Abstract:

Thereafter, Western polynucleotides are designed to activate T cells and for adoptive cell transfer of the constitutively activated T cells in the treatment of cancer.

Title: CONSTITUTIVELY ACTIVATED T CELLS FOR USE IN ADOPTIVE CELL THERAPY

Abstract: Polynucleotides encoding chimeric proteins comprising constitutively active TLR4, a constitutively active member of the T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family or a membrane-bound cytokine, or combinations thereof, are provided. The polynucleotides are useful for transfecting T cells and thereby propagate them without the need for soluble cytokines, for constitutively activate the T cells and for adoptive cell transfer of the constitutively activated T cells in the treatment of cancer.
CONSTITUTIVELY ACTIVATED T CELLS FOR USE IN ADOPTIVE CELL THERAPY

TECHNICAL FIELD
[0001] The present invention relates in general to central nervous system (CNS) injuries and, in particular, to sub-populations of human monocytes useful in the treatment of CNS injuries, methods for isolation of these sub-populations and treatment of patients suffering from spinal cord injury.

[0002] The present invention relates to the field of adoptive cell therapy and the use of activated T cells expressing constitutively active (ca) CD40 molecules, caTLR4 molecules, membrane-bound cytokines and combinations thereof.

BACKGROUND ART
[0003] The discovery that tumors express a wide variety of proteins that can be recognized by the immune system, have led to the development of several immunotherapeutic approaches, some of which are already established as a central component of different cancer treatment regimens. Cellular approaches are divided into active vaccination and adoptive cell therapy. Active immunotherapy, using antigen loaded dendritic cells (DCs), was initially considered to be the most promising approach, but the objective clinical responses to antigen loaded DC remain anecdotal with objective response rates not exceeding 5-10%. Factors that may account for these disappointing outcomes may include low affinity and avidity of tumor antigens, improper DC activation and the suppressive environment at tumor sites. Passive immunization through the adoptive transfer of a large number of tumor-reactive lymphocytes, known as adoptive cell therapy (ACT), is an alternative therapeutic approach; ACT involves the administration of large numbers of highly selective cells with high avidity for tumor antigens. These T cells can be programmed and activated ex vivo to exhibit antitumor effector functions. Furthermore, T cell infusion may be preceded by 'conditioning' of the patient with lymphodepleting chemotherapy or total body irradiation, which enables the diminution of immunosuppressive cell types/factors followed by the infusion of tumor-specific T cells.

[0004] Several basic approaches for the derivation, activation and expansion of functional anti-tumor effector T cells have been described in the last two decades. These include: autologous cells, such as tumor-infiltrating lymphocytes (TILs); T cells activated...
ex-vivo using autologous DCs, lymphocytes, artificial antigen-presenting cells (APCs) or beads coated with T cell ligands and activating antibodies, or cells isolated by virtue of capturing target cell membrane; allogeneic cells naturally expressing anti-host tumor T cell receptor (TCR); and non-tumor-specific autologous or allogeneic cells genetically reprogrammed or "redirected" to express tumor-reactive TCR or chimeric TCR molecules displaying antibody-like tumor recognition capacity known as "T-bodies". These approaches have given rise to numerous protocols for T cell preparation and immunization. A

