present disclosure.

The vector constructs, compositions, cells and methods described herein for delivering IL-12 are highly effective and are readily applied to a variety of cancers.

Definitions

The term "a cell" as used herein includes a plurality of cells.

The term "ALL" as used herein refers to acute lymphoblastic leukemia is a rapidly growing leukemia wherei the malignant hematopoietic cells are lymphoid precursor cells. Cytogenetic abnormalities occur in ~70% of cases of ALL in adults but are not associated with a single translocation event.

The term "allogenic" also referred to as "allogeneic" as used herein means cells, tissue, DNA, or factors taken or derived from a different subject of the same species. For example in the context where allogenic transduced cancer cells are administered to a subject with cancer, cancer cells removed from a patient that is not the subject, are transduced or transfected with a vector that directs the expression of IL-12 and the transduced cells are administered to the subject. The phrase "directs expression" refers to the polynucleotide comprising a sequence that encodes the molecule to be expressed. The polynucleotide may comprise additional sequence that enhances expression of the molecule in question.

The term "AML" as used herein refers to acute myeloid leukemia, a rapidly progressing disease in which too many immature non-lymphocyte white blood cells are present in the blood and bone marrow. Also called acute myelogenous leukemia, acute myeloblastic leukemia, acute nonlymphocytic leukemia, and ANLL.

The term "antibody" as used herein is intended to include monoclonal antibodies, polyclonal antibodies, and chimeric antibodies. The antibody may be from recombinant sources and/or produced in transgenic animals. The term "antibody fragment" as used herein is intended to include without limitations Fab, Fab', F(ab')₂, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, and multimers thereof, multispecific antibody fragments and Domain Antibodies. Antibodies can be fragmented using conventional techniques. For example, F(ab')₂ fragments can be generated by treating the antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the

formation of Fab fragments. Fab, Fab' and F(ab')₂, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques. The term also includes antibodies or antibody fragments that bind to the detecting cassette polypeptides disclosed herein.

By "at least moderately stringent hybridization conditions" it is meant that conditions are selected which promote selective hybridization between two complementary nucleic acid molecules in solution. Hybridization may occur to all or a portion of a nucleic acid sequence molecule. The hybridizing portion is typically at least 15 (e.g. 20, 25, 30, 40 or 50) nucleotides in length. Those skilled in the art will recognize that the stability of a nucleic acid duplex, or hybrids, is determined by the T_m , which in sodium containing buffers is a function of the sodium ion concentration and temperature ($T_m = 81.5^\circ\text{C} - 16.6 (\text{Log}_{10} [\text{Na}^+]) + 0.41(\%(\text{G}+\text{C}) - 600/l)$, or similar equation). Accordingly, the parameters in the wash conditions that determine hybrid stability are sodium ion concentration and temperature. In order to identify molecules that are similar, but not identical, to a known nucleic acid molecule a 1% mismatch may be assumed to result in about a 1°C decrease in T_m , for example if nucleic acid molecules are sought that have a >95% identity, the final wash temperature will be reduced by about 5°C . Based on these considerations those skilled in the art will be able to readily select appropriate hybridization conditions. In preferred embodiments, stringent hybridization conditions are selected. By way of example the following conditions may be employed to achieve stringent hybridization: hybridization at 5x sodium chloride/sodium citrate (SSC)/5x Denhardt's solution/1.0% SDS at $T_m - 5^\circ\text{C}$ based on the above equation, followed by a wash of 0.2x SSC/0.1% SDS at 60°C . Moderately stringent hybridization conditions include a washing step in 3x SSC at 42°C . It is understood, however, that equivalent stringencies may be achieved using alternative buffers, salts and temperatures. Additional guidance regarding hybridization conditions may be found in: Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 2002, and in:

Sambrook et al., *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, 2001.

The term "autologous" as used herein refers to cells, tissue, DNA or factors taken or derived from an individual's own tissues, cells or DNA. For example in the context where autologous transduced cancer cells are administered to a subject with cancer, cancer cells removed from the subject are transduced or transfected with a vector that directs the expression of IL-12 and the transduced cells are administered to the subject.

The phrase "cancer burden" refers to the quantum of cancer cells or cancer volume in a subject. Reducing cancer burden accordingly refers to reducing the number of cancer cells or the cancer volume in a subject.

The phrase "cancer that is characterized by periods of remission" refer to cancers that may respond to a treatment but wherein the cancer recurs at some later time suggesting that not all cancer cells were eradicated by the treatment. An example of such a cancer is CLL.

The term "cancer cell" as used herein refers to any cell that is a cancer cell or is derived from a cancer cell e.g. clone of a cancer cell.

The term "cassette" as used herein refers to a polynucleotide sequence that is to be expressed. The cassette can be inserted into a vector. The cassette optionally includes regulatory sequence to direct or modify its expression.

The phrase "cell surface protein" or "cell surface polypeptide" as used herein refers to a polypeptide that is expressed, in whole or in part on the surface of a cell. This optionally includes polypeptide fragments that are presented on cells as well as polypeptides or fragments thereof that are naturally found on the surface of a cell. In the context of a cell modified to express a vector construct comprising a detection cassette polypeptide, wherein the detection cassette polypeptide is a cell surface polypeptide, the cell surface marker need not be native to the cell it is being expressed on.

The term "CLL" refers to chronic lymphocytic leukemia, a slow growing type of leukemia. CLL is the most common leukemia of adults with an expectation of ~16500 cases in North America in 2008. Remissions can be achieved with purine analogues and monoclonal antibody therapy however the disease invariably progresses. CLL is also referred to as chronic lymphoblastic leukemia. B-CLL is a subset of CLL.

The term "clinical grade vector" as used herein refers to a vector manufactured using near-GMP or GMP procedures and quality assurance tested.

The term "CML" refers to chronic myeloid leukemia, a slowly progressing leukemia wherein excessive white blood cells are made in the bone marrow. The hallmark of this disease is the reciprocal translocation between chromosomes 9 and 22 leading to the formation of the Bcr-Abl oncogene. This is manifested by a rapid expansion of bone marrow-derived hematopoietic cells of the myeloid lineage. CML is also referred to as chronic myelogenous leukemia, and chronic granulocytic leukemia.

A "conservative amino acid substitution" as used herein, is one in which one amino acid residue is replaced with another amino acid residue without abolishing the protein's desired properties. Conservative amino acid substitutions are known in the art. For example, conservative substitutions include substituting an amino acid in one of the following groups for another amino acid in the same group: alanine (A), serine (S), and threonine (T); aspartic acid (D) and glutamic acid (E); asparagine (N) and glutamine (Q); arginine (R) and lysine (L); isoleucine (I), leucine (L), methionine (M), valine (V); and phenylalanine (F), tyrosine (Y), and tryptophan (W).

The term "detection cassette" as used herein refers to a polynucleotide that directs expression of a molecule that is useful for enriching, sorting, tracking and/or killing cells in which it is expressed. The detection cassette encodes a polypeptide that is expressed in the transduced or transfected cell and can as a result be used to detect and/or isolate transduced or transfected cells. The detection cassette is optionally used to determine the efficiency of cell transduction or transfection.

As used herein, the phrase "effective amount" or "therapeutically effective amount" or a "sufficient amount" of composition, vector construct, virus or cell of the present application is a quantity sufficient to, when administered to the subject, including a mammal, for example a human, effect beneficial or desired results, including clinical results, and, as such, an "effective amount" or synonym thereto depends upon the context in which it is being applied. For example, in the context of treating cancer, it is an amount of the composition, vector construct, virus or cell sufficient to achieve a treatment response as compared to the response obtained without administration of the composition, vector construct, virus or cell. The amount of a given compound of the present application that will correspond to such an amount will vary depending upon various factors, such as the given agent, the pharmaceutical formulation, the route of administration, the type of disease or disorder, the identity of the subject (e.g. age, sex, weight) or host being treated, and the like, but can nevertheless be routinely determined by one skilled in the art. Also, as used herein, a "therapeutically effective amount" of a composition, vector construct, virus or cell of the present disclosure is an amount which results in a beneficial or desired result in a subject as compared to a control. As defined herein, a therapeutically effective amount of a composition, vector construct, virus or cell of the present disclosure may be readily determined by one of ordinary skill by routine methods known in the art. Dosage regime may be adjusted to provide the optimum therapeutic response.

The term "hybridize" refers to the sequence specific non-covalent binding interaction with a complementary nucleic acid.

An "immune modulatory cassette" as used herein, means a polynucleotide that directs expression of a molecule or polypeptide that enhances the anti-tumor effect of an IL-12 transduced cell. One class of immune regulatory molecules is cytokines. Also included are compounds that inhibit molecules that antagonize IL-12 response. For example, IL-10 can inhibit IL-12, compounds that inhibit the antagonistic effect of IL-10 would positively modulate the immune response.

The term "immune response" as used herein can refer to activation of either or both the adaptive and innate immune system cells such that they shift from a dormant resting state to a state in which they are able to elaborate molecules typical of an active immune response.

The phrase "inducing an immune response" as used herein refers to a method whereby an immune response is activated. The phrase "enhancing an immune response" refers to augmenting an existing but immune response.

The term "increased risk of cancer" as used herein means a subject that has a higher risk of developing a particular cancer than the average risk of the population. A subject may have a higher risk due to previously having had said particular cancer and or having a genetic risk factor for said particular cancer.

The term "kills" with respect to transfected or transduced cells refers to inducing cell death through any of a variety of mechanisms including apoptosis, necrosis and autophagy. For example an agent that is cytotoxic kills the cells.

The term "leukemia" as used herein refers to any cancer or precancerous syndrome that initiates in blood forming tissues such as the bone marrow. A number of leukemias have been characterized including ALL, AML, CLL, and CML. Delivery of a LV/IL-12 construct to engineer IL-12 expression in dendritic cells or other efficient antigen-presenting cells could also be effective in a pre-cancerous state if dominant tumor-associated antigens had been identified for the future cancer in that case and the host immune response re-directed against that antigen.

The term "polynucleotide" and/or "nucleic acid sequence" as used herein refers to a sequence of nucleoside or nucleotide monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term also includes modified or substituted sequences comprising non-naturally occurring monomers or portions thereof. The nucleic acid sequences of the present application may be deoxyribonucleic acid sequences (DNA) or ribonucleic acid sequences (RNA) and may include naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The sequences

may also contain modified bases. Examples of such modified bases include aza and deaza adenine, guanine, cytosine, thymidine and uracil; and xanthine and hypoxanthine.

The term "polypeptide" as used herein refers to a sequence of amino acids consisting of naturally occurring residues, and non-naturally occurring residues.

The term "promoter" as used herein refers to a recognition site on DNA that is bound by an RNA polymerase. The polymerase drives transcription of the transgene.

The term "sequence identity" as used herein refers to the percentage of sequence identity between two polypeptide sequences or two nucleic acid sequences. To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., $\% \text{ identity} = \frac{\text{number of identical overlapping positions}}{\text{total number of positions}} \times 100\%$). In one embodiment, the two sequences are the same length. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. U.S.A. 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990, J. Mol. Biol. 215:403. BLAST nucleotide searches can be performed with the NBLAST nucleotide program

parameters set, e.g., for score=100, wordlength=12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present application. BLAST protein searches can be performed with the XBLAST program parameters set, e.g., to score=50, wordlength=3 to obtain amino acid sequences homologous to a protein molecule of the present invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997, *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-BLAST can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., of XBLAST and NBLAST) can be used (see, e.g., the NCBI website). The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

The term "subject" as used herein includes all members of the animal kingdom including mammals, suitably humans including patients.

The term "subject in need thereof" refers to a subject that could benefit from the method, and optionally refers to a subject with cancer, such as leukemia, or optionally a subject with increased risk of cancer, such as a subject previously having cancer, a subject with a precancerous syndrome or a subject with a strong genetic disposition.

The term "transduction" as used herein refers to a method of introducing a vector construct or a part thereof into a cell. Wherein the vector construct is comprised in a virus such as for example a lentivirus, transduction refers to viral infection of the cell and subsequent transfer and integration of the vector construct or part thereof into the cell genome.

The term "treating" or "treatment" as used herein means administering to a subject a therapeutically effective amount of the compositions, cells or vector constructs of the present application and may consist of a single administration, or alternatively comprise a series of applications.

As used herein, and as well understood in the art, "treatment" or "treating" is also an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Further any of the treatment methods or uses described herein can be formulated alone or for contemporaneous administration with other agents or therapies.

The term "vector construct" as used herein means a recombinant polynucleotide comprising a vector alternatively referred to as a vector backbone and at least one coding cassette. . A vector construct is optionally comprised in a virus, such as a lentivirus. The term "vector" has used herein refers to a means by which polynucleotides can be introduced into a cell or host.

Vector Constructs and Virus

The application provides in one aspect a vector construct or virus such as a lentivirus comprising a delivery vector and and IL-12 expression cassette. In one embodiment the delivery vector is a lentivirus or lentiviral vector (LV) backbone.

Interleukin-12 (IL-12) Expression Cassette

Interleukin-12 is a heterodimeric cytokine with multiple biological effects on the immune system. It is composed of two subunits, p35 and p40, both of which are required for the secretion of the active form of IL-12, p70. Interleukin-12 acts on dendritic cells (DC), leading to increased maturation

and antigen presentation, which can allow for the initiation of a T cell response to tumor specific antigens.

In one embodiment the IL-12 expression cassette comprises a polynucleotide that directs expression of IL-12 polypeptide. Any IL-12 polypeptide including variants and derivatives of known IL-12 molecules can be used. In a preferred embodiment, the IL-12 is human IL-12. In another embodiment, the IL-12 is murine IL-12.

In one embodiment the polynucleotide comprises the sequence of both IL-12 subunits, p35 and p40, separated by an IRES sequence which permits expression of multiple transgenes from a single transcript. In other embodiments, the polynucleotide directs expression of an IL-12 fusion polypeptide that retains IL-12 activity. In one embodiment, the polynucleotide that directs the expression of IL-12 comprises a cDNA encoding a human IL-12 polypeptide fusion obtained from InVivoGen (pORF with IL-12elasti(p40::p35)). In one embodiment, the polynucleotide directs the expression of an IL-12 polypeptide comprising all or part of SEQ ID NO:4 or 5, and/or a variant of a fragment thereof that retains IL-12 activity. In another embodiment, the polynucleotide directs expression of an IL-12 fusion polypeptide that has at least 70%, 70-80%, 80-90%, 90-95%, 95-99.9% or more to the IL-12 portion of SEQ ID NO:4 or 5 and retains IL-12 activity. IL-12 activity is determined for example by assessing activation of the IL-12 receptor in a cell based assay.

A person skilled in the art will understand that non-critical residues can be deleted, and or mutated without effect on IL-12. Polynucleotides directing expression of IL-12 polypeptide analogs are also contemplated.

Delivery Vectors

It will be appreciated by one skilled in the art that a variety of delivery vectors and expression vehicles are usefully employed to introduce a modified DNA molecule into a cell. Vectors that are useful comprise lentiviruses,

oncoretroviruses, expression plasmids, adenovirus, and adeno-associated virus. Other delivery vectors that are useful comprise herpes simplex viruses, transposons, vaccinia viruses, human papilloma virus, Simian immunodeficiency viruses, HTLV, human foamy virus and variants thereof. Further vectors that are useful comprise spumaviruses, mammalian type B retroviruses, mammalian type C retroviruses, avian type C retroviruses, mammalian type D retroviruses, HTLV/BLV type retroviruses, and lentiviruses.

Vectors such as those listed above have been employed to introduce DNA molecules into cells for use in gene therapy. Examples of vectors used to express DNA in cells include vectors described in: Kanazawa T, Mizukami H, Okada T, Hanazono Y, Kume A, Nishino H, Takeuchi K, Kitamura K, Ichimura K, Ozawa K. Suicide gene therapy using AAV-HSVtk/ganciclovir in combination with irradiation results in regression of human head and neck cancer xenografts in nude mice. *Gene Ther.* 2003 Jan;10(1):51-8. Fukui T, Hayashi Y, Kagami H, Yamamoto N, Fukuhara H, Tohnai I, Ueda M, Mizuno M, Yoshida J Suicide gene therapy for human oral squamous cell carcinoma cell lines with adeno-associated virus vector. *Oral Oncol.* 2001 Apr;37(3):211-5.

Retroviral Vectors

In one embodiment, the delivery vector is a retroviral vector. In a further embodiment, the delivery vector is a lentiviral vector. Lentiviral vectors (LVs), a subset of retroviruses, transduce a wide range of dividing and non-dividing cell types with high efficiency, conferring stable, long-term expression of the transgene²⁵⁻²⁷.

The use of lentivirus-based gene transfer techniques relies on the *in vitro* production of recombinant lentiviral particles carrying a highly deleted viral genome in which the transgene of interest is accommodated. In particular, the recombinant lentivirus are recovered through the *in trans* coexpression in a permissive cell line of (1) the packaging constructs, i.e., a vector expressing the Gag-Pol precursors together with Rev (alternatively

expressed in trans); (2) a vector expressing an envelope receptor, generally of an heterologous nature; and (3) the transfer vector, consisting in the viral cDNA deprived of all open reading frames, but maintaining the sequences required for replication, incapsidation, and expression, in which the sequences to be expressed are inserted.

In one embodiment the lentiviral vector comprises one or more of a 5'-Long terminal repeat (LTR), HIV signal sequence, HIV Psi signal 5'-splice site (SD), delta-GAG element, Rev Responsive Element (RRE), 3'-splice site (SA), Elongation factor (EF) 1-alpha promoter and 3'-Self inactivating LTR (SIN-LTR). The lentiviral vector optionally comprises a central polypurine tract (cPPT; SEQ ID NO: 2) and a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE; SEQ ID NO: 3). In a further embodiment, the lentiviral vector comprises a pHR' backbone. In certain embodiments, the pHR' back bone comprises for example as provided below.

In one embodiment the Lentigen lentiviral vector described in Lu, X. et al. Journal of gene medicine (2004) 6:963-973 is used to express the DNA molecules and/or transduce cells.

In one embodiment the lentiviral vector comprises a 5'-Long terminal repeat (LTR), HIV signal sequence, HIV Psi signal 5'-splice site (SD), delta-GAG element, Rev Responsive Element (RRE), 3'-splice site (SA), Elongation factor (EF) 1-alpha promoter and 3'-Self inactivating LTR (SIN-LTR). It will be readily apparent to one skilled in the art that optionally one or more of these regions is substituted with another region performing a similar function.

In certain embodiments the IL-12 is required to be expressed at sufficiently high levels. Transgene expression is driven by a promoter sequence. Optionally, the lentiviral vector comprise a CMV promoter. In another embodiment, the promoter is Elongation factor (EF) 1-alpha promoter. A person skilled in the art will be familiar with a number of promoters that will be suitable in the vector constructs described herein.

Enhancer elements can be used to increase expression of modified DNA molecules or increase the lentiviral integration efficiency. In one embodiment the lentiviral vector further comprises a nef sequence. In a

preferred embodiment the lentiviral further comprises a cPPT sequence which enhances vector integration. The cPPT acts as a second origin of the (+)-strand DNA synthesis and introduces a partial strand overlap in the middle of its native HIV genome. The introduction of the cPPT sequence in the transfer vector backbone strongly increased the nuclear transport and the total amount of genome integrated into the DNA of target cells. In an alternate preferred embodiment, the lentiviral vector further comprises a Woodchuck Posttranscriptional Regulatory Element (WPRE). The WPRE acts at the transcriptional level, by promoting nuclear export of transcripts and/or by increasing the efficiency of polyadenylation of the nascent transcript, thus increasing the total amount of mRNA in the cells. The addition of the WPRE to lentiviral vector results in a substantial improvement in the level of transgene expression from several different promoters, both in vitro and in vivo.. In a further preferred embodiment, the lentiviral vector comprises both a cPPT sequence and WPRE sequence. In yet a further embodiment, the lentiviral vector comprises a sequence having at least 70%, 70-80%, 80-90%, 90-95%, 95-99.9% or more sequence identity to SEQ ID NO:2 and/or SEQ ID NO:3. The vector also comprises in an alternate embodiment an internal ribosome entry site (IRES) sequence that permits the expression of multiple polypeptides from a single promoter.

In addition to IRES sequences, other elements which permit expression of multiple polypeptides are useful. In one embodiment the vector comprises multiple promoters that permit expression more than one polypeptide. In another embodiment the vector comprises a protein cleavage site that allows expression of more than one polypeptide. Examples of protein cleavage sites that allow expression of more than one polypeptide comprise those listed in the following articles which are incorporated by reference: Retroviral vector-mediated expression of HoxB4 in hematopoietic cells using a novel coexpression strategy. Klump H, Schiedlmeier B, Vogt B, Ryan M, Ostertag W, Baum C. *Gene Ther.* 200;8(10):811-7; A picornaviral 2A-like sequence-based tricistronic vector allowing for high-level therapeutic gene expression coupled to a dual-reporter system Mark J. Osborn, Angela

Panoskaltis-Mortari, Ron T. McElmurry, Scott K. Bell, Dario A.A. Vignali, Martin D. Ryan, Andrew C. Wilber, R. Scott McIvor, Jakub Tolar and Bruce R. Blazar. *Molecular Therapy* 2005; 12 (3), 569-574; Development of 2A peptide-based strategies in the design of multicistronic vectors. Szymczak AL, Vignali DA. *Expert Opin Biol Ther.* 2005; 5(5):627-38; Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. *Nat Biotechnol.* 2004;22(5):589-94. It will be readily apparent to one skilled in the art that other elements that permit expression of multiple polypeptides which identified in the future are useful and may be utilized in the vectors of the invention.