ACT is currently the most effective treatment for patients with metastatic melanoma, and is extensively explored for the treatment of other human cancers. However, the encouraging clinical achievements of particular ACT procedures are confronted with major obstacles which limit the clinical benefit and broader application of this approach. Whereas some of the intrinsic difficulties are attributable to the particular method employed for isolation, propagation or generation of the effector lymphocytes, others, such as the exhaustion of the proliferative and survival potential of fully differentiated T cells, seem to be a more general phenomena related to the effector phenotype. Other difficulties arise from extrinsic suppressive mechanisms exerted at the tumor site, which are mediated either by direct cell-to-cell contact with tumor cells, stromal cells and regulatory T cells (Tregs), or by inhibitory cytokines such as TGF-β. As a result, the administered T cells exhibit decreased intratumoral persistence and impaired functionality, and often fall short from executing a detectable tumoricidal effect. Different strategies are currently pursued, both in the pre-clinical and in the clinical settings, to evade or subvert these mechanisms and augment the curative outcome of ACT. These include the administration of Th1-promoting cytokines such as interleukin (IL)-2, IL-12 and IL-15, inhibition of contact-dependent T cell suppression by CTLA-4 or PD-1, depletion or attenuation of Tregs, blockade of TGF-β, triggering costimulatory signaling pathways such as CD28, 4-IBB and OX40, introduction of anti-apoptotic genes (e.g. Bcl-2, Bcl-xL) and the addition of TLR agonists. However, these effectors are highly toxic and are associated with life threatening side effects. Selective potentiation of anti-tumor CTLs is currently a major focus of the immunotherapy research.
SUMMARY OF INVENTION

[0006] The present invention relates, in one aspect, to polynucleotides comprising a sequence encoding a chimeric protein comprising a cytoplasmic domain of a T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family that is linked to a heterologous polypeptide comprising at least one self-assembly domain, said chimeric peptide being linked to an integral membrane or a transmembrane oligopeptide that allows the anchorage of the chimeric polypeptide to the cell membrane.

[0007] In another aspect, the invention relates to polynucleotides comprising a sequence encoding a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through its amino terminus to an integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane, and said transmembrane oligopeptide, if existent, is linked, optionally via a bridge peptide, to a peptide tag or oligopeptide tag.

[0008] In an additional aspect the invention relates to polynucleotides comprising a sequence encoding a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA.

[0009] In certain embodiments, the polynucleotide encoding a chimeric protein comprising a cytoplasmic domain of a T cell costimulatory receptor of the TNFR family or the chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 is each linked to an integral membrane or a transmembrane oligopeptide that allows the anchorage of the chimeric polypeptide to the cell membrane, wherein said transmembrane oligopeptide is linked via a flexible bridge peptide to the carboxyl terminus of a cytokine.

[0010] In still other aspects, the present invention relates to expressions vectors and compositions comprising the above-identified polynucleotides or any combination thereof, and to activated T cells expressing at least one chimeric protein encoded by these polynucleotides.

[0011] In yet an additional aspect, the present invention relates to methods for treating cancer, comprising administering to a cancer patient a therapeutically effective amount of activated tumor specific T cells expressing the chimeric proteins of the present invention;
and to methods for preparing activated T cells comprising transfecting said T cells with a polynucleotides or expression vectors as defined herein, whereby the polynucleotide is expressed within said T cells, thus obtaining the activated T cells.

[0012] In still an additional aspect, the present invention provides a method for propagating T cells, comprising transfecting said T cells with a polynucleotide comprising a sequence encoding a chimeric protein comprising IL-2 linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the IL-2 molecule to a cell membrane.

BRIEF DESCRIPTION OF DRAWINGS

[0013] Fig. 1 depicts flow cytometry analysis showing expression of GFP in human CD8 T cells following mRNA transfection. Fluorescence was analyzed 24 hours after transfection against the same cells undergoing an identical treatment but receiving no RNA (filled black histogram). MI, Marker i: the range used to calculate specific, relative to total, fluorescence and MFI (mean fluorescence intensity).

[0014] Figs. 2A-B depict schemes of the basic genetic construct (A) and the resulting MHC-I molecules at the cell surface (B). Abbreviations: pr, promoter; lead, leader peptide; p, antigenic peptide; li, linker; br, bridge. Major restriction sites are indicated.