In certain embodiments, the lentiviral vector is a clinical grade vector.

Viral Regulatory Elements

The viral regulatory elements are components of delivery vehicles used to introduce nucleic acid molecules into a host cell. The viral regulatory elements are optionally retroviral regulatory elements. For example, the viral regulatory elements may be the LTR and gag sequences from HSC1 or MSCV. The retroviral regulatory elements may be from lentiviruses or they may be heterologous sequences identified from other genomic regions.

One skilled in the art would also appreciate that as other viral regulatory elements are identified, these may be used with the nucleic acid molecules of the invention.

Detection Cassette

In certain embodiments, the vector construct comprises a detection cassette. The detection cassette comprises a polynucleotide that directs expression of a molecule that is useful for enriching, sorting, tracking and/or killing cells in which it is expressed. The detection cassette encodes a polypeptide that is expressed in the transduced or transfected cell and can as a result be used to detect and/or isolate transduced or transfected cells. The

detection cassette is optionally used to determine the efficiency of cell transduction or transfection.

In one embodiment, the detection cassette encodes a polypeptide that protects from a selection drug such as neomycin phosphotransferase or G418. In another embodiment, the detection cassette encodes a fluorescent protein such as GFP. Other fluorescent proteins can also be used. In a further embodiment, the detection cassette is a cell surface marker such as CD19, truncated CD19, CD20, human CD24, murine HSA, human CD25 (huCD25), a truncated form of low affinity nerve growth factor receptor (LNGFR), truncated CD34 or erythropoietin receptor (EpoR). In certain embodiments the detection cassette polypeptide is substantially overexpressed in transduced cells such that these cells are preferentially targeted. In other embodiments, the detection cassette polypeptide is not appreciably expressed on the cell type to be transduced or transfected.

As described below, the detection cassette polypeptide can be used to isolate transduced cells by methods such as flow cytometry.

In one embodiment, the detection cassette comprises a CD19 molecule or fragment thereof. In another preferred embodiment the construct comprises a detection polynucleotide incorporated into pHR'-cppt-EF-IRES-W-SIN, pHR'-cppt-EF-huCEA-IRES-hCD19-W-SIN or pHR'-cppt-EF-HER/neuIRES-hCD19-W-SIN. Additionally it will be readily apparent to one skilled in the art that optionally one or more of these elements can be added or substituted with other regions performing similar functions.

Immune Modulatory Cassette

Enhanced antitumor effect is obtainable with the use of specific immune modulatory molecules. One class of immune regulatory molecules is cytokines. Cytokines are integral to both the innate and acquired immune systems. They can alter the balance of cellular and humoral responses, alter class switching of B lymphocytes and modify innate responses.

In one embodiment, the immune modulatory cassette comprises a polynucleotide that encodes a polypeptide that modulates an immune cell, optionally a dendritic cell or a T cell, optionally a CD4+ T cell.

In one embodiment, the immune modulatory molecule useful for promoting anti-tumor effect is RANKL. RANKL is a molecule that extends the lifespan of DCs in an autocrine fashion. CD40L which enhances the stimulatory capacity of DCs, is also useful for promoting the anti-tumor effect of DC and tumor cell vaccines. In addition a number of other cytokines are useful including IL-2, IL-7, IL-15, IL-18, and IL-23. A person skilled in the art would recognize that other immune modulatory molecules, including molecules that promote APC function are suitable for use in constructs of the present application.

In another embodiment, the immune modulatory cassette comprises a polynucleotide that encodes or directs expression of a molecule that inhibits IL-12 down modulation, for example inhibits IL-10. In one embodiment, the molecule is a dominant negative IL-10 polypeptide. In another embodiment the molecule is a small molecule inhibitor. In another embodiment, the molecule is a siRNA or shRNA molecule that knocks down IL-10 gene expression.

Safety Components

The Cell Surface Protein – Use of Immunotoxin to Kill Transduced Cells

In certain embodiments of the invention, a cell surface protein (marker) herein referred to as a detection cassette, such as CD19, CD20 HSA, truncated LNGFR, CD34, CD24 or CD25 is delivered into target cells which further selectively clears these cells *in vitro* and *in vivo* by administering an immunotoxin (antibody conjugated to a toxin) directed against the cell surface protein. The term "immunotoxin" as used herein means an antibody or fragment thereof that is cytotoxic and/or an antibody or fragment thereof that is fused to a toxic agent. Immunotoxins are described in this application and known in the art, for example, in US patent application no. 20070059275.

Many immunotoxins are approved for use in humans. In one embodiment the immunotoxin is a murine anti-Tac (AT) monoclonal antibody¹⁹ fused to saporin (SAP)¹⁰⁰ a toxin that irreversibly damages ribosomes by cleaving adenine molecules from ribosomal RNA.²¹ The inventors have demonstrated both *in vitro* and *in vivo* that the AT-SAP (ATS) complex specifically target and kill retrovirally transduced cells that express huCD25. Use of immunotoxins to kill transduced cells are described in CA application Vector Encoding Therapeutic Polypeptide and Safety Elements to Clear Transduced Cells, filed March 27, 2007 which is incorporated herein by reference.

Activator Polynucleotides

Other safety components that can be introduced into the vector constructs disclosed are described in US application 11/559,757, THYMIDYLATE KINASE MUTANTS AND USES THEREOF and U.S. Application No. 12/052,565 which are incorporated herein by reference. In one embodiment, the lentiviral construct further comprises an activator polynucleotide encoding a polypeptide that converts a prodrug to a drug, optionally a modified tmpk polynucleotide. In one embodiment, the activator polynucleotide comprises a tmpk polynucleotide with at least 80% sequence identity to a modified tmpk polynucleotide, optionally the sequences listed below.

The safety facet of suicide gene therapy relies on efficient delivery and stable, consistent expression of both the therapeutic and the safety component genes.

Expression Cassette Variants and Analogs

In the context of a polypeptide, the term "analog" as used herein includes any polypeptide having an amino acid residue sequence substantially identical to any of the wild type polypeptides expressed by the expression cassette for example, IL-12 or mutant IL-12, in which one or more

residues have been conservatively substituted with a functionally similar residue and which displays the ability to activate in the context of IL-12, the IL-12 receptor similar to wild-type IL-12 or to IL-12 mutants. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as alanine, isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another. The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such polypeptide displays the requisite activity.

In the context of a polypeptide, the term "derivative" as used herein refers to a polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5 hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions or residues relative to the wild type sequence, so long as the requisite activity is maintained.

The methods of making recombinant proteins are well known in the art and are also described herein.

The nucleic acids described herein can also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. The nucleic acid can also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an nucleic acid.

The nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The nucleic acid molecules of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules.

Isolated Virus

The retroviral and lentiviral constructs are in one embodiment, packaged into viral particles. Methods for preparing virus are known in the art and described herein. In one embodiment, the application provides an isolated virus, optionally a lentivirus comprising the vector construct.

Methods of isolating virus are also known in the art and further described herein.

Methods of Expressing IL-12 in Cells and Cell Isolation

In one aspect, methods for expressing IL-12 in cells at or above a threshold level are provided. Accordingly in one aspect, the application provides a method of expressing IL-12 in a cell above a threshold level.

The polynucleotides may be incorporated into an appropriate expression vector which ensures good expression of the IL-12 and/or other expression cassettes herein described. For example, vectors described herein are suitable.

Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression vectors are "suitable for

transformation of a host cell", which means that the expression vectors contain a nucleic acid molecule and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked or operably linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The application therefore includes a recombinant expression vector containing a nucleic acid molecule disclosed herein, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence.

Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990)). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector.

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. The terms "transformed with", "transfected with", "transformation" "transduced" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector or vector construct) into a cell by one of many possible techniques known in the art. The phrase "under suitable conditions that permit transduction or transfection of the cell" refers to for example for ex vivo culture conditions, such as selecting an appropriate medium, agent concentrations and contact time lengths which are suitable for transfecting or transducing the particular host. Suitable conditions

are known in the art and/or described herein. The term "transformed host cell" or "transduced host cell" as used herein is intended to also include cells capable of glycosylation that have been transformed with a recombinant expression vector disclosed herein. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. For example, nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratory Press, 2001), and other laboratory textbooks. Suitable methods for transducing cells are known in the art and are also described herein.

Vector constructs are introduced into cells that are used for transplant or introduced directly *in vivo* in mammals, preferably a human. The vector constructs are typically introduced into cells *ex vivo* using methods known in the art. Methods for introducing vector constructs comprise transfection, infection, electroporation. These methods optionally employ liposomes or liposome like compounds. Introduction *in vivo* optionally includes intravenous injection and/or intratumoral injection. These methods are described more fully elsewhere

In certain embodiments, the cell is contacted with a composition vector construct and/or isolated virus described herein, for example an isolated virus comprising a lentiviral vector and a IL-12 expression cassette, under conditions that permit transduction or transfection of the cell. Methods of transducing cells are well known in the art.

In one embodiment, the method of expressing IL-12 in a cell comprises contacting the cell with a composition and/or vector construct described herein, for example comprising a lentiviral vector and an IL-12 expression cassette, under conditions that permit transduction or transfection of the cell.

In other embodiments, the cells are optionally transduced with retroviral constructs that drive expression of IL-12 and/or additional expression

cassettes described herein. Methods of transducing cells are well known in the art. Methods of transducing cells with lentiviral vectors are also described herein.

In another embodiment, the method further comprises isolating the transduced cell or a population of transduced cells.

After transduction or transfection with vector constructs comprising an IL-12 expression cassette, and/or detection cassette polynucleotide, cells expressing these molecules are optionally isolated by a variety of means known in the art. In certain embodiments, the cells are isolated by cell sorting or flow cytometry using an antibody to the detection cassette encoded selection marker. Additionally cell sorting is useful to isolate modified cells where the detection cassette is a fluorescent protein such as EGFP.

In one embodiment cells are isolated from the transduction or transfection medium and/or the viral preparation. For example the cells may be spun down and/or washed with a buffered saline solution. Accordingly, the cells can comprise a population of cells comprising transduced and untransduced cells. In certain embodiments, the population of cells comprises at least 1%, 2-5%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-95%, 95-99% or more than 99% IL-12 transduced or transfected cells.

Cells expressing polynucleotides of the invention are, in an alternate embodiment, isolated using magnetic sorting. Additionally, cells may be isolated by drug selection. In one embodiment, a vector comprising a drug resistance gene and a polynucleotides of the invention is introduced into cells. Examples of drug resistance genes include, but are not limited to, neomycin resistance gene, blasticidin resistance gene (Bsr), hygromycin resistance gene (Hph), puromycin resistance gene (Pac), Zeocin resistance gene (Sh ble), FHT, bleomycin resistance gene and ampicillin resistance gene. After transduction or transfection, modified cells including the drug resistance gene are selected by adding the drug that is inactivated by the drug resistance gene. Cells expressing the drug resistance gene survive while non-

transfected or non-transduced cells are killed. A person skilled in the art would be familiar with the methods and reagents required to isolate cells expressing the desired polynucleotides.

In a further embodiment, the transduced cells are growth arrested. Several methods can be used to growth arrest cells. In one embodiment, the transfected or transduced cells are growth arrested by irradiation. The term "growth arrested" refers to being inhibited for cell division. A person skilled in the art would recognize that the suitable irradiation dose to growth arrest a cell or population of cells may vary upon the cell type and/or number of cells. In one embodiment, the dose is about 75-150G. In another embodiment, for AML the dose of radiation is about 75G.

Host Cells

The disclosure also provides in one aspect a cell (including for example an isolated cell *in vitro*, a cell *in vivo*, or a cell treated *ex vivo* and returned to an *in vivo* site) expressing and/or secreting IL-12 above a threshold limit. In one embodiment, the cell is transduced with a vector construct, virus or composition described herein.

Cells transfected with a nucleic acid molecule such as a DNA molecule, or transduced with the nucleic acid molecule such as a DNA or RNA virus vector, are optionally used, for example, in bone marrow or cord blood cell transplants according to techniques known in the art.

Any cell may be used for transduction with the vector constructs described herein to obtain a cell expressing IL-12 above the threshold level. In one embodiment, the cell is a cancer cell. In one embodiment, the cancer cell is a primary cancer cell. In a further embodiment, the primary cancer cell is derived from a subject. The cancer cell is optionally an allogenic or autologous cell. The cancer cell to be transduced is optionally derived from, propagated from or cloned from a cancer cell obtained from a subject. The cancer cell is in one embodiment obtained from the subject by biopsy.

Alternatively, the cancer cell can be obtained from a blood sample, for example in the case of a leukemia, where the disease cell type is present in the peripheral blood. Methods for isolating cancer cells from a blood sample are known in the art and/or described herein.

Any cancer cell that can be transduced or transfected is a suitable host for transduction or transfection using a composition or vector construct of the application. In one embodiment the cancer cell is a leukemia cell. In one embodiment the leukemia cell is an acute lymphoblastic leukemia (ALL) cell, a chronic lymphoblastic leukemia (CLL) cell, chronic myeloid leukemia (CML) cell, or acute myeloid leukemia (AML) cell. In certain embodiments, the cancer cell is derived from a cancer that is characterized by or can exhibit periods of remission. In certain embodiments, the cancer cell is a metastatic cancer cell. In other embodiments, the cancer cell is a lymphoma, myeloma, tumor of the lung, ovary, prostate, breast, melanoma, colon, bladder, liver, pancreas, thyroid, head or neck cancer cell. The immune system is able to seek out cells residing in nearly all parts of the body and therefore all cancers could be susceptible to this approach including: leukemias, lymphoma, myelomas, tumors of the lung, ovary, prostate, breast, melanoma, colon, bladder, liver, pancreas, thyroid, head and neck.

Cell lines are optionally transduced or transfected. For example human T cell leukemia Jurkat T cells, human erythro-leukemic K562 cells, CES1, OCIAML1, OCIAML2, and Raji cells are optionally transduced or transfected with polynucleotides of the described herein. Raji is a burkitts lymphoma line, OCI AML 1 and 2 are acute meylogenous leukemia lines, CES1 is a chronic myelogenous leukemia

A cancer cell expresses tumor associated antigens and introduction of IL-12 and optionally immune modulatory molecules that augment the immune response when the tumor cell is introduced into the subject as demonstrated by the inventors. In one embodiment, the tumor cell is transduced with a lentiviral construct comprising an IL-12 cassette and optionally an immune modulatory cassette, wherein the immune modulatory cassette comprises a polynucleotide that encodes a molecule that induces DC cells and/or T cells.

Cancer cells are attractive vehicles for expressing IL-12 as the immune response is self limiting. Transduced cancer cells elicit an immune response that leads to the eradication of the initiating cell. IL-12 levels are thereby self-limited.

Compositions and vector constructs described herein are usefully introduced into any cell type *ex vivo*. *The compositions and vector constructs described herein may also be introduced into any cell type in vivo.*

Threshold Level

The inventors have demonstrated that a minimum number of cancer cells expressing at least a threshold amount of IL-12 can induce and/or enhance an immune response in a subject. The immune response in some embodiments, leads to loss of non-transduced cancer cells.

In one embodiment, the threshold level is at level is at least 1500 pg/mL/10⁶ cells/2hrs of IL-12. In another embodiment, the threshold level is at least 1500-2500 pg/mL/10⁶ cells/2hrs, 2500-5000 pg/mL/10⁶ cells/2hrs, 5000-7500 pg/mL/10⁶ cells/2hrs, 7500-10000 pg/mL/10⁶ cells/2hrs, 10000-12500 pg/mL/10⁶ cells/2hrs, 12500-15000 pg/mL/10⁶ cells/2hrs, 15000-17500 pg/mL/10⁶ cells/2hrs, 17500-20000 pg/mL/10⁶ cells/2hrs or at least 20000 pg/mL/10⁶ cells/2hrs of IL-12.

In another embodiment, a population of cells comprises transduced cells that secrete at least about 1500-2500 pg/mL/10⁶ cells/2hrs, 2500-5000 pg/mL/10⁶ cells/2hrs, 5000-7500 pg/mL/10⁶ cells/2hrs, 7500-10000 pg/mL/10⁶ cells/2hrs, 10000-12500 pg/mL/10⁶ cells/2hrs, 12500-15000 pg/mL/10⁶ cells/2hrs, 15000-17500 pg/mL/10⁶ cells/2hrs, 17500-20000 pg/mL/10⁶ cells/2hrs or at least 20000 pg/mL/10⁶ cells/2hrs of IL-12. In other embodiments, the population of cells comprise transduced cells that secrete at least about 20,000-40,000 pg/mL/10⁶ cells/2hrs of IL-12. A person skilled in the art would understand that each cell would secrete varying amounts of IL-12. The population may include cells secreting less or more than the numbers

herein listed or a given threshold. The transduced cells as a whole comprise a sufficient number of IL-12 secreting cells, secreting IL-12 above the threshold level such that DC are activated.

The population of cells can comprise transduced and non-transduced and/or transfected and non-transfected cells. In one embodiment, at least 0.5%, 1%, 2-5%, 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, 90-95%, 95-99% or more than 99% of cells in the population of cells are transduced or transfected and/or express IL-12.

In a preferred embodiment, the population of cells comprises 1% transduced cells secreting 20,000pg/10⁶cells/2 hrs.

The level of IL-12 expression can be determined by a number of methods including methods known in the art and methods described herein. For example IL-12 levels can be determined by ELISA, cytokine bead assay, intracellular staining, HPLC and MS/MS, or ELISPOT.

Compositions

The application describes compositions comprising an IL-12 expression cassette and a lentiviral vector as described herein. The vector is for providing a coding nucleic acid molecule (eg. the expression cassette) to a subject such that expression of the molecule in the cells provides the biological activity of the polypeptide encoded by the coding nucleic acid molecule to those cells. A coding nucleic acid as used herein means a nucleic acid or polynucleotide that comprises nucleotides which specify the amino acid sequence, or a portion thereof, of the corresponding protein. A coding sequence may comprise a start codon and/or a termination sequence.

In other embodiments, the composition comprises cells modified with the vector constructs described herein. Such modified cells can be administered intravenously using methods known in the art i.p., i.v., intratumorally, stereotactic injections to a variety of sites, direct injections, intramuscularly, etc.

Pharmaceutical Compositions

The pharmaceutical compositions of this invention used to treat patients having diseases, disorders or abnormal physical states could include an acceptable carrier, auxiliary or excipient.

The pharmaceutical compositions are optionally administered by *ex vivo* and *in vivo* methods such as electroporation, DNA microinjection, liposome DNA delivery, and virus vectors that have RNA or DNA genomes including retrovirus vectors, lentivirus vectors, Adenovirus vectors and Adeno-associated virus (AAV) vectors, Semliki Forest Virus. Derivatives or hybrids of these vectors are also useful.

Dosages to be administered depend on patient needs, on the desired effect and on the chosen route of administration. The expression cassettes are optionally introduced into the cells or their precursors using *ex vivo* or *in vivo* delivery vehicles such as liposomes or DNA or RNA virus vectors. They are also optionally introduced into these cells using physical techniques such as microinjection or chemical methods such as coprecipitation.

The pharmaceutical compositions are typically prepared by known methods for the preparation of pharmaceutically acceptable compositions which are administered to patients, and such that an effective quantity of the nucleic acid molecule is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA).

On this basis, the pharmaceutical compositions could include an active compound or substance, such as a nucleic acid molecule, in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and isoosmotic with the physiological fluids. The methods of combining the expression cassettes with the vehicles or combining them with diluents is well known to those skilled in the art. The

composition could include a targeting agent for the transport of the active compound to specified sites within cells.

Methods of Inducing/Enhancing Immune Responses and Methods of Treatments

The methods disclosed herein are useful for inducing and enhancing an immune response in a subject. In one embodiment, the subject has cancer. In another embodiment, the subject is in remission. In a further embodiment, the subject has an increased risk of cancer.

In one embodiment, the application provides a method of inducing or enhancing an immune response in a subject comprising administering a transduced cell or population of cells described herein or a composition comprising said cells.

In another embodiment, the application provides a method of inducing or enhancing a memory immune response in a subject.

In one embodiment, the immune response induced or enhanced is a CD4+ T cell mediated immune response.

The application also provides a method of delivering IL-12 to a subject for enhancing cancer treatment comprising:

generating an IL-12 secreting cell wherein IL-12 secreted per cell is above a threshold level; and

introducing an effective number of the generated IL-12 secreting cells to the subject.

In another embodiment, the application provides a method of of sustaining IFN γ levels induced by IL-12 in a host comprising:

generating an IL-12 secreting cell wherein IL-12 secreted per cell is above a threshold level; and

introducing an effective number of the generated IL-12 secreting cells to the subject.

In one embodiment, transduced cells, a population of cells and/or a composition comprising said cells are administered to a subject. In another embodiment, the cells, population of cells and/or composition are administered with an adjuvant. For example, in one embodiment incomplete Freund's adjuvant is used. In addition, the cells, population of cells and/or composition is administered once, or repeated. For example, the cells and or population of cells are administered a second time to boost the immune response and/or increase the amount of IL-12 delivered or IFN γ sustained.

In one embodiment, cancer cells are obtained from a subject, and genetically modified to express and/or secrete IL-12 above a threshold level. The transduced cells or population of cells comprising transduced cells is irradiated and administered to the subject. Accordingly in certain embodiments, clinical use of the modified cells is restricted to the subject from whom the cancer cell was derived.

Wherein cells additionally express an activator polynucleotide encoding a polypeptide that converts a prodrug to a drug, for example a modified tmpr polynucleotide, cells are optionally not irradiated. Any unwanted cells can be killed upon administration of the prodrug. For example, in some cases, irradiation may negatively effect the ability of the transduced cells to induce an immune response eg irradiation may cause cell death in certain cell populations. Use of an activator polynucleotide or other mechanism to remove unwanted cells transplanted into the subject is alternatively used in such situations.

The methods disclosed herein are useful for treating a variety of cancers. The inventors have shown that leukemias of a variety of types are amenable to IL-12 treatment.

Residual disease which can lay dormant during remissions may be targeted by the method disclosed herein. The delayed disease progression of

many leukemias provides a critical window of opportunity for immune-based approaches. The present immunotherapy may also rid quiescent cells such as cancer initiating "stem" cells because it does not require biochemically or genetically active targets. Further the present immunotherapy may also lead to eradicating metastatic disease.

The methods described herein are also useful to treat solid cancers. For example the methods may be used to treat melanoma, renal cancer and prostate cancer.

The cells may be introduced by a variety of routes as disclosed elsewhere including intraperitoneal injection or intravenous infusion. Alternatively, a vector construct, isolated virus or composition comprising said construct or virus can be injected intratumorally such that transduction takes place in vivo

The number of cells injected or administered is in one embodiment an effective number to induce an immune response. An immune response can be detected using a number of methods known in the art including detecting host T cell recognition of tumor cells in vitro. Alternatively, an immune response can be detected by assessing cytokine profile changes. For example increased expression of IFN-gamma is indicative of an immune response.

In certain embodiments, the methods further comprise monitoring cancer progression. Cancer progression can be monitored using known methods.

In one embodiment, compositions and vectors of the invention are used to treat cancer by adoptive therapy. In one embodiment, cytotoxic lymphocyte cells are expanded using LV-IL-12 transduced cells in vitro. Adoptive therapy or adoptive (immuno)therapy refers to the passive transfer of immunologically competent tumor-reactive cells into the tumor-bearing host to, directly or indirectly, mediate tumor regression. The feasibility of adoptive (immuno)therapy of cancer is based on two fundamental observations. The

first of these observations is that tumor cells express unique antigens that can elicit an immune response within the syngeneic (genetically identical or similar especially with respect to antigens or immunological reactions) host. The other is that the immune rejection of established tumors can be mediated by the adoptive transfer of appropriately sensitized lymphoid cells. Clinical applications include transfer of peripheral blood stem cells following non-myeloablative chemotherapy with or without radiation in patients with lymphomas, leukemias, and solid tumors.

In one aspect of the present invention, donor T cells or stem cells (either embryonic or of later ontogeny) are transduced with vectors of the invention. Cells expressing these vectors are isolated and adoptively transferred to a host in need of treatment. In one embodiment the bone marrow of the recipient is T-cell depleted. Methods of adoptive T-cell transfer are known in the art (J Translational Medicine, 2005 3(17): doi;0.1186/1479-5876-3-17, Adoptive T cell therapy: Addressing challenges in cancer immunotherapy. Cassian Yee). This method is used to treat solid tumors and does not require targeting the vector-transduced expressing T-cells to the tumor since the modified T-cells will recognize the different MHC class molecules present in the recipient host resulting in cytotoxic killing of tumor cells.

In one embodiment, autologous DC and T cells are contacted ex vivo with IL-12 transduced cancer cells and/or expanded ex vivo and administered to a subject in need thereof with or without LV-IL-12 secreting cells.

The compositions and vectors are also useful for the reduction of cell proliferation, for example for treatment of cancer. The present disclosure also provides methods of using compositions and vectors of the disclosure for expressing IL-12 for the reduction of cell proliferation, for example for treatment of cancer.

The application also provides a method of reducing the number of tumor cells or cancer burden in a subject with cancer, or having an increased

likelihood of developing cancer comprising administering a transduced cell, population of cells, or a composition comprising said cells to the subject.

In another embodiment, the application provides a method of treating a subject with cancer or an increased risk of developing cancer comprising administering a transduced cell, population of cells, or a composition comprising said cells to the subject.

Vector constructs containing the nucleic acid molecules of the disclosure and isolated viruses are typically administered to mammals, preferably humans, using techniques described below. The polypeptides produced from the nucleic acid molecules are also optionally administered to mammals, preferably humans. The invention relates to a method of medical treatment of a mammal in need thereof, preferably a human, by administering to the mammal a vector construct described herein or a cell containing the vector construct.

One aspect relates to methods for providing a coding nucleic acid molecule to the cells of an individual such that expression of the coding nucleic acid molecule in the cells provides the biological activity or phenotype of the polypeptide encoded by the coding nucleic acid molecule. The method also relates to a method for providing an individual having a disease, disorder or abnormal physical state with a biologically active polypeptide by administering a nucleic acid molecule of the present invention. The method may be performed *ex vivo* or *in vivo*. Gene therapy methods and compositions are demonstrated, for example, in U.S. Patent Nos. 5,869,040, 5,639,642, 5,928,214, 5,911,983, 5,830,880, 5,910,488, 5,854,019, 5,672,344, 5,645,829, 5,741,486, 5,656,465, 5,547,932, 5,529,774, 5,436,146, 5,399,346 and 5,670,488, 5,240,846. The amount of polypeptide will vary with the subject's needs. The optimal dosage of vector may be readily determined using empirical techniques, for example by escalating doses (see US 5,910,488 for an example of escalating doses).

The method also relates to a method for producing a stock of recombinant virus by producing virus suitable for gene therapy comprising modified DNA encoding a gene of interest. This method preferably involves

transfecting cells permissive for virus replication (the virus containing therapeutic gene) and collecting the virus produced.

Cotransfection (DNA and marker on separate molecules) may be employed (see eg US 5,928,914 and US 5,817,492). As well, a detection cassette or marker (such as Green Fluorescent Protein marker or a derivative) may be used within the vector itself (preferably a viral vector).

Combination Treatments

In certain embodiments, the vector constructs, transduced cells, population of cells and or compositions comprising these, are administered in combination with other therapies. For example, the the vector constructs, transduced cells, population of cells and or compositions comprising these may be administered before or after chemotherapy suitable for the cancer being treated. In other embodiments wherein the cancer is a solid cancer, the vector constructs, transduced cells, population of cells and or compositions comprising these are administered before or after surgery.

In one embodiment, cancer cells are harvested from a subject's blood before the combination treatment, optionally chemotherapy, is started. The cancer cells are then transduced with a LV- IL-12. Transduced cells are frozen for later use and administered when the subject is in remission.

Dosing

The methods provide in certain embodiments, that a composition, transduced cell, population or cells, or vector construct described herein is administered to the subject. The compositions, cells or vector constructs of the present application may be administered at least once a week in one embodiment. However, in another embodiment, the composition, transduced cell, population or cells, or vector construct may be administered to the subject from about one time per week, one time per 14 days, or 28 days. The administration may be repeated 1, 2, 3, 4, 5, 6 or more times. In another embodiment, administration is about once daily for a given treatment, for example for rIL-12 therapy. The length of the treatment period depends on a variety of factors, such as the severity of the disease, the age of the patient,

the concentration and the activity of the compounds of the present application, or a combination thereof. In one embodiment, the treatment is chronic treatment and the length of treatment is 1-2 weeks, 2-4 weeks or more than 4 weeks. The treatment regimen can include repeated treatment schedules. It will also be appreciated that the effective amount or dosage of the compound used for the treatment or prophylaxis may increase or decrease over the course of a particular treatment or prophylaxis regime. Changes in dosage may result and become apparent by standard diagnostic assays known in the art. In some instances, chronic administration may be required.

The number of cells administered varies with the expression level of the transduced cell or population of cells. For example, where the IL-12 expressing cells express over 20000 pg/mL/10⁶ cells/2hrs IL-12, as few as 5000 or 0.5% of a population of cells comprising IL-12 expressing cells may be sufficient for the methods described herein. However where the IL-12 expressing cells express only 2000 pg/mL/10⁶ cells/2 hrs IL-12, greater than 100000 or 10% of a population of cells comprising IL-12 expressing cells may be needed.

In one embodiment, 1-5, 5-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100 or more than 100 x10⁶ cells are administered. In another embodiment, 10⁶ – 10⁹ cells are administered. Where the cells produce greater than 2000 pg/ml/10⁶ cells/2hrs greater than 10% of the population of cells express IL-12. Wherein the cells express 20,000pg/ml/10⁶cells/2hrs, at least 0.5% of the population of cells express IL-12.

Polypeptide Production and Research Tools

A cell line (either an immortalized cell culture or a stem cell culture) transfected or transduced with a polynucleotide of the invention (or variants) is useful as a research tool to measure levels of expression of the coding nucleic acid molecule and the activity of the polypeptide encoded by the coding nucleic acid molecule.

The invention includes a method for producing a recombinant host cell capable of expressing a nucleic acid molecule of the invention comprising introducing into the host cell a vector of the invention.

The invention also includes a method for expressing a polypeptide in a host cell of the invention including culturing the host cell under conditions suitable for coding nucleic acid molecule expression. The method typically provides the phenotype of the polypeptide to the cell.

Another aspect of the invention is an isolated polypeptide produced from a nucleic acid molecule or vector of the invention according to a method of the invention.

Another aspect relates to a system or model for testing the mechanism of IL-12 mediated rejection of cancer. In one embodiment the system is an *in vitro* system. Understanding the underlying mechanism that leads to an effective anti-leukemia immune response is greatly facilitated by establishing *in vitro* assays which mimic *in vivo* observations. This is useful for comparing and adapting murine models to human disease. In one embodiment, the *in vitro* system comprises murine bone marrow derived DCs (grown for 6-9 days in GM-CSF) induced to mature (increased expression of CD80) in the presence of both spleen cells + 70Z/3-IL-12 producing cells (but not with either alone). Maturation does not occur if non-transduced 70Z/3 cells are substituted for the 70Z/3-IL-12 cells. Selected populations from the spleen are added and/or removed (immature T cells, CD4⁺ T cells, CD8⁺ T cells, NKT cells, NK cells, DC precursors) to define the critical cell types that are required for 70Z/3 - IL-12 mediated DC maturation.

In one embodiment the system comprises human leukemia cells expressing IL-12 and/or a mouse model susceptible to developing cancer to determine the mechanism by which Interleukin -12 (IL-12) provokes an immune response which, in mice, results in complete rejection of leukemia. In one embodiment, the system permits analysis of the interactions of T cells, dendritic cells (DC), leukemia cells and the cytokines that they produce in

established murine *in vitro* and *in vivo* systems. In another embodiment, the system permits optimization of the parameters essential for engineering primary samples of human leukemia cells to express quantities of IL-12 above necessary thresholds established in the murine system. In a further embodiment, the system is useful to establish *in vitro* conditions to determine how primary human leukemia cells expressing IL-12 interact with the autologous DCs and T cells.

EXAMPLES

Example 1

Direct injection of recombinant IL-12 has shown effectiveness in some mouse models of leukemia.[9-13] while initial human trials employing this approach were less promising ([14-17]and discussed in [4]). It is well recognized in the literature that IL-12-induced anti-leukemia activity is largely mediated by the secondary secretion of IFN- γ . [13] Gollob *et al.*, in particular, have suggested that the induction and maintenance of IL-12-induced IFN- γ was a key component of effective therapy in patients with metastatic renal cell cancer.[18] However the concomitant induction of antagonistic effects with elevated IFN- γ levels continues to pose a challenge and is the impetus for a number of groups to continue testing the efficacy of recombinant IL-12 following different dose and time protocols[7, 8, 19-21] and to evaluate the therapeutic potential of cell-based IL-12 gene therapy ([22-27]and discussed in [4, 13]) in order to overcome this.

More recent clinical trials have included approaches such as intraleukemial injection of IL-12 secreting fibroblasts and dendritic cells, methods that have proven effective in mouse models. To date, these approaches have not had a significant impact on patient survival[15-17]. Finding the reason for this disconnect is of paramount importance.

The inventors recently published a model of ALL in which one variant of the 70Z/3 murine pre-B cell leukemia line, 70Z3-L, is lethal in syngeneic mice while another variant, 70Z/3-NL, elicits a protective immune response (27). The 70Z/3-L cells, although unable to initiate immunity, were readily rejected when an immune response was first initiated against 70Z/3-NL cells. Therefore, our model is amenable to testing whether IL-12 can initiate a specific immune response, recognition of 70Z/3-L and survival of challenged animals. 70Z/3 leukemia is reminiscent of human ALL with neoplastic lesions arising in the liver, spleen, lymph nodes, bone marrow and rarely the central nervous system. Among the most common physical manifestations of the disease are ascites and splenomegaly.

Materials and Methods

Animals.

Female (C57Bl/6xDBA/2)F1 mice (referred to as BDF₁), 8-12 weeks, old were purchased from the Jackson Laboratories (Bar Harbor, Ma). Mice were kept under sterile conditions in the specific pathogen free (SPF) animal facility at the Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada. Mice are fed an irradiated diet and autoclaved tap water. Animals are terminated by CO₂ asphyxiation and cervical dislocation. The Animal Care Committee of the Ontario Cancer Institute approved all experimental protocols employed.

Tumor cells.

Leukemia cells.

70Z/3-L leukemia cells (described in[28]), derived from BDF₁ mice, were maintained in IMDM with 5% heat inactivated fetal bovine serum (HYCLONE, South Logan, UT, USA), 100µg/mL penicillin-streptomycin or 100µg/mL kanamycin (GIBCO-Invitrogen), and 5.5x10⁻⁵ M β-mercaptoethanol (referred to as complete IMDM) in a humidified atmosphere at 37°C and 5% CO₂. Cell concentrations were kept at 5-10x10⁵ cells/mL.

Lentiviral vector construction.

Lentiviral vectors expressing IL-12 cDNA were constructed by a method similar to that described by Yoshimitsu *et al.*[29] with modification. Plasmid pORF-mIL12 (IL-12elasti(p35::p40) Mouse (p35::p40)) (InvivoGen, San Diego, CA) was modified by creating *EcoRI* and *BamHI* restriction enzymes sites, upstream and downstream of the IL-12 gene respectively using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). This resulting construct was then digested with *EcoRI/BamHI* (New England Biolabs). Murine IL-12 cDNA was purified after electrophoresis on a 1% agarose gel, and then subcloned into the pHR' LV backbone downstream of the elongation factor 1 alpha (EF1 α) promoter. Positive plasmid clones for pHR-cPPT-EF1 α -mIL-12-WPRE (i.e. LV-mIL-12) were identified by diagnostic restriction enzyme digestion analyses and subsequent DNA sequencing (InnobioTech, Toronto, ON, Canada).

Viral production and transduction of the cells.

Concentrated LVs were produced by a transient triple-transfection method using pHR-cPPT-EF1 α -mIL-12-WPRE and accessory plasmids onto 293T monolayers by calcium phosphate.[30, 31] An approximate vector titre was estimated based on LV/enGFP[29] production and testing on naïve 293T cells that occurred in parallel. The murine pre-B leukemic cell line, 70Z3-L, was then transduced with an approximate multiplicity of infection (MOI) of 20. Single cell clones, obtained by limiting dilution in 96 well plates at population densities of less than 0.3 cells/well, were then quantitated for IL-12 production/ 10^6 cells/mL/2hrs using a commercially available IL-12 ELISA kit (BD Biosciences, San Jose, CA).

In vivo challenge experiments.

In vivo challenge experiments.

Leukemia cells and transduced cells were grown in complete IMDM and were washed 3 times with 30mL of phosphate buffered saline (PBS) with Ca²⁺ and Mg²⁺. The cells were resuspended at $5-10 \times 10^6$ cells/mL in PBS and injected into the animals in a volume of 100-200 μ L. Mice received IP injections that were performed on the right side of the abdomen using a 1mL syringe with a 26-gauge needle.

Serum collection.

Serum collection in live mice was achieved by puncturing the saphenous vein with a sterile needle and collecting the blood in a serum separator tube (BD, NJ, USA). These tubes were centrifuged at 10,000 RPM for 5 minutes, the serum was then transferred to a micro centrifuge tube and frozen at -20°C until use.

Intraperitoneal administration of rIL-12.

Recombinant mouse IL-12 was purchased from R&D Systems, Minneapolis, USA. Mice were injected IP with 10^6 70Z/3-L cells in 100-200 μL PBS on day 0 followed by daily injections of 0.1-20ng/mouse/day rIL-12 in PBS for a period of 14 days. A secondary challenge consisted of IP injection of 10^6 70Z/3-L cells 70 days after primary challenge, carried out in the manner just described. For the delayed rIL-12 treatments mice received an IP injection of 10^4 70Z/3-L cells in 100-200 μL PBS on day 0. Thereafter groups of 4 or 5 mice received 14 successive rIL-12 IP injections of 20ng/mouse/day but the initiation of these injections was delayed by between 0 and 5 days. The animals were monitored daily for the appearance of symptoms both during the injection period and following the end of the injections.

Intraperitoneal administration of leukemia cell-produced IL-12.

Interleukin-12 secreting cells were produced as described above. Mice were injected IP with 10^6 transduced cells or a mixture of transduced and naïve cells in various proportions in 100-200 μL PBS. A secondary challenge consisted of IP injection of 10^6 70Z/3-L cells or 10^6 L1210 cells more than 110 days after primary challenge carried out in the manner just described. The animals were monitored daily for the appearance of symptoms following injection.

Challenge in-depleted animals.

Mice were depleted of CD4^+ , CD8^+ or both T cell subsets as well as NK cells and IFN- γ using specific antibodies. The hybridoma GK1.5 is directed against CD4^+ T cells, YTS169 against CD8^+ T cells, HB170 (R4-6A2) against IFN- γ and the hybridoma HB9419 was used to produce an

isotype control antibody. All hybridomas were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The lines were grown in 2.5 litres of complete IMDM or OptiMEM in Lifecell culture bags (Lifecell Tissue Culture, Baxter Corporation, Concord, Ontario, Canada) in a humidified atmosphere at 37°C and 5% CO₂ until a live cell count (using trypan blue exclusion) revealed 30% dead cells in the culture. The media was then centrifuged and filtered to remove cells and cellular debris. The antibodies were purified from the media using an affinity column of packed sepharose beads (Gammabind G, Amersham Biosciences Corp, Piscataway, NJ, USA) and concentrated with Centriprep YM-30 columns (Millipore, Billerica, Massachusetts, USA) before dialysis in PBS. NK cells were depleted using an anti-asialoGM1 antibody produced by Wako Bioproducts (Richmond, VA). Rabbit IgG (Sigma-Aldrich) was used as a control for the anti-asialoGM1 antibody. The T cell subset and IFN- γ antibodies were injected on days -1, 3, 7, 10 and 14. The doses used were 1mg of antibody on day -1 and 500 μ g for the remaining injections. The NK cell depleting antibody was injected on days -1, 4, 9 and 14 using the recommended dilution.[32] Control isotype antibodies were injected following the same dose and schedule as their corresponding depleting antibodies. The depletion potential of the antibodies was demonstrated *in vivo* prior to their use in our experiments by injecting mice with a range of concentrations and subsequently examining tissues by flow cytometry to quantify cellular subsets, or examining serum for the presence of cytokines by ELISA. This experiment was conducted twice to test both model systems. In each case, cells were injected on day 0: either 10⁶ 70Z/3-L cells followed by 14 daily injections of rIL-12 (10 or 20ng/mouse/day) or 10⁶ 70Z/3-L vector-transduced cells of the LV12.2 clonal line. Controls included mice injected with 70Z/3-L alone and mice injected with PBS alone according to the appropriate injection schedule.

Bead assay for cytokine levels in the serum.

Mice were injected IP on day 0 with 10⁶ 70Z/3-L cells in 100 μ L PBS and treated daily with 100 μ L preparations of PBS alone or containing low doses of rIL-12 (10 or 20ng/mouse/day) for 14 days. Control groups

included mice injected daily for 14 days with PBS in the absence of 70Z/3-L cells and rIL-12, and a group that was left entirely untreated. Alternatively, for the leukemia cell-mediated IL-12 therapy experiment, mice were injected on day 0 with 10^6 70Z/3-L cells in 100 μ L PBS containing various proportions of the 70Z/3-L vector-transduced cell line LV12.2 (0.5%, 1% and 10%). Control groups included mice injected with 70Z/3-L cells alone or PBS alone. Serum was non-terminally collected from all groups on days 7, 10 and 20 before their daily injection by puncture of the saphenous vein as described above. All mice from the group receiving 70Z/3-L cells alone in the leukemia cell-produced IL-12 therapy model had perished by day 20 such that serum was not collected from this group. Serum samples were diluted 1/5 and stained according to the protocol provided with the Mouse Inflammation Cytometric Bead Array Kit (BD, San Diego, CA, USA). Standards were prepared in triplicate from independent dilutions and flow cytometry was done using a FACScan (Becton Dickinson, Oakville, ON). Acquisition was performed using CellQuest software version 3.1.

Southern blot to determine gene copy number.