[0015] Fig. 3 shows a bar graph demonstrating that human peripheral blood lymphocyte (PBL)-derived CD4 and CD8 T cells produce interferon-γ (IFN-γ) following transfection with 5 μg caTLR4- (but not caTLR2- or EGFP-) encoding mRNA. NT, non-treated; LPS, lipopolysaccharide; Pam3, Pam3CysSK4;

[0016] Figs. 4A-B demonstrates that as little as 1 μg caTLR4 mRNA activates human CD4 (A) and CD8 (B) T cells derived from PBLs of healthy HLA-A2+ (A2+) and HLA-A2- (A2-) donors. Transfection of either peptide-p2m-TLR4 (construct 500) or β2m-TLR4 only (construct 931) were performed with 1 or 5 μg in-vitro-transcribed mRNA. Non-treated cells or cells similarly transfected with EGFP-encoding mRNA (GFP) served as a negative control, and cells transfected with EGFP mRNA and treated with LPS (1 μg/ml) served as a reference.

[0017] Figs. 5A-B show a flow cytometry analysis for the expression of CD25 by the tumor specific CTL clone, 1C9, (A) and the expression of CD69 by the primary TIL 431-4 (B), following transfection of 5μg mRNA encoding either p2m-HLA-A2 (construct 541),
caTLR4 (construct 931) or GFP (construct 540; irrelevant RNA). GFP served as an indicator for transfection efficiency (upper panel). NKG2D, an activating receptor that is expressed by T cells, as well as by natural killer (NK) cells and macrophages; CD137, a lymphocyte activation marker also known as 4-IBB.

[0018] Figs. 6A-C depict the design of oligomeric derivatives of hCD40. (A) The basic scheme of the genetic construct. (B) The expected organization of the GCN4 coiled coil motifs. (C) The expected oligomerization of the encoded polypeptides.

[0019] Fig. 7 depicts a scheme of the chimeric gene encoding for single chain IL-12. Pr, Promoter; Spe I, Xma I, Xho I and Not I, restriction sites.

[0020] Fig. 8 shows CFSE dilution assay for cell proliferation. CD8 T cells were prepared from PBLs of a healthy donor, grown for 4 days in OKT3 and IL-2 and separated to CD8 and CD4 T cells. Following 24 h rest were transfected or treated as indicated. Results are presented as % of live cells with decreased CFSE staining relative to the initial histogram.

[0021] Fig. 9 shows that there is functional synergy between genetic adjuvants. Melanoma TILs, grown the presence of 2,000 U/ml IL-2 for 4 days, were electroporated with 10 \( \mu \)g each mRNA. 24 hours post-transfection growth medium was collected for IFN-\( \gamma \) ELISA. No, non-treated cells; N.R., irrelevant mRNA. GFP mRNA served as an additional negative control and as a reference for transfection yield.

[0022] Figs. 10A-B show restoration of TIL-425 functional activity by the genetic adjuvants. Anti-melanoma CD8 TILs were transfected with mRNA encoding caCD40, memIL\( \frac{3}{4} \) caTLR4, their indicated combinations and an irrelevant mRNA as a negative control. 3 days post transfection the cells were incubated with relevant M427 melanoma (A) or irrelevant M171 melanoma (B). Flow cytometry analysis for IFN-\( \gamma \) (intracellular staining, upper panels) and cytolysis-associated degranulation (CD107, lower panels).

**DETAILED DESCRIPTION OF THE INVENTION**

[0023] As mentioned above, passive immunization through the adoptive transfer of a large number of tumor-reactive lymphocytes, known as adoptive cell therapy (ACT), is one of the most promising approaches being developed for treating cancer. However, a network of suppressive mechanisms operating at the tumor site, T cell exhaustion, down regulated effector mechanisms and low persistence of the transferred cells are critical factors which limit clinical efficacy and broader use of ACT. The present invention is based on a novel
genetic approach designed to overcome these complications and maximize the curative potential of tumor-reactive T cells in ACT.

[0024] The central components of this approach are based on the concept of severing T cell activation from the ligand-dependent activation of T cell activating factors by expressing in the T cells constitutively active factors; and on the concept of confining the activity of T cell promoting cytokines, which are naturally secreted from the cells, preventing their undesired consumption by competing cells (mainly Tregs) and coupling their activity to that of other T cell activating factors, by expressing these cytokines in T cells as integral membranal proteins.