The mull-12 gene copy number of vector-transduced 70Z3-L clones was determined by Southern blot as described before.²⁴ Briefly, 5 μ g of genomic DNA extracted from vector-transduced or naïve control 70Z3-L cells was treated with both EcoRI and HindIII (New England Biolabs) and electrophoresed onto a 0.8% agarose gel. Next the agarose gel was washed and transferred onto a positively charged nylon membrane (Bio-Rad).²⁵ A 746 bp fragment containing the WPRE sequence of LV-mull-12 to be used as the hybridization probe was amplified by PCR (Forward primer; 5'-tgctcctttacgctatgtgg-3', Reverse primer; 5'-tcgttgggagtggaattagcc-3') employing the PCR DIG Probe Synthesis kit (Roche). Southern hybridization was performed using the DIG Luminescent Detection kit (Roche), according to the manufacturer's instructions. Serial dilutions of the LV-mull-12 plasmid (see above) in mouse genomic DNA were used as WPRE standards. The results were analyzed using NIH image software and presented as copies / genome.

RESULTS

Intraperitoneal administration of rIL-12 protects mice challenged with 70Z/3-L.

Interleukin-12 is known to be a potent modulator of the immune response attributed with a number of anti-leukemia effects including, but not limited to, T cell-mediated antigen-specific leukemia clearance. This molecule has been approved for clinical use but optimum delivery programs have yet to be defined. In an attempt to alter the course of 70Z/3-L leukemia, we began by testing the effect of IP administration of rIL-12 on the appearance of morbidity after IP injection of 10^6 70Z/3-L cells. Doses of 0.1-20ng/mouse/day for 14 days, which are at least 20-fold below the maximum tolerated dose in mice were tested. In Figure 1a, the inventors show that doses above 10ng were sufficient to significantly improve the survival of animals ($p = 0.002$).

Intraperitoneal administration of rIL-12 leads to long-term protective immunity against the 70Z/3-L leukemia.

Next addressed is whether the results observed above were due solely to the acute effects of IP administered rIL-12 on innate responses or to the induction of a long-term adaptive immune response in the mice. To accomplish this, mice received IP injections of 10^6 70Z/3-L cells, were treated for 14 days with 20ng/mouse/day rIL-12, subsequently challenged 70 days later by IP injection of 10^6 70Z/3-L cells and monitored for the appearance of symptoms. A group of naïve mice was included to control for the efficiency of the cells to cause disease. Figure 1bii shows that all animals first treated with IP administration of rIL-12 (Figure 1bi) survived a secondary challenge with 70Z/3-L cells in the absence of further IL-12 therapy. Thus, IP administration of rIL-12 not only protected against the primary 70Z/3-L challenge but also established long-term protective immune memory.

Intraperitoneal administration of rIL-12 protects animals with pre-established 70Z/3-L leukemia.

To determine if IP administration of rIL-12 can lead to leukemia clearance as well as protection from a developing neoplasm, treatment initiation was delayed to allow for dissemination of the disease. These

experiments were conducted starting with 10^4 70Z/3-L cells injected IP because of their rapid growth. This dose is still lethal to 100% of mice in approximately 20 days. Initiation of rIL-12 administration was delayed by 0 to 5 days and continued for 14 days following the first injection. We found that the initiation of rIL-12 therapy could be delayed by 5 days and still achieve significant protection against the leukemia (Figure 1c). The differences between the survival curves of the six treatment groups are not statistically significant and longer delays were not tested.

CD4⁺ and CD8⁺ T cells are required for the rIL-12-mediated rejection of 70Z/3-L cells after IP administration.

Depleting antibodies were used to determine which cell types mediate the rIL-12-induced rejection of 70Z/3-L leukemia after IP administration. Figure 1d shows that both CD4⁺ and CD8⁺ T cells are important as depletion of either population eliminates immune protection in all animals. The mean survival was 14 days for mice depleted of CD8⁺ T cells, 23 days for mice depleted of CD4⁺ T cells and 13 days for mice depleted of both T cell subsets. The three curves are not statistically different from each other. Neutralizing antibodies against IFN- γ were included to examine its role in the rejection response. This abrogated the protective effects of IP administered rIL-12 demonstrating that IFN- γ plays an essential role in leukemia rejection. Although the importance of NK cells has been shown in other models of IL-12 therapy[33, 34] changes in rejection of the 70Z/3-L leukemia were not observed when NK cells were depleted in this treatment modality (Figure 1d).

Generation of IL-12 secreting leukemia cells by implementation of lentiviral transduction.

In light of these results, to the option of developing a leukemia cell-mediated approach for the delivery of IL-12 treatment was explored. Figure 2a shows the lentiviral construct with an IL-12 fusion transgene under control of the EF-1 α promoter that was generated. After transducing 70Z/3-L cells with an approximate MOI of 20, single cell clones were derived as described in Materials and Methods. Supernatants from these clonal cell lines were

tested for the production of IL-12. The range of secretion from selected clones varied from approximately 250 to 91,000pg/mL/10⁶ cells/2hrs and these levels remained stable over time as shown in Figure 2b. Furthermore, the different levels of IL-12 measured did not seem dependent on cell growth kinetics, nor on survival, as the *in vitro* growth properties of the vector-transduced clones were similar as measured by thymidine incorporation and visual inspection. Southern Blot analysis demonstrated that no clone had more than 7 proviral integration events.

Only a small proportion of vector-transduced 70Z/3-L cells producing IL-12 are required to confer immunity.

Whether the production of IL-12 by vector-transduced 70Z/3-L cells would elicit a protective immune response was determined by injecting 10⁶ cells of each of 12 clones, spanning a range of secretion levels, into the abdominal cavity of BDF₁ mice. The three lowest producing clones (range: 200–1,000pg/mL/10⁶ cells/2hrs) failed to elicit an immune response and mice injected with these cells progressed towards death. In contrast, all mice injected with 10⁶ cells of the ten highest producing clones (range: from 1500 – 40000pg/mL/10⁶ cells/2hrs) survived (Figure 3). To date, the majority of the mice included in this study have survived past 2 years post-injection.

One 70Z/3-L transduced clone, LV12.1 which produces approximately 21,500pg/mL/10⁶ cells/2hrs, was mixed with naïve 70Z/3-L cells to determine if the inclusion of IL-12 producing vector-transduced cells would result in the elimination of non-producing cells also. As little as 2% of the vector-transduced cells were sufficient to confer complete protection (Figure 4a). To further examine the efficacy of producer/non-producer proportions, two other 70Z/3-L transduced clones were selected that differed in IL-12 production by 10-fold (clone LV12.3: 2,000pg/mL/10⁶ cells/2hrs vs. clone LV12.2: 20,000pg/mL/10⁶ cells/2hrs). In this case, as few as 0.5% (i.e. 5,000 LV12.2 cells in 10⁶ total cells) of the higher producing clone was sufficient to confer protection to 80% of the mice but 0.1% failed to protect any mice. However,

even 10% (i.e. 100,000 LV12.3 cells in 10^6 total cells) of the lower producing clone was insufficient to protect, indicating that a threshold of IL-12 production per vector-transduced cell is required to elicit an effective immune response (Figure 4b).

Leukemia cell-mediated IL-12 therapy leads to specific long-term protective immunity against the 70Z/3-L leukemia.

More than 110 days post IP injection with 10^6 LV12.2 cells, mice were challenged with either 10^6 cells of the parental leukemia line 70Z/3-L or another well-characterized B-cell leukemia, L1210, and monitored for the appearance of symptoms. Groups of naïve mice were included to control for the efficiency of both the 70Z/3 and L1210 cells to cause disease. Figure 5 shows that all animals to survive the initial insult with LV12.2 were immune to subsequent challenge with 70Z/3-L but not L1210. Thus, cell-mediated IL-12 therapy leads to specific long-term protective immunity.

CD4⁺ T cells are primarily required for leukemia cell-mediated rejection of 70Z/3-L cells.

Depleting antibodies were used to determine which cell types mediate the IL-12-induced rejection of 70Z/3 leukemia. Figure 6 shows that the CD4⁺ T cell subset is of primary importance unlike in the IP administered rIL-12 therapy model above. The mean survival of leukemia challenged mice was 37 days for animals depleted of CD4⁺ T cells and 18 days for those depleted of both T cell subsets. The curves are statistically different ($p=0.003$), suggesting an important role for CD8⁺ T cells but only in the absence of CD4⁺ T cells. The CD8⁺ T cell subset alone is not sufficient to confer protection. Furthermore, the neutralization of IFN- γ did not diminish the protective effect as was seen with IP administered rIL-12 therapy (Figure 6). This was a surprising result and prompted us to further interrogate the regulation of IFN- γ and various other inflammatory cytokines in each model.

In vivo cytokine regulation.

Interleukin-12 induces the secretion of other cytokines that can have agonistic, antagonistic or synergistic effects and can influence the specific

immune response that is initiated.[6-8, 18, 35-38] It was therefore important to measure the regulation of some of these cytokines *in vivo* to better understand how leukemia rejection is accomplished and shed some light on the results of our neutralization experiments. For this purpose we employed a flow cytometry technique that detects a panel of inflammatory cytokines, including IL-12 p70, TNF- α , IFN- γ , MCP-1, IL-10 and IL-6 in serum. Mice received IP injection of 10^6 70Z3-L cells on day 0 and daily IP injections of either 10 or 20ng rIL-12/mouse/day for 14 days. Serum samples were collected on days 7, 10 and 20. Alternatively, mice were challenged with an IP injection of 10^6 70Z3-L cells on day 0 spiked with various proportions (0.5%, 1% and 10%) of vector-transduced cells and serum samples were collected according to the same schedule as described above. The results of these two assays are shown in Figure 7.

The levels of IL-10 induced on day 20 are significantly higher after leukemia cell-mediated therapy as compared to IP administered rIL-12 therapy ($p < 0.0017$) but are not significantly different between IL-12 treated and control groups for either mode of delivery at any time point. Likewise, the levels of IFN- γ and TNF- α are significantly higher in response to IL-12 secreted from vector-transduced cells ($p < 0.0015$ and 0.0110 respectively). Of note, however, is that leukemia cell-mediated treatment groups show significantly higher levels of IFN- γ than the control groups on day 7 ($p = 0.0007$) but resolve to near basal level by day 20.

DISCUSSION

The inventors demonstrate that IP administered low dose rIL-12 therapy can elicit a protective immune response in leukemia-bearing mice and that an effective approach to deliver IL-12 is via the leukemia cells themselves. Remarkably few transduced leukemia cells are needed to achieve protection provided a sufficient amount of IL-12 is produced per cell, and that protection is achieved in a manner distinct from that with IP administered rIL-12 therapy.

Given the key role that IL-12 plays in the initiation of effective immune responses in various leukemia models, the potential for cytokine therapy

using a murine model of ALL was re-examined. It had previously been found that 70Z/3-L cells lead to the rapid death of mice injected with as few as 10^2 cells. In contrast, variants of this line that are recognized by the immune system and subsequently rejected were established. Mixing as few as 10^5 of these non-leukemic variants with 10^6 70Z/3-L cells resulted in complete rejection of all 70Z/3 cells.[39] While why these variants are recognized by the immune system has not yet been determined, these experiments revealed that 70Z/3-L cells can be rejected if the immune system can be modulated appropriately; making this experimental system amenable to the study of IL-12-induced anti-leukemia activity.

Interleukin-12-based therapies have not become front line cancer treatments in part because studies often report low response rates among patients.[6-8] The poor outcomes associated with IL-12 treatment in these clinical studies can be explained by the physiological response to IL-12-induced IFN- γ . For example high levels of IL-12, and consequently IFN- γ , have been shown to induce IL-10 and lead to downmodulation of IL-12 responsiveness in the host.[6] However, Gollob et al report chronic T helper type-1-like immune activation involving IFN- γ production is necessary for rIL-12-induced antitumor effects.[18]

Previous groups have demonstrated that administration of IL-12 at doses significantly below the maximum tolerated dose can avoid the induction of antagonistic mechanisms.[20] The inventors demonstrated that IP administration of a dose as low as 10-20ng of rIL-12 daily for 14 days, equivalent to 500-1,000ng/kg, is sufficient to significantly increase the survival of mice injected with 70Z/3-L. This dose is effective against an established leukemia burden and rejection leads to long-term immune memory in a T cell-dependent manner.

Other strategies for delivery of IL-12 were investigated 70Z/3-L cells can be readily transduced with our novel lentiviral construct. Different vector-transduced clones produce varied amounts of IL-12. This appears to be a stable trait as we have measured similar levels of secreted IL-12 for each clone on 2-5 independent occasions. The vector copy number in these clones

was determined but this alone does not explain the variable secretion levels, nor does their rate of proliferation. One possible explanation, however, is that the variable secretion is a result of different integration sites and the effect of different genes controlling transgene regulation.

The establishment of clones that produce different levels of IL-12 has allowed examination of the relationship between IL-12 production and the proportion of IL-12⁺ vector-transduced vs. IL-12⁻ naïve 70Z/3-L cells necessary for immune activation. To date, this potentially critical aspect of cell-mediated cytokine therapy has not been thoroughly examined. A very small proportion of IL-12 producing vector-transduced 70Z/3-L cells are sufficient to trigger a protective immune response. For one clone, LV12.2, 5,000 such vector-transduced cells (but not 1,000) were sufficient to save 80% of the mice injected with 10^6 70Z/3-L cells. This result could indicate either that a critical number of "hits" or a sufficient amount of IL-12 is required to trigger an immune response. A reasonable interpretation of "hit" might be an encounter between an IL-12 producing vector-transduced 70Z/3-L cell and an appropriate APC, such as a DC. The alternative explanation proposed is that these 5,000 vector-transduced cells simply deliver a sufficient quantity of IL-12 into the system to trigger an immune response in a more direct fashion. To determine which of these explanations is correct, a different clone, LV12.3, that produces 10-fold less IL-12 per cell was employed. Titrated numbers of vector-transduced cells were injected along with 10^6 70Z/3-L naïve cells. Even 100,000 of such vector-transduced cells failed to confer protection. This represents twenty-fold more cells and twice the potential IL-12 released into the system. Together, these results suggest that it is the number of "hits" that matter rather than the absolute amount of IL-12, but that to qualify as a "hit", the vector-transduced 70Z/3-L cell must produce IL-12 above a certain threshold.

These findings have important implications for clinical trial design and may explain at least part of the differences observed between murine studies, in which IL-12 can initiate a curative immune response, and human studies, in which the immune response is modest and patient survival is normally

unaffected. The protocols used in mouse studies usually involve selection of clones that secrete relatively high levels of IL-12 and frequently the preparation administered consists of 100% IL-12 secreting cancer cells. In contrast, human studies generally rely on freshly obtained populations of cancer cells that are difficult to clone. Therefore bulk populations of cells are transduced and average amounts of IL-12 produced by these populations are measured. In cases reported to date, these average amounts are far below what is predicted to be necessary to elicit protective immunity and there is no information on the distribution of production levels within these populations.

The IL-12-induced anti-leukemia activity in our two models is T cell-dependent but the subsets that are critical differ depending on the mode of IL-12 delivery. The role of IFN- γ also appeared to differ, prompting us to look at its *in vivo* regulation along with a number of other inflammatory cytokines. This was done using a flow cytometry based cytokine bead assay. The regulation of IL-10, IFN- γ and TNF- α are of particular interest in our model systems because IL-10 is known to be the most biologically relevant antagonist of IL-12,[4] IFN- γ may mediate the effects of IL-12[4, 13] and a combination of IFN- γ and TNF- α is required for the development of CD4⁺ CTLs.[5]

The fact that IL-10 production was not elevated above background in any of our treatment groups suggests that the amount of IL-12 administered was sufficiently low as to avoid the induction of antagonistic molecules and dampening of the biologic effect. Measured levels of IFN- γ were significantly higher in the treated groups receiving leukemia cell-produced IL-12 as compared to controls on day 7 but were not significant by day 10 and returned to near baseline by day 20. Furthermore, IFN- γ production was significantly greater in the leukemia cell-mediated model in general. In light of these results, it is probable that the leukemia cell-mediated IL-12 therapy neutralization experiment did not demonstrate a critical role for IFN- γ simply because the neutralizing antibody was overwhelmed by the levels produced. There is ample literature describing how IL-12 leads to the increased maturation of DCs, the production of IFN- γ and more efficient antigen

presentation by the IFN- γ -dependent up-regulation of MHC-II and co-stimulatory molecule expression. T-helper lymphocytes are driven by IFN- γ to differentiate with a type-1 functional profile and subsequently promote the strong CD8⁺ CTL response that we saw with IP administered rIL-12 therapy. However, there is also a literature describing a role for CD4⁺ CTLs in models of infection [5, 40, 41] and more recently in tumor immunology [42-46]. It is possible that the IFN- γ and TNF- α rich environment resulting from leukemia cell-mediated therapy led to the development of an effector CD4⁺ population. This could account for the differential importance of T cell subsets in our two models and explain the distinct results of the neutralization experiments. The major thrust of tumour vaccination research has traditionally focused on targeting CD8⁺ CTLs, which require stimulation by a CD4⁺ helper T cell population, to affect tumour clearance but the clinical response has been limited. Directly targeting CD4⁺ effector cells may be important to achieve a more robust anti-tumour response.

Despite the beneficial effects of IFN- γ that we have highlighted above, a dampening of the response with repeated administration is still of concern in models of IL-12 therapy. An important attribute of our leukemia cell-mediated model is that a sufficient immune response is initiated and the leukemia cleared but the signal is self-limiting because the source of IL-12 into the system is the cancer cells that are, themselves, the target of therapy. As the leukemia cells are rejected, the source is reduced and IFN- γ levels return to baseline without a significant increase in the antagonistic molecule IL-10.

IL-12, given at doses below the level leading to the induction of antagonistic mechanisms, is sufficient to launch a protective immune response against 70Z/3-L ALL cells and complete clearance of the leukemia. The mode of IL-12 delivery can have a profound impact on the nature of the immune response that is mounted and demonstrates a critical role for CD4⁺ cells in our leukemia cell-mediated model that apparently does not exist in our IP administration model. Although previous studies have been concerned with the counter-productive side effects resulting from elevated levels of IL-12-induced IFN- γ , several critical and beneficial roles for this cytokine have been

demonstrated. Moreover in our model, a potentially problematic dampening of the immune response was not observed, possibly due to the self-limiting nature of the leukemia cell-mediated therapy approach employed.

This work in a murine model of ALL using a LV constructed that engineers expression of murine IL-12 has demonstrated that animals can be completely protected from leukemia-induced death when certain levels of IL-12 are produced by the transplanted cells

Example 2

Acute Myeloid Leukemia

The following myeloid leukemia lines were transduced with the murine LV IL-12 construct.

A lentiviral vector, (pHR-cPPT-EF1 α -mIL-12-WPRE) that engineers expression of murine interleukin-12 (mIL-12) was constructed and characterized. Plasmid pORF-mIL12 (IL-12elasti(p35::p40) Mouse (p35::p40)) was modified by creating *EcoRI* and *BamHI* restriction enzymes sites, upstream and downstream of the mIL-12 gene, respectively, using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). This resulting construct was then digested with *EcoRI/BamHI* (New England Biolabs). Murine IL-12 cDNA was purified after electrophoresis on a 1% agarose gel, and then subcloned into the pHR' LV backbone downstream of the elongation factor 1 α promoter (EF1 α). Positive plasmid clones for pHR-cPPT-EF1 α -mIL-12-WPRE (i.e. LV-mIL-12) were identified by diagnostic restriction enzyme digestion analyses and subsequent DNA sequencing (Innobiotech, Toronto, ON, Canada).

Lentivirus was produced by transfecting 293T cells with the plasmids pCMV Δ R8.91, pMDG and either control enGFP lentivector (pHR-Cppt-'EF-GW-SIN). Viral supernatants were collected at 48 hours post-transfection, filtered and concentrated by ultracentrifugation. Concentrated virus was serially diluted and the efficiency of viral production was assessed by detection of p24 antigen by ELISA.

To determine the transduction efficiency of mIL-12 lentivirus murine myeloid leukemia lines MMB3.19 and C1498 (1 million cells/ml) were infected in vitro with mIL-12 or enGFP lentivirus at a multiplicity of infection (M.O.I) of 1. The infected cells were maintained at 37°C and the media changed 24 hours after culture. The supernatant was collected 48 hours later and the levels of IL-12 measured by ELISA.

Cells transduced in vitro with lentivirus encoding mIL-12 produce efficiently high concentrations of IL-12 [~214ng/ml and 7.5ng/ml for MMB3.19 and C1498 cells, respectively). MMB3.19-IL-12 and C1498-IL-12 cells secreted 214 and 7.5 times more IL-12, respectively, than the enGFP transduced cells. The results are illustrated in the chart below and in Figure 8a.

	GFP	MIL-12
MMB3.19	0.9	214.7
C1498	0.9	7.5

Example 3

Human IL-12

I. Lentiviral vector construction.

Lentiviral vectors expressing human IL-12 cDNA were constructed by a method similar to that described for mouse IL-12 construct. The cDNA of human IL-12 was obtained as a fusion form from InvivoGen (pORF-hIL12 (IL-12elasti(p35::p40)). The open reading frame of the gene was amplified by the following PCR primers: hIL-12 ORF Fwd, 5'-TTGGCGCGCCACCATGGGTCACCAGC-3'; and hIL-12 ORF Rev, 5'-TTGGCGCGCCTTAGGAAGCATTAGATAGCTCATCACTC-3'. The PCR product was then subcloned into our Lentiviral backbone (pHR'-cPPT-EF1a-WPRE). The construct was confirmed by diagnostic restriction enzyme digestion analyses and subsequent DNA sequencing.

II. Transfection experiment

To assess the pHR'-cPPT-EF1a-hIL12-WPRE construct, 1×10^6 293T

cells were transfected with the construct, the human IL-12 template pORF-hIL12 or empty lentivector pHR'-cPPT-EF1a-WPRE. Cell supernatant was collected 24 and 48 hours after transfection. The hIL-12 level was measured by ELISA (BD pharmingen, San Diego, CA) (Chart below; Figure 8b).

III. Transduction to 293 T cells

Lentivirus carrying hIL-12 open reading frame (LV- hIL-12) were produced by a transient triple-transfection method using pHR-cPPT-EF1 α -hIL-12-WPRE and accessory plasmids onto 293T monolayers by polyethylenimine. Virus supernatant was collected 24 and 48 hours after transfection. To test the transduction ability of the LV-hIL1, 1x10⁶ 293T cells were transduced with the virus supernatant. hIL-12 expression level in the cell supernatant was measured by the same ELISA assay as mentioned above (Chart below Figure 8b).

IV. Transduction to AML.1 cells

200-fold concentration of LV-hIL12 virus was obtained by ultracentrifuge. To test the transduction ability of the virus to other tumor cell lines, 0.5 or 1 million of AML.1 cells (an acute leukemia cell line) were transduced with 1/100 diluted concentrated LV-hIL12 virus. hIL-12 expression level in the cell supernatant was measured by the same ELISA assay as mentioned above (Figure 8c).

	24h		48h	
	Ave (pg/ml)	SD	Ave (pg/ml)	SD
LV-hIL12	1010.052	33.145	840.397	24.184
pHR-hIL12	774.131	340.254	933.513	50.522
pORF-hIL12	1079.439	62.461	959.165	19.813
pHR vector	0	1.762	0	3.98

LV-hIL12 will be used to transduce other human leukemic cell lines and primary cancer cells derived from subjects with leukemia.

Example 4

Chronic Myeloid Leukemia in Humans

Immunotherapy offers a method to improve the treatment of leukemias, in particular in combination with other treatment modalities. Indeed, maybe only potent immune system-invoking therapy will be effective at fully eradicating leukemia since residual disease often exists in patients that are in remission, which can be re-activated later. This is especially true for chronic myeloid leukemia (CML), a clonal disorder involving the Philadelphia chromosome, which represents 15% of all adult leukemias. On the other hand, this delayed disease progression provides a key window of opportunity for immunotherapy. Since immunotherapy is not dependent on abrogating cell functions by interrupting signaling or on intercalation into DNA by small molecules, for example, it can also be effective on transformed cells that are quiescent or inhabit inaccessible locales. Of importance, immunotherapy may be an effective way to target true cancer stem cells. Lastly, due to the circulating and surveillance nature of the immune system, existing metastatic disease even in primary CML patients could be treated by this approach.

Approximately 4500 new patients are diagnosed with CML in North America every year. Onset of the most prevalent form of CML is associated with a reciprocal translocation between chromosomes 9 and 22 leading to the formation of Bcr-Abl oncogene. This is manifested by a rapid expansion of bone marrow-derived hematopoietic cells of the myeloid lineage. Current first-line therapy involves treatment of CML patients with imatinib mesylate (Gleevec®), a small-molecule tyrosine kinase inhibitor of the Bcr-Abl product. Unfortunately, this is not a curative treatment. In fact, 4% of early-stage and a full 50% of advanced-stage CML patients develop resistance to imatinib mainly due to ABL1 mutations (1). Imatinib another treatment, is also costly and requires life-long ingestion of the drug; effects of prolonged administration (or of others of this class) are not known. This strategy is also not likely to impact the cancer stem cell, which may be relatively quiescent and thereby resistant to metabolic modulation. Also the lack of inhibitor specificity for only the Bcr-Abl product means that other tyrosine kinases can also be affected. As such, imatinib has shown some serious side effects; a recent study has

shown that mice and human patients receiving imatinib demonstrate severe cardiotoxicity (2).

A wide range of immunotherapy strategies have been envisioned. Indeed, it has been known for years that the immune system is capable of recognizing and clearing cancer cells in some instances and yet not in others. Cytokines have pleiotropic effects on the immune system. One cytokine that has received a lot of attention towards amelioration of cancer is interleukin-12 (IL-12). IL-12 is heterodimeric and acts to increase antigen presentation by dendritic cells (DCs) and to induce their maturation. The basic concept behind therapy using IL-12 is that it alerts the immune system to a higher degree of vigilance and if this attention can be directed against cancer cells, elimination by the immune system may be possible. IL-12 has been given as a systemic bolus for treatment of leukemias but clinical outcomes have been quite modest. This may be due to difficulties in establishing appropriate dosing per patient and the severe peripheral toxicities observed.

Interleukin-12 (IL-12). IL-12 is a heterodimeric cytokine with multiple biological effects on the immune system. It is composed of two subunits, p35 and p40, both of which are required for the secretion of the p70 active form. IL-12 acts on DCs, leading to increased maturation and antigen presentation, which can allow for the initiation of a T cell response against tumor specific antigens. It also drives the secretion of IL-12 by DCs, creating a positive feedback mechanism to amplify the response. Once a response is initiated, IL-12 directs the immune system towards a Th1 cytokine profile, inducing CD4⁺ T cells to secrete IFN- γ and leading to a CD8⁺ cytotoxic T cell response (3). However, IL-12 is also a strong pro-inflammatory cytokine that leads to the secretion of other cytokines including TNF- α which, combined with IFN- γ , is a prerequisite for the development of CD4⁺ cytotoxic T lymphocytes (CTL; ref. 4). Furthermore, IL-12 can promote the activation of macrophages and eosinophils through induction of IFN- γ and other cytokines. This leads to IL-12 secretion and further amplification of both the innate and acquired responses (3). However, high levels of IL-12, and consequently IFN- γ , have also been associated with induction of antagonistic molecules such as

IL-10 and the depletion of signalling molecules downstream of IL-12, such as STAT4 (3, 5-7).

Lentiviruses (LVs) and Gene Therapy. The first approved gene therapy clinical trial was published in 1989. Since then >2500 patients worldwide have received gene therapy to date.

Safety is a high priority. One major vector system that has been responsible for generating renewed enthusiasm is based on LVs. LV are most-commonly derived from HIV-1 (17). Substantial segments of the viral genome have been deleted and additional safety elements, such as self-inactivating LTRs, have been added (18). Moreover, these vectors are now produced in ways to reduce the possibility of recombination developing replication competent lentivirus (RCL). Indeed, substantial effort has gone into testing the safety and efficacy of this platform; for example, LVs offer stable integration but with less insertion into promoters that can disrupt cell functions than occurs with onco-retroviruses. LVs also allow the ability to engineer co-expression of more than one gene. A number of these bicistronic constructs have been generated by the inventors (see ref. 19, 20). The LV constructs comprise a novel suicide control system. This enzyme/prodrug combination employs a modified human enzyme engineered to respond to AZT (ref. 21; see Comment (ref. 22)). This safety system offers the ability to control the fate of transduced cells and will be practical to use in any setting involving transplant of tumor cells, stem cells, and the like.

Safety improvements and the efficiency of LVs have recently led to clinical trials. The first LV trial was been completed in 2006 and involved anti-sense RNA sequences as transgenes that targeted HIV (23). This study was performed in AIDS patients with high viral loads; some reductions in these viral loads were observed. More importantly, no RCL was found between the recombinant vector and the endogenous wild-type virus. These results have now led to at least 6 other LV protocols being initiated for indications including cancer and inherited disease. Such outcomes have also led to a renaissance in corporate interest in gene therapy that still has a large but untapped potential to treat a variety of disorders.

As the inventors have found, localized concentration of IL-12 at the tumor/DC/T cell interface may be relevant for up-regulation of the immune response, and effective dosing at that site is not being generated in the clinical protocols.

State-of-the-art gene transfer techniques (lentiviruses; LVs) were used to quantitatively modulate the expressed IL-12 profile by the tumor cell itself. LVs are very efficient at stably transferring genes into cells.

The inventors have generated a novel clinically-oriented LV that engineers expression of human IL-12. Virus has been produced and virus and the vector have been validated in established human cancer cell lines by quantitating titer and human IL-12 production (see Figure 8b). Human primary CML cells will be transduced which will produce varying levels of human IL-12. The cells will be analysed to demonstrate that the human IL-12 produced by the transduced cells is functional. A pre-clinical xenograft model will be adapted to examine maintenance of the transduced CML cells. The kinetics of human IL-12 produced in vivo will be measured.

Gleevec is the treatment of choice; however side effects, resistance, the need for long-term therapy, and high cost are associated with Gleevec use.

Murine models of CML. Two established CML lines were tested and show differential production of IL-12 in vitro in transduced populations derived from these lines.

CML and ALL are similar in that high remission rates in adults are followed by high relapse rates. This clinical course not only provides initial material suitable for infecting with the vector constructs described herein but a rationale for subsequent treatment. Importantly, CML shows this bi/tri-phasic progression and some initial response to imatinib that allows time to develop immune modulating tumor cells following vector transductions.

LVs offer some real advantages over other gene transfer methods that seek to generate stable cell lines secreting IL-12 for such applications: for example - plasmid transfection is very inefficient and adenovirus- or AAV-mediated gene delivery do not lead to appreciable vector integration, which

will provide variable levels of IL-12 over time. The inventors have shown that transduced murine cells stably express transgenes ~2 years after initial infection (24).

Synthesis of human vector. A recombinant LV that engineers stable expression of human IL-12 was generated. The cDNA for human IL-12 was obtained as a fusion form from InVivoGen (pORF with IL-12elasti(p40::p35)). This cDNA was subcloned as above into the pHR' LV backbone. Diagnostic restriction digests and sequencing of both DNA strands was performed to confirm the fidelity of the new construct. This first construct will be monocistronic; other constructs may employ our suicide strategy involving mutated thymidylate kinase mentioned above (21) that would add another layer of safety.

Generation of high-titer vector stocks. High titer recombinant virion stocks were generated and titered in vitro. High titer vector stocks were established by ultracentrifugation of collected and pooled supernatants after triple plasmid transfections of 293T cells as done before (20). The vector was pseudotyped with the VSV-g glycoprotein which allows a wide range of cells to be infected. After sufficient titer of the pHR'human IL-12 delivery vector is obtained, pooled vector stocks will be tested by a 'Direct' assay to ensure that RCL has not been generated. In this assay, recipient 293T cells are infected a single time and then grown out for a number of passages. After 4-6 weeks, supernatants from these infected cells are collected and used to infect naïve cells. These cells are grown out and then assayed by functional assays and PCR on isolated genomic DNA to determine if vector has been functionally transmitted to these secondary recipient targets.

Testing in 293T cells. The level of human IL-12 produced in comparison to vector copy number in infected cells will be determined. Firstly, 293T cells will be infected at a range of modest MOIs from about 0.1 to 100. Supernatants from pools of infected cells, done in triplicate, will be examined for human IL-12 production by ELISAs. Next, individual cell clones will be established by

limiting dilution. These cell lines will be examined for human IL-12 production relative to copies of integrated provirus – as measured by Southern blots. Controls will be comprised of 293T cells infected with a LV/eGFP virus previously constructed (19). This information will provide information relating to the relative MOIs to be used and allows correlation of the secretion of this human form of IL-12 with relative vector copy number. Use of this stable cell line will provide a reference point for titrating all future viral preparations that are made with the intent of infecting patient CML cells, which may have considerable variability in sample-to-sample infection frequencies.

Testing in Human CML. Firstly, established CML cell lines will be infected at various MOIs and clonal populations will be assessed for IL-12 expression in relation to vector copy number. It has been shown by the inventors that K562 (a CML line) is readily and productively infected with recombinant LVs (21). Numerous clones from each pool will be derived and examined for vector copy and relative human IL-12 production. Cell viability of clones producing various levels of human IL-12 over time will be measured by thymidine incorporation assays. Cells will be cultured for many weeks and compared with original clones frozen initially after limiting dilution to determine if human IL-12 production changes over time. Vector stability will also be measured in these cells by repeat Southern Blot analyses. Secondly, primary human CML cells will be obtained from a minimum of 3-5 CML donors initially to reduce reliance on a single sample. Here cells will be infected at 2 or 3 different MOIs. Cells from each donor will be handled separately to give information on the variability that can be expected. As above, human IL-12 production will be measured by ELISA in relation to vector copy number.

Additional pre-clinical data will be obtained. From a number of transduced K562 and Jurkat clonal lines, the sequence of the human IL-12 cDNA from the integrated provirus in genomic DNA will be determined after PCR amplification and subcloning to a stable plasmid. This will provide information on the stability of the vector itself and whether recombinations are occurring that could decrease protein expression levels from a given vector copy

number. If consistent alterations are observed in a variety of clones such sequences could be mutated to reduce overlap or alter secondary mRNA structure to favor maintenance of fidelity. Further the vector integration site of cell populations by LM-PCR will be analysed to determine clonality. It will also be important to determine that the human IL-12 secreted by the transduced CML clones is functional. For this primary human DC cultures will be used to examine stimulation and the enhancement of T cell proliferation compared to controls.

It will be determined whether vector-transduced primary CML cells that have undergone growth arrest (by very high dose irradiation, for example) in preparation for safe clinical infusions into patients are still able to secrete similar levels of human IL-12 compared to control cells. No differences are expected as others have shown stable expression of GM-CSF and CD40L, for example, in patient leukemia cells after irradiation (25). One group even reported enhanced transgene expression in leukemia cells after γ -irradiation (26). Also, the suicide gene component mentioned above may be added, and killing efficiency of bicistronically transduced primary CML cells producing human IL-12 will be assessed after AZT addition at concentrations we have used before (21).

Test CML cell growth in vivo. The cell lines are assessed for growth in vivo. Cells will be introduced in immune deficient NOD/SCID mice and mice will be examined for the persistence of transduced CML cell lines and primary patient cells in vivo in this xenograft model. This model shows stable engraftment of human hematopoietic cells, especially when an antibody is given to reduce murine NK cell activity. Anti-CD122 antibody (24) from a hybridoma cell line is purified in milligram quantities. Anti-CD122 antibody increases human cell engraftment in NOD/SCID mice. NOD/SCID mice were either not pre-treated (n=3) or pre-treated with anti-CD122 (200 μ g; i.p. injection; n=3). 24 hrs later, mice were irradiated (350 cGy; ^{133}Cs source) and injected i.v. with 7×10^5 purified cord blood-derived human CD34⁺ cells. At 7 weeks post-transplant, bone marrow was harvested, and human cell engraftment was determined by flow cytometry using anti-human CD45 PE. Two of three control recipients lacked long-term human cell engraftment, as defined by $\leq 1\%$ CD45⁺ events. Both growth-arrested cells and un-manipulated transduced cells will be given at various doses to recipient

NOD/SCID mice. Persistence of transduced CML cells will be determined by conventional assays involving flow cytometry for human cell surface antigens (such as CD45/CD71) along with RT-PCR analyses for the LV as has been done for the Bcr-Abl oncogene fusion (27). These studies will be important to prove that the CML cells comprise the primary populations in the xenografted animals. As well, circulating levels of human-specific IL-12 will be determined by ELISA; production of secondary cytokines such as IFN- γ is also measured.

Where the bicistronic vector that engineers expression of the novel suicide gene is employed, the effectiveness of transduced cell killing in vivo can be measured after the addition of AZT to animals - dosing that is below the level of systemic toxicity is described in (21). A fully adaptive transplant system in this xenograft model is developed wherein matching genetically modified cells are returned to animals previously reconstituted with autologous patient hematopoietic components. The optimal dose of IL-12 relative to immune response is determined. The effect of the addition of other co-stimulatory molecules or alternative cytokines that perturb the immune response invoked either positively or negatively are assessed. Lentivectors that express shRNAs that downregulate expression of important genes that may effect stimulation such as IL-10 are also assessed. The contribution of various populations of hematopoietic cells themselves using depletion and sorting-mediated add-back studies are also assessed.

Example 5

Leukemia cells from 4 donors from each group (CML, AML, CLL, ALL) will be enriched following Ficoll centrifugation by established protocols. Initially, for AML and ALL we will carefully select patients with high leukocyte (>60k) and high % blast counts in which case we expect enrichments to exceed 95% purity. For CML, patients in blast crisis will be selected to achieve the same result. For CLL mature CLL lymphocytes from patients with very high leukocyte counts (>100k) will be achieved to achieve this enrichment. In each experiment, the leukemia cell population will be infected at 3 different MOIs using our LV/huIL-12 construct and a LV/enGFP control. An enzyme-linked immunospot (ELISPOT) assay for use as a readout in these experiments is being developed. The cloned, stable, murine lines

produce a range of IL-12 from 200-40000pg/10⁶/ml/2hrs and serve to calibrate the ELISPOT assay by correlating spot size to known secretion levels at the signal cell level. A similar calibration set will be created with human established cell lines by subcloning after the primary LV/huIL12 transduction. The ELISPOT assay will allow us to quantify not only the percentage of primary leukemia cells expressing IL-12 from the transduced IL-12 vector, but also will provide a distribution of IL-12 production levels. The assay will be developed to reliably yield at least 10% of the leukemia cells expressing at least 20000pg/10⁶/ml/2hr. Primary cells will be frozen and thawed and retested to determine the stability of this distribution. Primary cells will also be irradiated and retested for the production and distribution of IL-12 levels. Clinical protocols using these populations would serve as autologous cell based vaccines to be used to prevent relapse in patients who achieve CR.

Example 6

Acute Lymphoblastic Leukemia (ALL)

Similarly as described for CML, ALL cells transduced with a LV IL-12 construct will be made and tested.

Testing in Human ALL cells. Firstly, established ALL cell lines will be infected at various MOIs and clonal populations will be assessed for IL-12 expression in relation to vector copy number. It has been shown by the inventors that Jurkat cells (an ALL line) are readily and productively infected with recombinant LVs (21). Numerous clones from each pool will be derived and examined for vector copy and relative human IL-12 production. Cell viability of clones producing various levels of human IL-12 over time will be measured by thymidine incorporation assays. Cells will be cultured for many weeks and compared with original clones frozen initially after limiting dilution to determine if human IL-12 production changes over time. Vector stability will also be measured in these cells by repeat Southern Blot analyses. Secondly, primary human ALL cells are obtained from a minimum of 3-5 ALL donors initially to reduce reliance on a single sample. Here cells are infected at 2 or 3 different MOIs. Cells from each donor are handled separately to give information on the variability that can be expected. As above, human IL-12 production will be measured by ELISA in relation to vector copy number.

Additional pre-clinical data will be obtained. From a number of transduced K562 and Jurkat clonal lines, the sequence of the human IL-12 cDNA from the integrated provirus in genomic DNA will be determined after PCR amplification and subcloning to a stable plasmid. This will provide information on the stability of the vector itself and whether recombinations are occurring that could decrease protein expression levels from a given vector copy number. If consistent alterations are observed in a variety of clones such sequences could be mutated to reduce overlap or alter secondary mRNA structure to favor maintenance of fidelity. Further the vector integration site of cell populations by LM-PCR will be analysed to determine clonality. It will also be important to determine that the human IL-12 secreted by the transduced CML clones is functional. For this primary human DC cultures will be used to examine stimulation and the enhancement of T cell proliferation compared to controls.

It will be determined whether vector-transduced primary ALL cells that have undergone growth arrest (by very high dose irradiation, for example) in preparation for safe clinical infusions into patients are still able to secrete similar levels of human IL-12 compared to control cells. No differences are expected as others have shown stable expression of GM-CSF and CD40L, for example, in patient leukemia cells after irradiation (25). One group even reported enhanced transgene expression in leukemia cells after γ -irradiation (26). Also, the suicide gene component mentioned above is optionally added, and killing efficiency of bicistronically transduced primary ALL cells producing human IL-12 will be assessed after AZT addition at concentrations we have used before (21).

Administering IL-12 expressing cells to an ALL Subject

Acute Lymphoblastic Leukemia: It is estimated that 5,200 new patients will be diagnosed with ALL in the US in 2007, and 1,420 will die of the illness. ALL is the most common type of leukemia in children with 61% of diagnoses made in individuals under age 20 (29). The overall 5-year relative survival rate for the period 1996-2003 was 64.0%. There was a slightly

positive annual percentage change (0.3%) in ALL incidence for the period of 1985-2005 (29).

The malignant hematopoietic cells are lymphoid precursor cells. Cytogenetic abnormalities occur in ~70% of cases of ALL in adults but are not associated with a single translocation event as in CML. The standard treatment course has been given the terms induction, consolidation, maintenance, and CNS prophylaxis - but even with intensive therapy only 20-40% of adults with ALL are cured with current regimens. Therapy for ALL includes conventional chemotherapy (vincristine, anthracycline, cyclophosphamide, L-asparaginase etc.), radiation therapy and bone marrow transplant. Newer drugs have been developed including clofarabine, nelarabine, and dasatinib, but here responses have been relatively modest and toxicities remain an issue.