[0025] One component of our approach is based on the fact that cellular activation of T cells, their differentiation, survival, resistance to Treg-mediated suppression and anti-tumor activity are influenced by members of the tumor necrosis factor receptor (TNFR) family, which attract great attention in tumor immunotherapy in recent years.

[0026] The present invention is thus directed to a polynucleotide comprising a sequence encoding a chimeric protein comprising a cytoplasmic domain of a T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family that is linked to a heterologous polypeptide comprising at least one self-assembly domain, said chimeric peptide being linked to an integral membrane or a transmembrane oligopeptide that allows the anchorage of the chimeric polypeptide to the cell membrane. The polypeptide encoded by the polynucleotides defined above is termed herein "self-aggregating TNFR polypeptide", for example as in "self-aggregating CD40", "constitutively active T cell costimulatory receptor of the TNFR family" or "caTNFR".

[0027] The candidates in this family that are most relevant to the present invention are CD40, CD27, 4-1BB (CD137), OX40 (CD134), herpesvirus entry mediator (HVEM; also known as TNFRSF14), CD30 and glucocorticoid-induced TNFR-related protein (GITR). For example, several clinical trials with anti-4-1BB and anti-OX40 mAbs in patients with melanoma and other solid tumors have been launched (see http://clinicaltrials.gov/).

[0028] 4-1BB. 4-1BB is expressed on activated human T cells. Its ligation by cognate ligand expressed on the surface of antigen-presenting cells (APCs) or by antibodies or soluble ligand promotes T cell survival by upregulating anti-apoptotic genes, induces cell division, augments Th1 cytokine production, protects T cells from antigen-induced cell death, induces memory formation and confers resistance to Treg suppression. Results from
different experimental systems have described the beneficial anti-tumor effect of 4-1BB stimulation in-vivo.

**0029** OX40. OX40 is a late costimulatory receptor expressed on activated CD4 and CD8 T cells. OX40 ligation promotes T cells survival, proliferation, migration to sites of inflammation, memory formation and abrogation of suppression. Similarly to 4-IBB, a large body of evidence maintains that OX40 stimulation augments anti-tumor T cell reactivity.

**0030** CD40. CD40 is also expressed on both CD8 and CD4 T cells, playing an intrinsic role in T cell costimulation, memory formation, rescue from exhaustion and contra-Treg activity.

**0031** Thus, in certain embodiments, the T cell costimulatory receptor of the TNFR family is selected from CD40, CD27, 4-IBB (CD137), OX40 (CD134), HVEM, CD30 or GITR, preferably the human homolog of these receptors.

**0032** The cytosolic portions of all seven TNFR members mentioned here bear structural similarities and they all signal through adaptor TNFR-associated factor (TRAF) proteins via the NF-kB, p38 MAPK or JNK/SAPK pathways. Signaling entails receptor homo-oligomerization, which is induced by engagement with their respective homo-oligomeric (typically homotrimERIC) ligands. A skilled artisan may easily assess if a cell expresses a molecule that has ligand-independent characteristic TNFR activity as measured for example by the activation of downstream signal pathways as recited above.

**0033** The self-assembly domain that mediates the aggregation of the chimeric protein comprising the full or partial intracellular domain of a TNFR molecule may be any such self-assembly domain known in the art, such as a coiled-coil domain. As taught by Testa et al (2009), coiled coils comprise two or more a-helices that supercoil around each other to form rope-like structures. At the sequence level, these motifs usually have a repeating pattern of seven residues called the heptad repeat, and often designated abcdefg. Residues at a and d positions are predominantly hydrophobic, which results in a hydrophobic stripe along each participating helix. These stripes come together to form the buried hydrophobic core of the coiled coil. Residues flanking the core also affect coiled-coil formation; charged amino acids at e and g positions can form salt bridges and electrostatic interactions to stabilize and specify coiled-coil structures further. An example of a coiled-coil is the leucine zipper motifs present in many DNA-binding proteins transcriptional regulators,
some of which are listed in Miller (2009), incorporated herein by reference. One example of a leucine zipper is that of the C/EBP a basic region: leucine zipper (bZIP) protein of the amino acid sequence of SEQ ID NO: 1; each seven amino acid residues of which forms a heptad. Non-limiting examples of human bZIP protein families mentioned in the Miller paper are C/EBPα, AP-1 (including the Jun subfamily, Fos subfamily and ATF2&4 subfamily), CREB/ATF, ATF6, PAR, MAF and CNC families. The self-assembly domain of each of these proteins, but not limited to them, is contemplated by the present invention for use in the chimeric polypeptide of the present invention.