Imatinib has also been used in Philadelphia chromosome positive ALL. Imatinib has limited effectiveness in ALL treatment when used as a single agent, but several studies have shown improved outcomes when it is combined with standard chemotherapy (30). Clofarabine (Clolar®) was approved in December of 2004 for pediatric patients with relapsed or refractory ALL overall response rates average 25% (30). Nelarabine (Arranon®) was approved as an orphan drug by the FDA in October, 2005 for treatment of T-cell ALL. Complete responses are reported in 54% of patients with T-cell ALL (30). Approximately 700 ALL patients per year in the US have T-cell ALL (30).

Drugs in development for ALL include Rituximab in Phase III, AMN107 and 852A both in Phase II, Nilotinib (Tasigna®) and AT9283 both in Phase I/II and KW-2449 in Phase I. Cell based therapies such as nonmyeloablative stem cell transplant and allogeneic umbilical cord blood transplantation are also in development. Drugs in trials for specific types of ALL include therapeutics directed to T-cell ALL (T-ALL) such as Alemtuzumab (Campath®), daclizumab and denileukin diftitox (Ontak®) all in Phase II and Similarly, a number of CML drugs in trials for Ph+ ALL such as MK0457 and Bortezomib (Velcade®) which are both in Phase II, SKI-606 in Phase I/II and INNO-406 in Phase I.

Clinical Use

50 ml of heparanized blood is collected from patients following REB approved informed consent. The blood is diluted with 110 ml of alpha medium and aliquoted in to 50 ml conical centrifuge tubes. Ficol hypaque is injected under the blood and the tubes are spun at 1600 rpm at 15 C for 20 minutes. The layer of mononuclear cells is removed and resuspended in 100 ml alpha medium with 5% FCS. The cells are spun at 1000 rpm for 10 minutes and then resuspended in 10 ml alpha medium with 5% FCS cells are then counted and then frozen for future use or distributed for fresh experiments. This would yield over 1×10^9 blasts from the peripheral blood of patients.

Blast cells are collected from the subject prior to chemotherapy when they are very high in numbers. The cells or a portion thereof are optionally frozen. The patient is treated with chemotherapy or other appropriate modality. Cells are then thawed if frozen, infected with LV IL-12 and analyzed for the required level of expression (e.g the threshold level). Cells meeting this criteria are optionally irradiated, and reintroduced into the patient.

Where the vector construct comprises a safety gene component, cells are optionally not irradiated.

Further cells are optionally infected prior to freezing.

Administering IL-12 expressing cells to a Subject with CML

Chronic Myeloid Leukemia: It is estimated that 4,570 people in the US will be diagnosed with CML and 490 will die of this illness 2007 (30). There was a negative annual change in incidence (-2.6%) of CML for the period of 1997-2004 (30).

Current preferred first-line therapy involves treatment of CML patients with imatinib mesylate (Gleevec®). It has been reported that 4% of early-stage CML patients and a full 50% of advanced-stage CML patients develop resistance to imatinib (32). Imatinib mesylate treatment also requires life-long medication; the full effects of such prolonged administration of this agent (or others of this class) are not yet known. Gleevec can cause severe side effects

such as cytopenias, particularly anemia, neutropenia, and thrombocytopenia; severe congestive heart failure and left ventricular dysfunction; severe hepatotoxicity; grade 3/4 hemorrhage and gastrointestinal perforations including some that have been fatal. Along those lines, a recent study has shown that mice and human patients receiving imatinib mesylate demonstrate cardiotoxicity (2); although the overall prevalence of this severely adverse event has not yet been systematically verified and accurately quantitated.

Dasatinib (Sprycel®) has recently been introduced as a therapy for CML patients that have failed treatment with imatinib. Dasatinib can also produce severe and sometimes fatal side effects: thrombocytopenia, neutropenia, and anemia (NCI CTC Grade 3 or 4); severe hemorrhages including fatalities have occurred in a significant percentage of patients (1-7% depending on site of hemorrhage). Most bleeding events were associated with severe thrombocytopenia. Other side effects include severe fluid retention and cardiac effects (QT prolongation) (33).

Nilotinib (Tasigna®) has very recently been approved in the US as a new anti-cancer therapy for CML patients who are resistant or intolerant to treatment with imatinib. Similar to dasatinib, nilotinib can cause neutropenia and thrombocytopenia. Nilotinib also prolongs the QT interval and sudden deaths have been reported (34).

Other treatment options for patients with CML include conventional cytotoxic chemotherapy, interferon-alpha, bone marrow transplant and allogeneic stem cell transplant.

Drugs in development for CML include Lonafarnib Phase III, LBH589 Phase II/III, AT9283 Phase I/II, MK0457 Phase II, Bortezomib (Velcade) Phase II, 852A Phase II, SKI-606 Phase I/II, allogeneic umbilical cord blood transplantation Phase II, XL228 Phase I, KW-2449 Phase I, INNO-406 Phase I and homoharringtonine (Ceflatonin®) which has recently completed Phase I/II (35).

Clinical Use

50 ml of heparanized blood is collected from patients following REB approved informed consent. The blood is diluted with 110 ml of alpha medium and aliquoted in to 50 ml conical centrifuge tubes. Ficol hypaque is injected under the blood and the tubes are spun at 1600 rpm at 15 C for 20 minutes. The layer of mononuclear cells is removed and resuspended in 100 ml alpha medium with 5% FCS. The cells are spun at 1000 rpm for 10 minutes and then resuspended in 10 ml alpha medium with 5% FCS cells are then counted and then frozen for future use or distributed for fresh experiments. This would yield over 1×10^9 blasts from the peripheral blood of patients.

Blast cells are collected from the subject prior to chemotherapy when they are very high in numbers. The cells or a portion thereof are optionally frozen. The patient is treated with chemotherapy or other appropriate modality. Cells are then thawed if frozen, infected with LV IL-12 and analyzed for the required level of expression (e.g the threshold level). Cells meeting this criteria are optionally irradiated, and reintroduced into the patient.

Where the vector construct comprises a safety gene component, cells are optionally not irradiated.

Further cells are optionally infected prior to freezing.

Administering LV IL-12 to a CLL patient

CLL B-CLL is the most common leukemia of adults with an expectation of ~16500 cases in NA this year (Estimates based on American Cancer Society and Canadian Cancer Society Reports). Remissions can be achieved with purine analogues and monoclonal antibody therapy however the diseases invariable progresses. Allogeneic stem cell transplants can be curative but many patients do not qualify for this treatment because of their age. The observation that GVL responses occur after stem cell transplantation confirms that an anti-leukemia immune response to CLL is possible. The slow progression of B-CLL also makes this disease attractive for immunotherapy approaches.

Clinical Use

50 ml of heparanized blood is collected from patients following REB approved informed consent. The blood is diluted with 110 ml of alpha medium and aliquoted in to 50 ml conical centrifuge tubes. Ficol hypaque is injected under the blood and the tubes are spun at 1600 rpm at 15 C for 20 minutes. The layer of mononuclear cells is removed and resuspended in 100 ml alpha medium with 5% FCS. The cells are spun at 1000 rpm for 10 minutes and then resuspended in 10 ml alpha medium with 5% FCS cells are then counted and then frozen for future use or distributed for fresh experiments.

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Where the vector construct comprises a safety gene component, cells are optionally not irradiated.

Further cells are optionally infected prior to freezing.

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Example 7

Treating Solid Tumors

Solid tumors are removed partially or fully from a subject. The solid tumor is optionally any respectable tumor. The tumor is optionally a renal cell cancer, melanoma or prostate cancer.

Single cell suspensions are obtained and cells are transduced or transfected with an IL-12 vector construct such as LV hIL-12. Transfected or transduced cells are optionally irradiated to induce growth arrest and prevent cell division. Transduced cells comprising vector constructs comprising an activator polynucleotide such as a modified tmpk molecule are not irradiated as cells expressing the activator polynucleotide can be killed by administration of the prodrug.

A population of cells including transduced cancer cells is administered to the subject from which the cancer was derived. The population of cells is administered, intradermally or subcutaneously about once a week, once every two weeks, or about once a month for a 3 month period. Approximately 1×10^6 to 1×10^8 cells are administered.

The subject is monitored for an anti-cancer immune response and cancer progression.

Example 8

Research Models and Systems

Determine the critical aspects of initiating anti-leukemia responses in the murine system. The *in vivo* induction of anti-leukemia immunity using *in vitro* models will be studied. DCs mature in culture when exposed to 70Z/3-IL-12 cells only in the presence of spleen cells. Untransduced 70Z/3 cells do not mirror this effect. Selected populations of spleen cells will be systematically removed to determine which spleen cells are responsible for the observed effects. Antibodies specific for subpopulations of T cells, NK cells, and macrophages, will be used in combination with either MACS or FACS for depletion and/or enrichment. These experiments will be conducted in transwell plates which allow the physical separation of the various cell types to identify critical cell-cell interactions. DC maturation (increased expression of CD80) as our prime read out has been used. However, it is possible that DC maturation in the presence of 70Z/3 cells will be followed by activation of specific T cell populations. The *in vitro* system will be used to determine if T cell responses are initiated and, if so, the nature of those responses. Cytokine production typical of Th1 induction (such as IFN γ) as well as the appearance CD4⁺ and or CD8⁺ mature T cells specific for 70Z/3 cells will be monitored. 70Z/3 specific T cell clones will be expanded and their cell surface phenotype will be characterized. Their cytotoxic potential in Cr⁵¹ release assays using 70Z/3 cells as targets will be tested.

The established *in vivo* model will also be used to explore the induction of protective immunity. In particular, adoptive transfer experiments will be undertaken to determine if CD4⁺ cells can confer immunity and if so if these cells are CD4⁺ CTL or NKT cells. These cells will be isolated and cloned *in vitro* after they arise in the mice to establish their growth properties and mechanism of cytotoxicity. By comparing the induction of immunity to AML to our current ALL model, we will study why some cancers are more immunogenic than others.

With this background knowledge we will initiate IL-12 transduction experiments using established human leukemia cell lines representing different classes of leukemia. These include K562, CES1, OCIAML1,

OCIAML2, Jurkat, Raji. The Medin lab has already shown that both K562 and Jurkat are readily infected with LV vectors in past experiments. The cell lines will be transduced in bulk culture after which clones will be selected by limit dilution. The clones will be examined for cell proliferation by thymidine incorporation assays and for IL-12 production by ELISA. The stability of the IL-12 production will be determined after extended cell culture times as well as after several freeze/thaw cycles. Repeat Southern blot analysis will be used to determine vector copy number and stability as well.

Human *in vitro* assay. Established cell lines and primary samples will also be used to develop *in vitro* assays similar to those underway in the murine system. *In vitro* culture conditions that support human DCs and T cell subsets have been developed. Using these as a starting point the effects of IL-12 producing cell lines and primary samples in short term assays will be monitored. We will establish the ability of IL-12 producing cell lines and primary leukemia samples to influence the maturation of human DCs in the presence and absence of selected T cell subsets. We will monitor cell surface markers such as CD80 for DC maturation and IFN γ secretion for induction of Th1 responses. If evidence of an IR is detected, CD4 and CD8 subsets will be isolated and tested for anti-leukemia cytotoxicity and specificity.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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Sequences

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pHR Backbone

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SEQ ID NO: 2 cPPT seq

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SEQ ID NO: 3

Woodchuck Hepatitis Virus wpre

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SEQ ID NO:4

pORF-hIL-12 sequence (5048 bp).
hIL-12 open reading frame in **bold**.
Elastin linker is underlined.

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SEQ ID NO: 5
pORF-mIL-12 (p35p40) sequence (4846 bp).
mIL-12 open reading frame in bold.
Elastin linker sequence is undelined.

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Ile Gly Lys Leu Leu Ser Ser Tyr Leu Gln Lys Lys Ser Asp Val Glu
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Asp His Ser Val His Leu Leu Phe Ser Ala Asn Arg Trp Glu Gln Val
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Pro Leu Ile Lys Glu Lys Leu Ser Gln Gly Val Thr Leu Val Val Asp
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Arg Tyr Ala Phe Ser Gly Val Ala Phe Thr Gly Ala Lys Glu Asn Phe
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 50 55 60
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 65 70 75 80
 Pro Leu Ile Lys Glu Lys Leu Ser Gln Gly Val Thr Leu Val Val Asp
 85 90 95
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 Ser Leu Asp Trp Cys Lys Gln Pro Asp Val Gly Leu Pro Lys Pro Asp
 115 120 125
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 130 135 140

Ala Phe Gly His Glu Arg Tyr Glu Asn Gly Ala Phe Gln Glu Arg Ala
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Leu Arg Cys Phe His Gln Leu Met Lys Asp Thr Thr Leu Asn Trp Lys
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 35 40 45

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195 200 205

Leu Gly Glu Leu Trp Lys
210

Claims:

1. A composition comprising:
 - a. a lentiviral vector;
 - b. an IL-12 expression cassette.
2. The composition of claim 1, wherein the IL-12 expression cassette comprises a polynucleotide optionally encoding a p35 polypeptide and a polynucleotide encoding a p40 polypeptide; or a polynucleotide encoding an IL-12 fusion polypeptide.
3. The composition of claim 2 wherein the IL-12 fusion polypeptide has at least 70% sequence identity to SEQ ID NO: 4 and binds an IL-12 receptor.
4. The composition of any one of claims 1 to 3, wherein the lentiviral vector optionally comprises one or more of a: 5'-Long terminal repeat (LTR), HIV signal sequence, HIV Psi signal 5'-splice site (SD), delta-GAG element, Rev Responsive Element (RRE), 3'-splice site (SA), Elongation factor (EF) 1-alpha promoter and 3'-Self inactivating LTR (SIN-LTR).
5. The composition of any one of claims 1 to 4, wherein the lentiviral vector comprises a central polypurine tract optionally SEQ ID NO:2 and/or a woodchuck hepatitis virus post-transcriptional regulatory element, optionally SEQ ID NO:3; or a sequence having at least 70% sequence identity to SEQ ID NO:2 and/or SEQ ID NO:3.
6. The composition of claim 1 to 4 wherein the lentiviral vector comprises a pHR' backbone.
7. The composition of any one of claims 1 to 6, wherein the lentiviral vector is a clinical grade vector.

8. The composition of any one of claims 1 to 7, further comprising an activator polynucleotide encoding a polypeptide that converts a prodrug to a drug, optionally a modified tmpk polynucleotide.
9. The composition of claim 8, wherein the activator polynucleotide comprises a tmpk polynucleotide with at least 80% sequence identity to a modified tmpk polynucleotide described herein.
10. The composition of any one of claims 1 to 9, further comprising a detection cassette.
11. The composition of claim 10, wherein the detection cassette comprises a CD19, truncated CD19, CD20, human CD24, murine HSA, human CD25 (huCD25), a truncated form of low affinity nerve growth factor receptor (LNGFR), truncated CD34, eGFP, eYFP, or any other fluorescent protein or erythropoietin receptor (EpoR) polynucleotide; or a polynucleotide with at least 70% sequence identity to said polynucleotide.
12. The composition of any one of claims 1 to 11 further comprising an immune modulatory cassette.
13. The composition of claim 12, wherein the immune modulatory cassette comprises a polynucleotide that encodes a polypeptide that modulates an immune cell, optionally a dendritic cell or a T cell, optionally a CD4+ T cell, optionally CD40L, IL-7, or IL-15.
14. The composition of any one of claims 1 to 13, wherein the composition is a pharmaceutical composition and further comprises a pharmaceutically acceptable carrier.
15. A vector construct comprising:
 - a. a lentiviral vector;
 - b. an IL-12 expression cassette.

16. An isolated virus comprising the vector construct of claim 15 or the composition of claim 1 to 14.
17. An isolated cell secreting IL-12 at the or above the threshold level, wherein the cell is optionally transduced with the composition of any one of claims 1 to 14, the vector construct of claim 15 or the isolated virus of claim 16 .
18. The isolated cell of claim 17 wherein the cell is a cancer cell, optionally an established cell line, optionally a primary cancer cell, optionally a cancer cell derived from a subject.
19. The isolated cell of claim 18 wherein the cancer cell is a leukemic cell, optionally an ALL cell or a CLL cell.
20. The isolated cell of any one of claims 17 to 19 wherein the threshold level is at least 1500 pg/mL/10⁶ cells/2hrs of IL-12, optionally at least 1500 pg/mL/10⁶ cells/2hrs, 1500-2500 pg/mL/10⁶ cells/2hrs, 2500-5000 pg/mL/10⁶ cells/2hrs, 5000-7500 pg/mL/10⁶ cells/2hrs, 7500-10000 pg/mL/10⁶ cells/2hrs, 10000-12500 pg/mL/10⁶ cells/2hrs, 12500-15000 pg/mL/10⁶ cells/2hrs, 15000-17500 pg/mL/10⁶ cells/2hrs, 17500-20000 pg/mL/10⁶ cells/2hrs or at least 20000 pg/mL/10⁶ cells/2hrs of IL-12
21. A population of cells comprising isolated cells of any one of claims 17 to 20 wherein the population of cells optionally comprises at least 0.1 to 1% IL-12 producing leukemic cells, optionally about 0.5%, about 1%, about 1-5%, 5-10%, 10-20% or more IL-12 producing leukemic cells, and wherein the population of cells secretes above the threshold level optionally the threshold level necessary to induce or enhance a CD4+ T cell dependent immune response, optionally at least 1500 pg/mL/10⁶ cells/2hrs, 1500-2500 pg/mL/10⁶ cells/2hrs, 2500-5000 pg/mL/10⁶ cells/2hrs, 5000-7500 pg/mL/10⁶ cells/2hrs, 7500-10000 pg/mL/10⁶ cells/2hrs, 10000-12500 pg/mL/10⁶ cells/2hrs, 12500-15000

pg/mL/ 10^6 cells/2hrs, 15000-17500 pg/mL/ 10^6 cells/2hrs, 17500-20000 pg/mL/ 10^6 cells/2hrs or at least 20000 pg/mL/ 10^6 cells/2hrs of IL-12.

22. The population of cells of claim 21 wherein the population of cells is derived from a clone that secretes IL-12 above the threshold level optionally at least 1500 pg/mL/ 10^6 cells/2hrs of IL-12.
23. A composition comprising the isolated virus, cell or population of cells of any one of claims 16 to 22.
24. A method of expressing IL-12 in a cell, optionally a cancer cell comprising contacting the cell with the composition of any one of claims 1 to 14, the vector construct of claim 15 or the isolated virus of claim 16 under conditions that permit transduction of the cell, thereby providing a transduced cell, optionally wherein the IL-12 is secreted.
25. The method of claim 24, further comprising a step of isolating the transduced cell or isolating a population of cells comprising the transduced cell.
26. The method of claim 24 or 25, further comprising:
 - a. growth arresting the transduced cell, the population of cells or composition; and
 - b. introducing the transduced cell, population of cells and/or composition in a subject.
27. A method of reducing the number of tumor cells or cancer burden in a subject in need thereof comprising administering to the subject the isolated virus of claim 16, the transduced cell of any one of claims 17 to 20, the population of cells of claim 21 or 22, or the composition of claim 23.

28. A method of treating a subject with cancer or an increased risk of cancer comprising administering the virus of claim 16, the transduced cell of any one of claims 17 to 20, the population of cells of claim 21 or 22, or the composition of claim 23.
29. The method of claim 27 or 28 further comprising monitoring cancer progression.
30. The method of any one of claim 27 to 29, wherein the cancer is leukemia, optionally ALL, AML, CML or CLL.
31. A method of inducing or enhancing an immune response in a subject optionally with cancer or an increased risk of cancer comprising administering the isolated virus of claim 16, the transduced cell of any one of claims 17 to 20, the population of cells of claim 21 or 22, or the composition of claim 23.
32. A method of inducing or enhancing a memory immune response in a subject, optionally with cancer or an increased risk of cancer, comprising administering the isolated virus of claim 16, the transduced cell of any one of claims 17 to 20, the population of cells of claim 21 or 22, or the composition of claim 23.
33. The method of claim 31 or 32 wherein the immune response comprises a CD4+ mediated immune response.
34. The method of any one of claims 27 to 33 wherein the transduced cell is growth arrested prior to administering to the subject.
35. The method of any one of claims 27 to 33 wherein the transduced cell is irradiated prior to administering to the subject.
36. A method of delivering IL-12 to a subject, optionally with cancer or an increased risk or cancer, optionally, for enhancing cancer treatment comprising:

- a. generating an IL-12 secreting cell wherein IL-12 secreted per cell is above a threshold level; and
 - b. introducing an effective number of the generated IL-12 secreting cells to the subject.
37. A method of sustaining IFN γ levels induced by IL-12 in a host comprising:
- a. generating an IL-12 secreting cell wherein IL-12 secreted per cell is above a threshold level; and
 - b. introducing an effective number of the generated IL-12 secreting cells to the patient.
38. The method of claim 36 or 37 wherein the threshold level of IL-12 secreted is at least 1.5 fg/ml/cell/2hrs.
39. The method of claim 36 wherein the threshold level of IL-12 secreted is at least 1.5 pg/ml cells/2hrs.
40. The method of claim 36 or 37, wherein the IL-12 secreting cell is generated by contacting the cell with a composition comprising a lentiviral delivery vector and an IL-12 expression cassette.
41. The method of any one of claims 36 to 40, wherein the cell is optionally a cancer cell, optionally derived from the subject with cancer.
42. The method of any one of claims 36 to 41 wherein the cells are introduced by IP injection, subcutaneously or intradermally.
43. The method of any one of claims 36 to 42, wherein the cancer is a leukemia, optionally ALL, AML, CML or CLL.
44. The method of any one of claims 36 to 423, wherein the IL-12 secreting cell is growth arrested prior to introducing to the subject.

45. The method of claim 31 or 32 wherein the response is initiated against a leukemia.
46. The method of claim 31 or 32 wherein the response is initiated substantially free of inducing or enhancing of a CD8⁺ T cell-dependent immune response.
47. The method of claim 31 or 32 wherein the immune response leads to long-term immune memory.
48. The method of claim 31 or 32 wherein the immune response does not induce or enhance antagonistic cytokines.
49. The method of any one of claims 24 to 48 wherein the level of IL-12 produced is above a threshold level that enhances dendritic cell maturation and/or antigen presentation.
50. Use of an isolated virus of claim 16, the transduced cell of any one of claims 17 to 20, the population of cells of claim 21 or 22, or the composition of claim 23 for reducing the number of tumor cells or cancer burden in a subject in need thereof.
51. Use of the virus of claim 16, the transduced cell of any one of claims 17 to 20, the population of cells of claim 21 or 22, or the composition of claim 23 for treating a subject with cancer.
52. The use of claim 50 or 51, wherein the cancer is leukemia, optionally ALL, AML, CML or CLL.
53. Use of the isolated virus of claim 16, the transduced cell of any one of claims 17 to 20, the population of cells of claim 21 or 22, or the composition of claim 23 for inducing or enhancing an immune response in a subject.
54. Use of the isolated virus of claim 16, the transduced cell of any one of claims 17 to 20, the population of cells of claim 21 or 22, or the

composition of claim 23 for inducing or enhancing a memory immune response in a subject.

55. The use of claim 53 or 54 wherein the immune response comprises a CD4+ T cell mediated immune response.

56. The use of any one of claims 50 to 55 wherein the transduced cell is growth arrested.

57. The use of any one of claims 50 to 55 wherein the transduced cell is irradiated.

58. Use of an IL-12 secreting cell for delivering IL-12 to a subject, optionally with cancer or an increased risk of cancer optionally for enhancing cancer treatment:

- a. generating an IL-12 secreting cell wherein IL-12 secreted per cell is above a threshold level; and
- b. isolating an effective number of the generated IL-12 secreting cells for introduction to the subject.

59. The use of claim 58 wherein the threshold level of IL-12 secreted is at least 1.5 fg/ml/cell/2hrs.

60. The use of claim 58 wherein the threshold level of IL-12 secreted is at least 1.5 pg/ml cells/2hrs.

61. The use of claim 58, wherein the IL-12 secreting cell is generated by contacting the cell with a composition comprising a lentiviral delivery vector and an IL-12 expression cassette.

62. The use of any one of claims 50 to 61, wherein the cell is optionally a cancer cell, optionally derived from the subject with cancer.

63. The use of any one of claims 50 to 61 wherein the cells are introduced by IP injection, subcutaneously or intradermally.

64. The use of any one of claims 50 to 61, wherein the cancer is a leukemia, optionally ALL AML, CML or CLL.
65. The use of any one of claims 50 to 61, wherein the IL-12 secreting cell is growth arrested prior to introducing to the subject.
66. The use of claim 53 or 54 wherein the response is initiated against a leukemia.
67. The method of claim 53 or 54 wherein the response is initiated substantially free of inducing or enhancing of a CD8⁺ T cell-dependent immune response.
68. The use of claim 53 or 54 wherein the immune response leads to long-term immune memory.
69. The use of claim 53 or 54 wherein the immune response does not induce or enhance antagonistic cytokines.
70. The use of any one of claims 50 to 69 wherein the level of IL-12 produced is above a threshold level that enhances dendritic cell maturation and/or antigen presentation.
71. Use of a composition of any one of claims 1 to 23, the vector construct of claim 24 or the virus of claim 25, or the transduced cell or the population of cells of claim 26, for treating a subject in need thereof, optionally a subject with cancer or an increased risk of developing cancer.
72. The method of any one of claims 24 to 49, wherein the number of cells administered ranges from 10⁵ cells to 10⁹ cells, optionally about 10⁵, about 10⁶ cells, about 10⁷ cells, about 10⁸ cells, or about 10⁹ cells.
73. The use of any one of claims 50 to 71, wherein the population of cells administered ranges from 10⁵ cells to 10⁹ cells, optionally about 10⁵ cells, about 10⁶ cells, about 10⁷, cells, about 10⁸ cells, or about 10⁹

cells.

FIGURE 1A

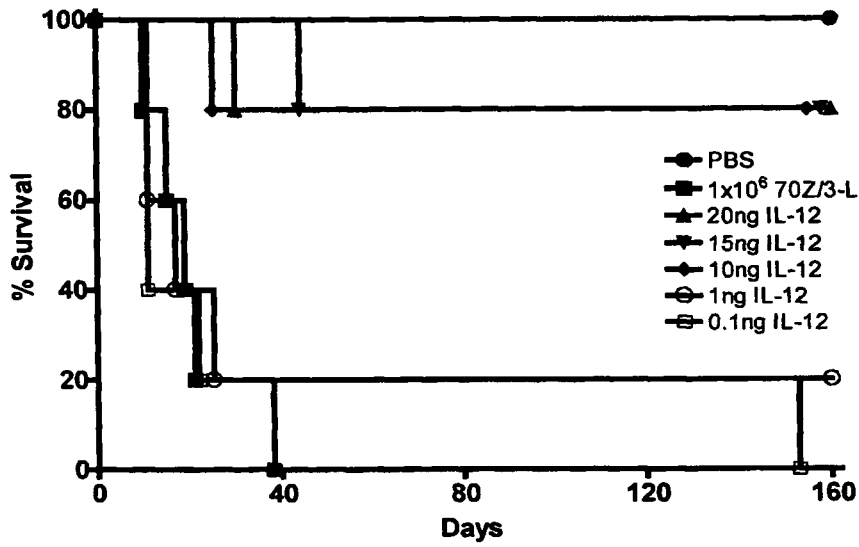


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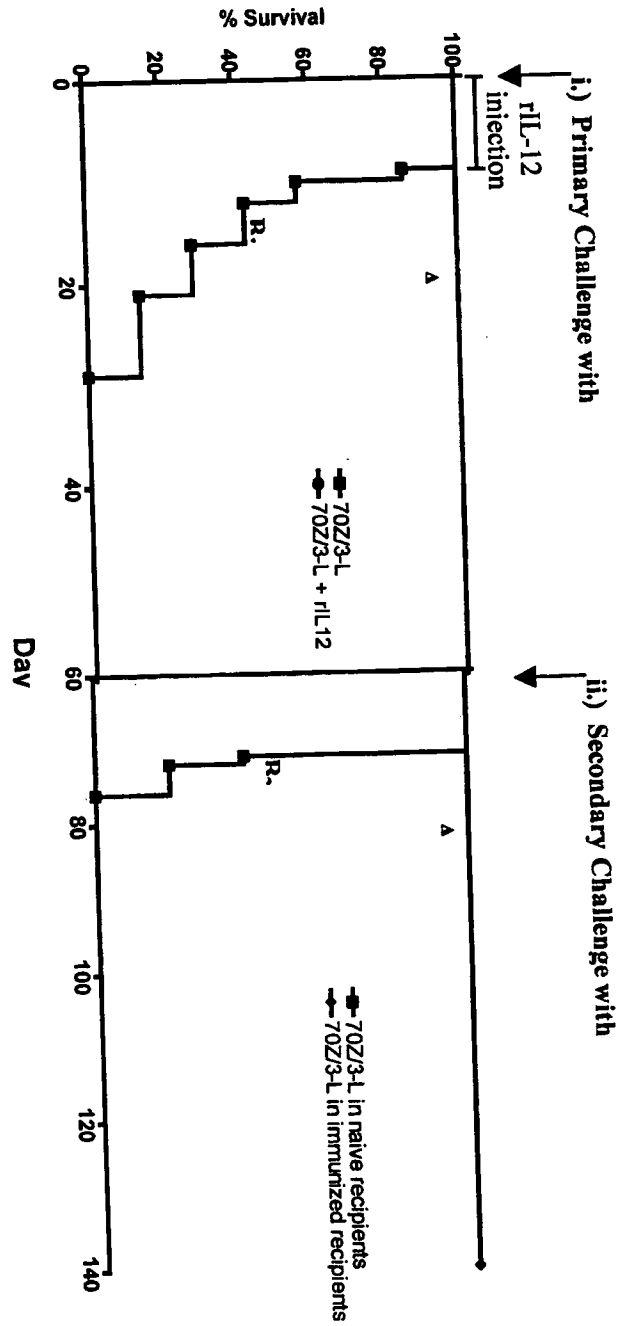


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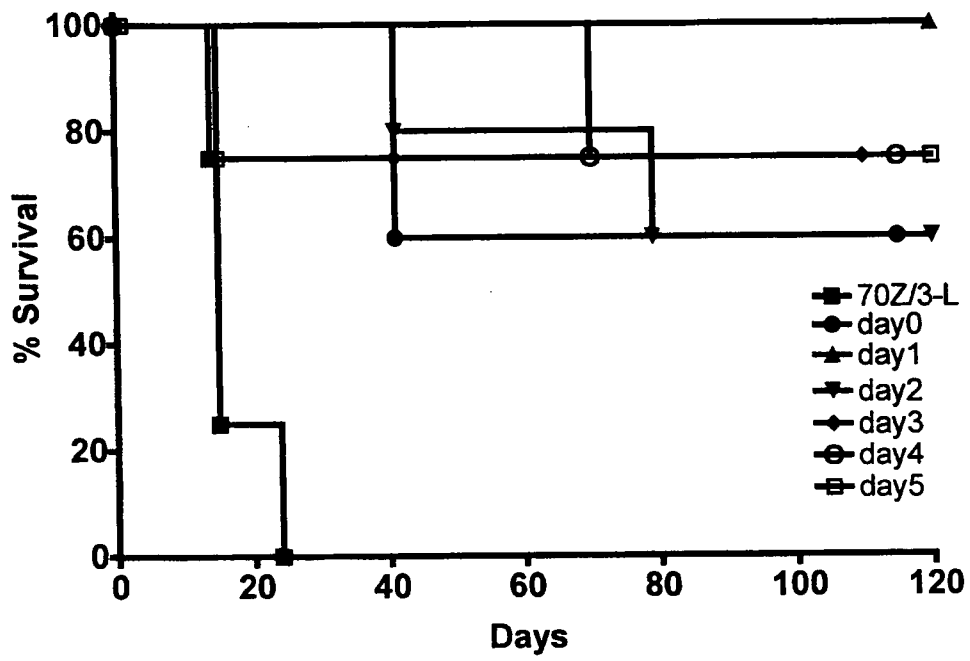


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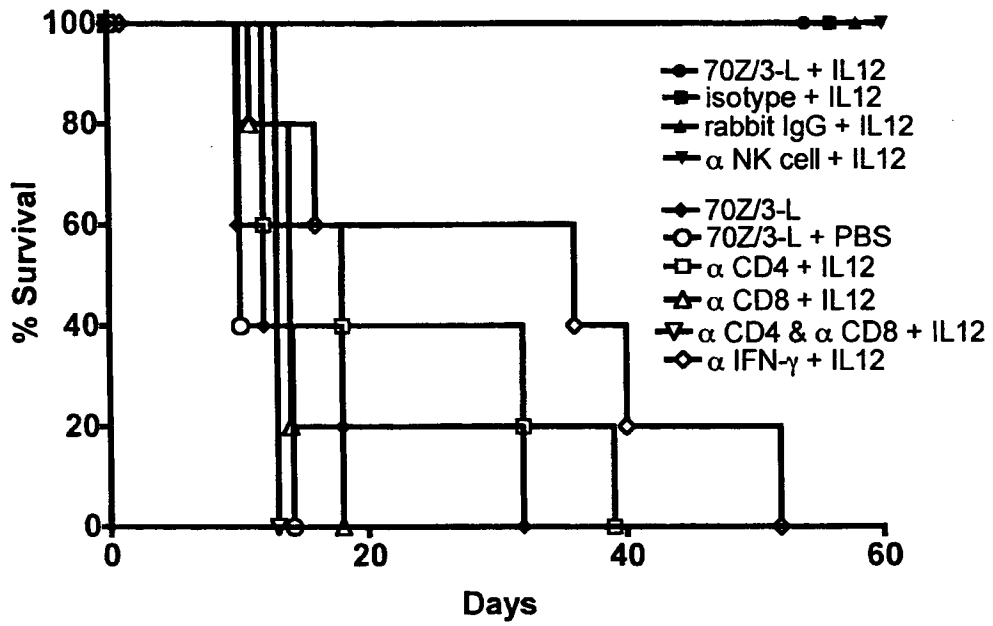


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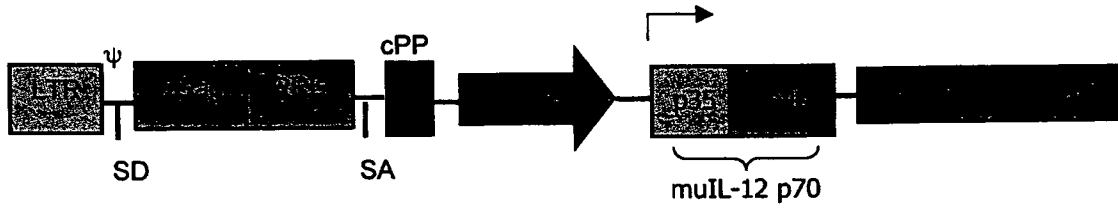


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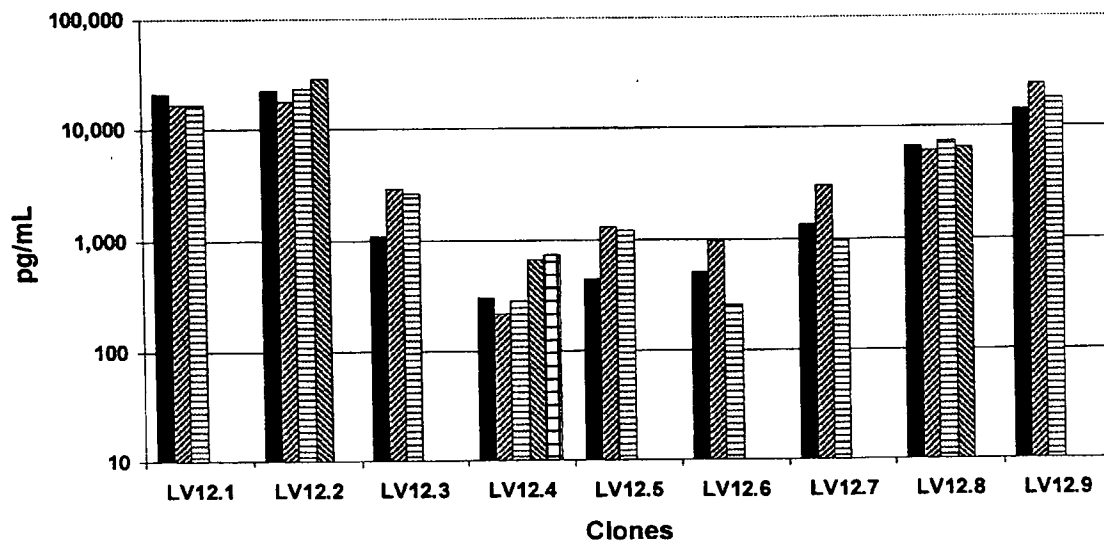


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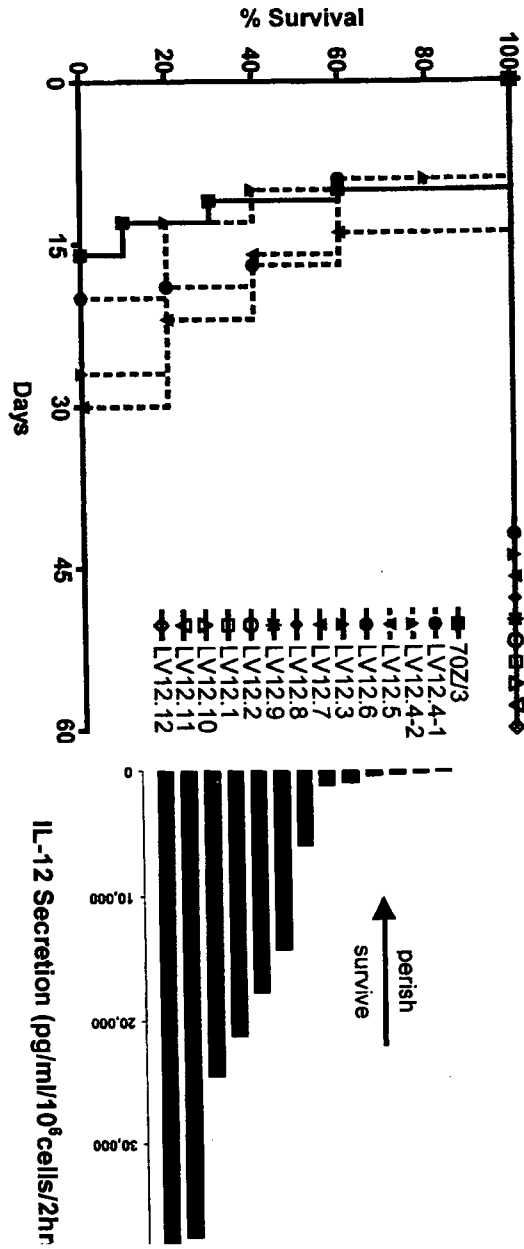
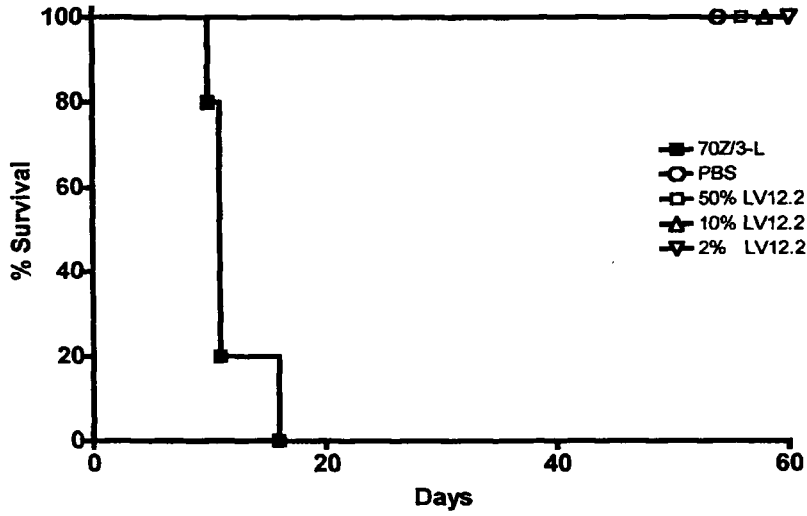


FIGURE 4

a.)



b.)