[0034] Other self-assembly domains that could be used in the chimeric proteins of the present invention are association domains (tetramerization domains) of the vanilla receptor as taught by García-Sanz et al. (2004).

[0035] The coiled-coil domain may be a yeast GCN4 leucine zipper DNA-binding motif, which depending on its particular amino acid sequence, is capable of forming homodimers, homotrimerers, or homotetramers. We have used a CD40 genetic construct as a template, and incorporated the GCN4 binding motifs in 3 configurations encoding dimers, trimers and tetramers, based on Harbury et al, (1993; 1995).

[0036] Thus, in certain embodiments, the self-assembly domain is a yeast GCN4 leucine zipper DNA-binding motif, in particular the yeast GCN4 leucine zipper DNA-binding motif of SEQ ID NO: 2 forming homodimers, SEQ ID NO: 3 forming homotrimerers or SEQ ID NO: 4 forming homotetramers.

[0037] The self-aggregating TNFR polypeptide could conceivably be anchored by any transmembrane oligopeptide known in the art (see below). The cytoplasmic constitutively active domain of the TNFR molecule of the chimeric protein of the present invention is linked to an integral membrane or transmembrane oligopeptide that allows the anchorage of the TNFR molecule to a cell membrane. In other words, the TNFR moiety may be linked to an oligopeptide which is buried in the lipid bilayer and does not protrude to the other side of the membrane.

[0038] In certain embodiments, the transmembrane oligopeptide of the TNFR chimeric protein is the transmembrane oligopeptide of CD40.

[0039] The term "contralateral" is used herein to describe the side of the membrane which is opposite to the side of the constitutively active TNFR signal conducting moiety. For example, for CD40, the contralateral side is the extracellular side. Accordingly, the
term "contralateral protein" is used herein to describe a protein or peptide attached to the transmembrane stretch on the contralateral side of the membrane. In this case the contralateral protein is a peptide tag or oligopeptide tag.

[0040] The contralateral protein to which the transmembrane stretch may be attached via the bridge peptide is not essential for the proper activation of the T cells by the TNFR molecule and could thus be chosen according to its desired function such as, but not limited to, detection or purification of the expressed protein (e.g. a His-tag (e.g. His$_6$), a (His-Asn)$_n$ tag, a Flag tag, a Ha tag, a fluorescent protein, such as enhanced green fluorescent protein (EGFP), or any other tag that may facilitate the purification and identification of the chimeric protein), or an enzymatic function.

[0041] The terms peptide, oligopeptide and peptide-tag as used herein are defined as a peptide or oligopeptide that has no functional activity and that can be detected with a specific detecting agent, such as a specific antibody. As exemplified herein below, the tag may be His$_6$.

[0042] In certain embodiments, the transmembrane oligopeptide of the TNFR chimeric protein oligopeptide is linked, optionally via a bridge peptide comprises an amino acid stretch of about 5 amino acids to about 15 amino acids, preferably 13 amino acids of the membrane-proximal amino acids of human HLA-A2, to a peptide tag.

[0043] In certain embodiments, the peptide tag may be selected from, but is not limited to, Ha tag, a His tag or a Myc tag.