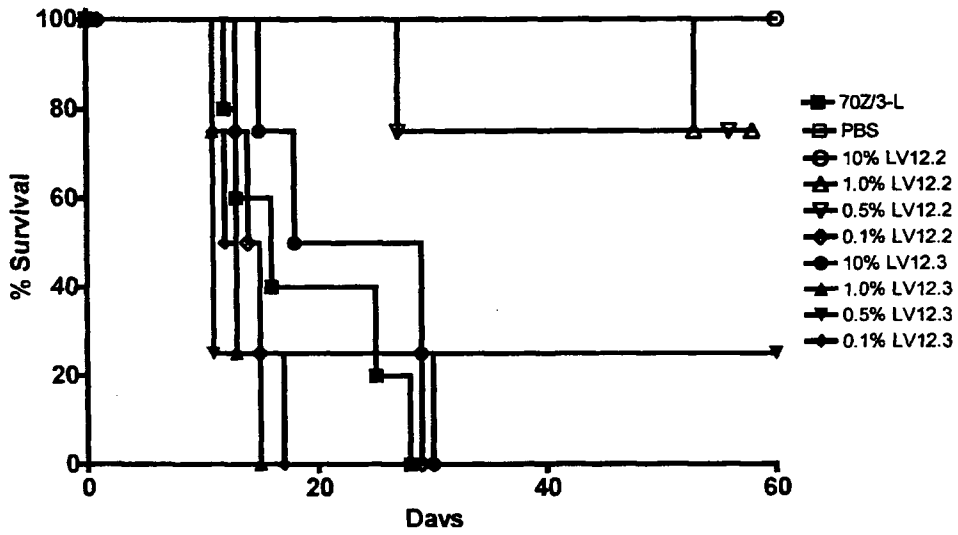


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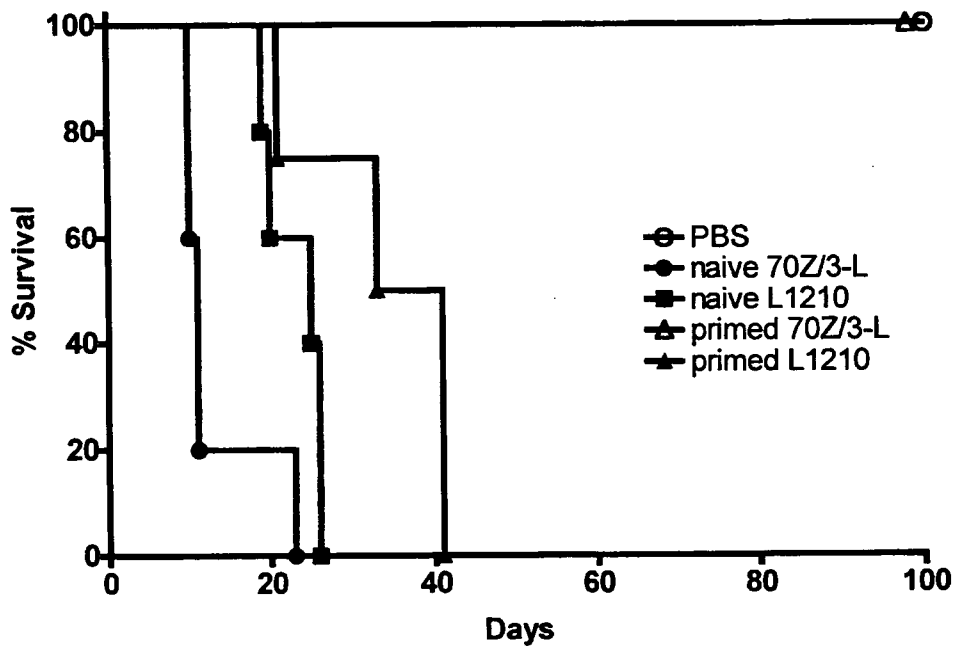


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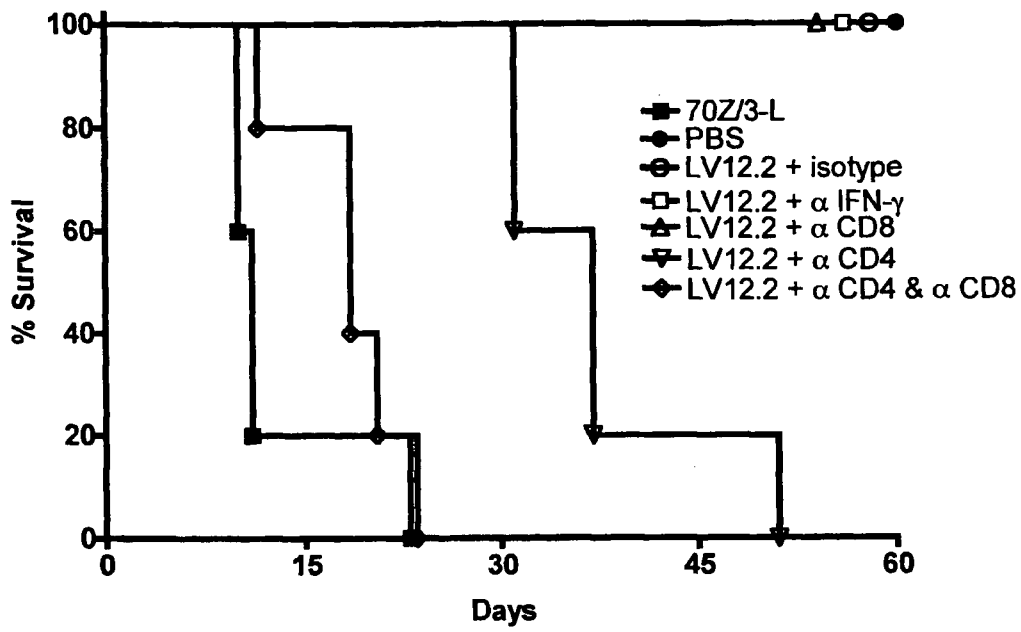


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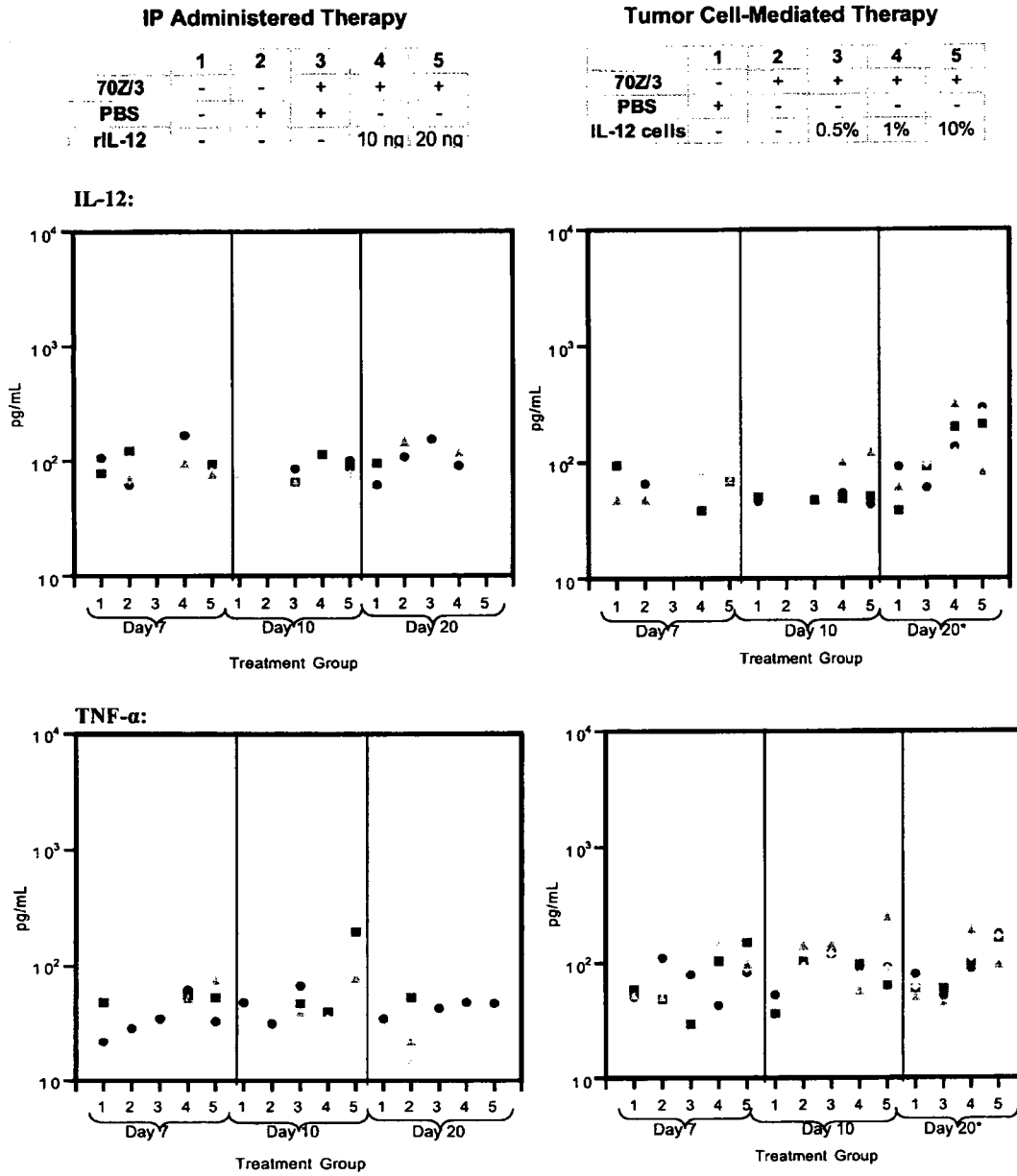


FIGURE 7 (CONTINUED)

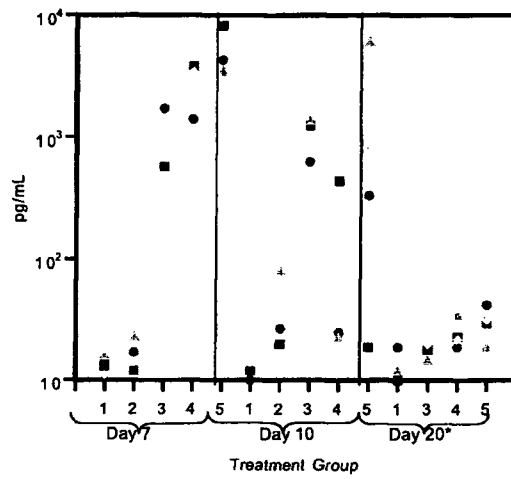
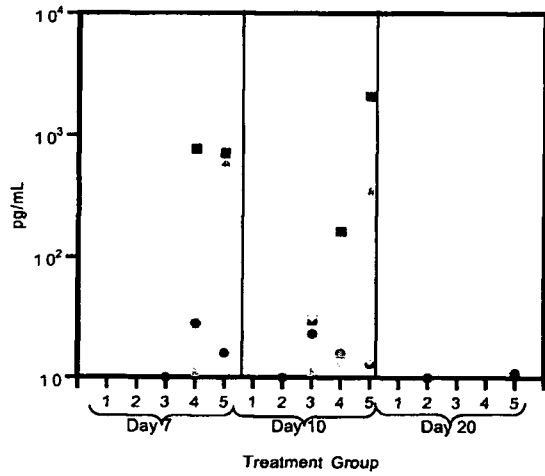
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	1	2	3	4	5
70Z/3	-	-	+	+	+
PBS	-	+	+	-	-
rIL-12	-	-	-	10 ng	20 ng

Tumor Cell-Mediated Therapy

	1	2	3	4	5
70Z/3	-	+	+	+	+
PBS	+	-	-	-	-
IL-12 cells	-	-	0.5%	1%	10%

IFN- γ :



MCP-1:

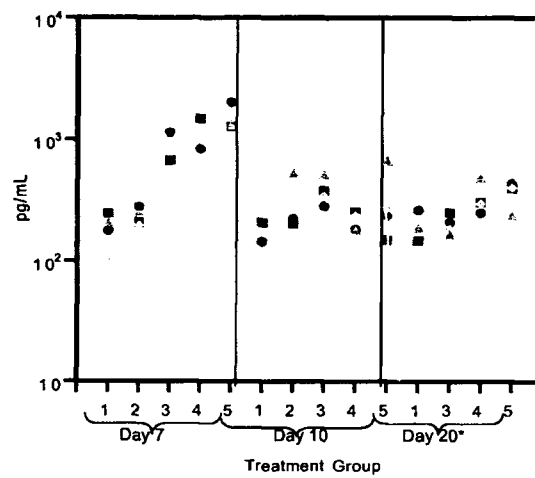
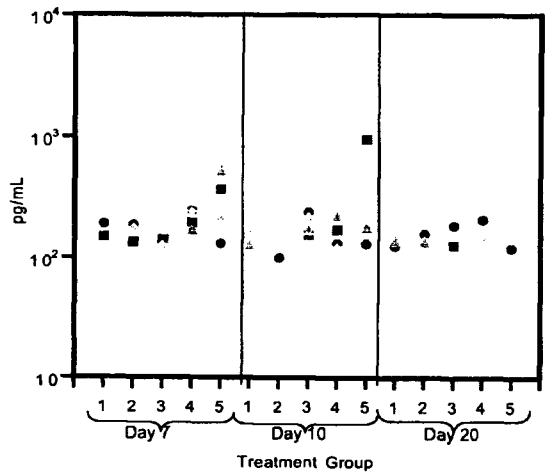


FIGURE 7 (CONTINUED)

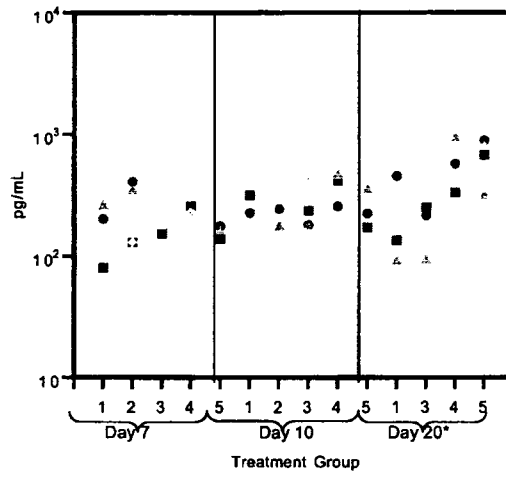
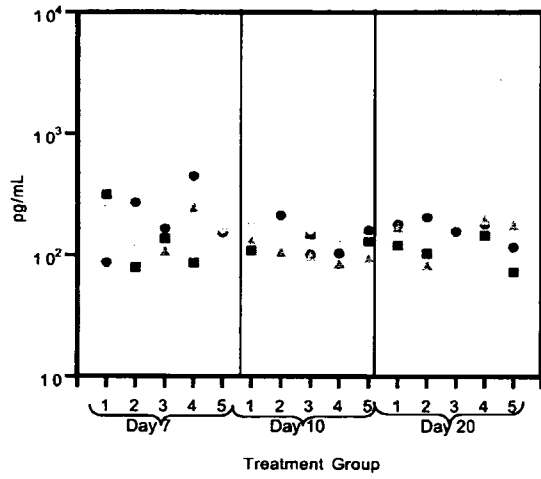
IP Administered Therapy

	1	2	3	4	5
70Z/3	-	-	+	+	+
PBS	-	+	+	-	-
rIL-12	-	-	-	10 ng	20 ng

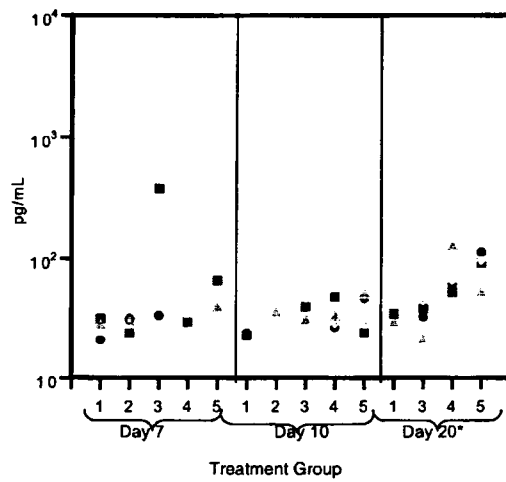
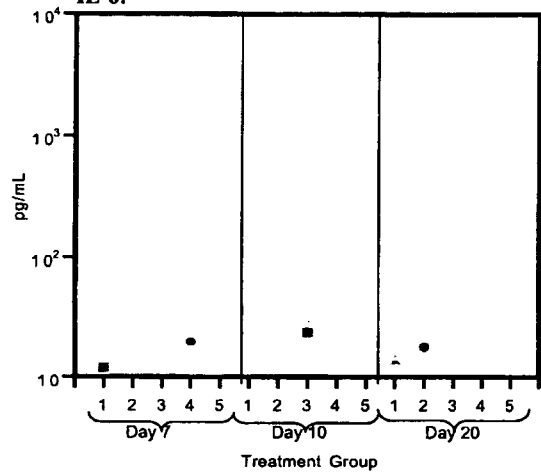
Tumor Cell-Mediated Therapy

	1	2	3	4	5
70Z/3	-	+	+	+	+
PBS	+	-	-	-	-
IL-12 cells	-	-	0.5%	1%	10%

IL-10:



IL-6:



INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/000849

<p>A. CLASSIFICATION OF SUBJECT MATTER</p> <p>IPC: <i>C12N 15/867</i> (2006.01) , <i>A61K 38/20</i> (2006.01) , <i>A61K 39/00</i> (2006.01) , <i>A61K 48/00</i> (2006.01) , <i>A61P 35/00</i> (2006.01) , <i>A61P 35/02</i> (2006.01) (more IPCs on the last page)</p> <p>According to International Patent Classification (IPC) or to both national classification and IPC</p>													
<p>B. FIELDS SEARCHED</p> <p>Minimum documentation searched (classification system followed by classification symbols)</p> <p>IPC: <i>C12N 15/867</i> (2006.01) , <i>A61K 38/20</i> (2006.01) , <i>A61K 39/00</i> (2006.01) , <i>A61K 48/00</i> (2006.01) , <i>A61P 35/00</i> (2006.01) , <i>A61P 35/02</i> (2006.01) (more IPCs on the last page)</p> <p>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched</p>													
<p>Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)</p> <p>Databases: Delphion, Scopus, Medline, Biosis, CAPlus</p> <p>Keywords: lentivirus, vector, IL-12, interleukin-12, cancer, tumour, tumor</p>													
<p>C. DOCUMENTS CONSIDERED TO BE RELEVANT</p> <table border="1" style="width:100%; border-collapse: collapse;"> <thead> <tr> <th style="width:10%;">Category*</th> <th style="width:60%;">Citation of document, with indication, where appropriate, of the relevant passages</th> <th style="width:30%;">Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td align="center">X</td> <td>CHEN X et al, "Alteration of T cell immunity by lentiviral transduction of human monocyte-derived dendritic cells", RETROVIROLOGY. 1 November 2004 (01-11-2004), Vol. 1, No. 1, pages 37-49. ISSN: 1742-4690. See whole document; particularly figure 3.</td> <td>1-8, 10, 12-73</td> </tr> <tr> <td align="center">X</td> <td>KUWATA T et al, "Construction of chimeric simian and human immunodeficiency viruses that produce interleukin 12", AIDS RESEARCH AND HUMAN RETROVIRUSES. 1 March 2000 (01-03-2000), Vol. 16, No. 5, pages 465-470. ISSN: 0889-2229. See whole document.</td> <td>1-7, 14-17, 20-26, 31-42, 46-49, 53-63, 65 and 67-73</td> </tr> <tr> <td align="center">X</td> <td>TAHARA H et al, "Effective eradication of established murine tumors with Il-12 gene therapy using a polycistronic retroviral vector" JOURNAL OF IMMUNOLOGY. 1995, Vol. 154, No. 12, pages 6466-6474. ISSN: 0022-1767 See whole document.</td> <td>17-60 and 62-73</td> </tr> </tbody> </table>		Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	CHEN X et al, "Alteration of T cell immunity by lentiviral transduction of human monocyte-derived dendritic cells", RETROVIROLOGY. 1 November 2004 (01-11-2004), Vol. 1, No. 1, pages 37-49. ISSN: 1742-4690. See whole document; particularly figure 3.	1-8, 10, 12-73	X	KUWATA T et al, "Construction of chimeric simian and human immunodeficiency viruses that produce interleukin 12", AIDS RESEARCH AND HUMAN RETROVIRUSES. 1 March 2000 (01-03-2000), Vol. 16, No. 5, pages 465-470. ISSN: 0889-2229. See whole document.	1-7, 14-17, 20-26, 31-42, 46-49, 53-63, 65 and 67-73	X	TAHARA H et al, "Effective eradication of established murine tumors with Il-12 gene therapy using a polycistronic retroviral vector" JOURNAL OF IMMUNOLOGY. 1995, Vol. 154, No. 12, pages 6466-6474. ISSN: 0022-1767 See whole document.	17-60 and 62-73
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<p><input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.</p>													
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>												
<p>Date of the actual completion of the international search</p> <p>28 July 2008 (28-07-2008)</p>	<p>Date of mailing of the international search report</p> <p>10 September 2008 (10-09-2008)</p>												
<p>Name and mailing address of the ISA/CA</p> <p>Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476</p>	<p>Authorized officer</p> <p>Kristoffer Wilde 819- 953-0551</p>												

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2008/000849**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

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

1. Claim Nos. : 24-49 and 72

because they relate to subject matter not required to be searched by this Authority, namely :

Although claims 24-49 and 72 are directed to methods of treatment of the human/animal body which this Authority is not obliged to search under Rule 39.1(iv) of the PCT, the search has been carried out based on the alleged effects of the compositions, constructs, cells or viruses referred to therein.

2. Claim Nos. :

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

3. Claim Nos. :

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

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

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

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

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

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

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

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

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

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/000849

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MILLER G et al, "Overexpression of interleukin-12 enables dendritic cells to activate NK cells and confer systemic antitumor immunity", THE FASEB JOURNAL. April 2003 (04-2003), Vol. 17, No. 6, pages 728-730. ISSN: 1530-6860. See whole document.	17, 20-60 and 62-73
X	MEKO J B et al, "High cytokine production and effective antitumor activity of a recombinant vaccinia virus encoding murine interleukin 12", CANCER RESEARCH. 1 November 1995 (01-11-1995), Vol. 55, pages 4765-4770. ISSN: 1557-3265. See whole document.	17-60 and 62-73
A	SUZUKI T et al, "Vaccination of dendritic cells loaded with interleukin-12-secreting cancer cells augments <i>in vivo</i> antitumor immunity: characteristics of syngeneic and allogeneic antigen-presenting cell cancer hybrid cells", CLINICAL CANCER RESEARCH. 1 January 2005 (01-01-2005), Vol. 11, No. 1, pages 58-66. ISSN: 1557-3265.	
A	ZITVOGEL et al, "Construction and characterization of retroviral vectors expressing biologically active human interleukin-12", HUMAN GENE THERAPY. December 1994 (12-1994), Vol 5, pages 1493-1506. ISSN: 1043-0342.	
A	ROBERTSON M J et al, "Interleukin 12: basic biology and potential applications in cancer treatment", THE ONCOLOGIST. 1 February 1996 (01-02-1996), Vol. 1, No. 1 & 2, pages 88-97. ISSN: 1549-490X.	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/000849

A61P 37/04 (2006.01) , *C07K 14/54* (2006.01) , *C12N 15/24* (2006.01) , *C12N 5/10* (2006.01) ,
C12P 21/02 (2006.01)