[0044] As shown herein below as a non-limiting example, the inventors have succeeded in creating constitutively active, ligand-independent CD40, which confers a constitutively activated state on transfected T cells, by linking CD40 to the GCN4 yeast transcriptional activator, which contains a leucine zipper DNA-binding motif that induces homophilic interactions, and thus induces self-oligomerizing. Since, as observed above, the TNFR members mentioned here bear structural similarities and they all signal through adaptor TNFR-associated factor (TRAF) proteins via the NF-0B, p38 MAPK, or JNK/SAPK pathways, one may assume that also the other TNFR members, when linked to a self-assembly domain, will become constitutively active, that are capable of conferring a constitutively activated state on transfected T cells. The terms "self-aggregating TNFR polypeptide", or "constitutively active T cell costimulatory receptor of the TNFR family" as used herein thus refer to any polypeptide with minor variations in its amino acid sequence as compared with the self-aggregating TNFR polypeptides defined herein, that
have ligand-independent characteristic TNFR activity as measured for example by the activation of downstream signal pathway elements such NF-κB, p38 MAPK or JNK/SAPK.

[0045] In certain embodiments, the polynucleotide encoding the constitutively active T cell costimulatory receptor of the TNFR family comprises the following nucleotide sequences: (a) the sequence of SEQ ID NO: 5 encoding for the leader peptide of the human β2m gene; (b) a sequence encoding for a peptide tag for detection selected from: (i) Ha tag of SEQ ID NO: 6; (ii) Myc tag of SEQ ID NO: 7; or (iii) His tag of SEQ ID NO: 8; (c) the sequence of SEQ ID NO: 9 encoding a bridge peptide; (d) the sequence of SEQ ID NO: 10 encoding for the transmembrane domain of human CD40; (e) the sequence of SEQ ID NO: 11 encoding for a GCN4-derived homo-oligomerizing domain forming homotrimers; and (f) a sequence encoding for the cytosolic signaling domain of a T cell costimulatory receptor of the TNFR family selected from: (iv) human CD40 of SEQ ID NO: 12; (v) human 4-IBB of SEQ ID NO: 13; (vi) human OX40 of SEQ ID NO: 14; (vii) human CD27 of SEQ ID NO: 15; or (viii) human GITR of SEQ ID NO: 16; or (g) nucleotide sequences substantially identical to the sequences of (a)-(f).

[0046] An indication that two nucleic acid sequences (i.e. polynucleotides) or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid has activity that is identical to, or characteristic of, the polypeptide encoded by the second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

[0047] The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10° C lower than the
thermal melting point (Tm) for the specific sequence at a defined ionic strength pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5xSSC, and 1% SDS, incubating at 42° C, or, 5xSSC, 1% SDS, incubating at 65° C, with wash in 0.2xSSC, and 0.1% SDS at 65° C.

[0048] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 1xSSC at 45° C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous reference, e.g., Current Protocols in Molecular Biology, ed. Ausubel, et al.

[0049] In a related embodiment, the nucleic acid sequences are at least about 80%, 90%, 95%, or 97-98%, or 100% identical to the sequences of SEQ ID NOs: 5-20 and 23-29, respectively, as assessed using a computer program such as BLAST (available at the National Center for Biotechnology Information, USA) that finds regions of similarity between biological sequences.

[0050] In certain embodiments, the polynucleotide encoding the constitutively active T cell costimulatory receptor of the TNFR family comprises the sequence of (iv) encoding for the cytosolic signaling domain of CD40.

[0051] The second component of our approach is based on the documented ability of toll-like receptor-2 (TLR2) and TLR9 ligands, but not TLR4 ligand, to act directly on CD4-ThT and on CD8 T cells and augment their effector functions.
The term "T cells" as used herein refers to T-lymphocytes taken directly from a living mammal; they do not include in their scope immortalized T cell lines, such as Jurkat cells.

Cumulative findings show that TLR2 engagement on these cells triggers interferon (IFN)-γ production, lowers the activation threshold, enhances cell proliferation and survival, maintains T cell memory, attenuates regulatory T cell (Treg)-mediated suppression and renders effector T cells resistant to this suppression.

Dendritic cells (DCs) and other antigen presenting cells (APCs) express a panel of TLRs, which recognize conserved microbial molecular motifs and products released from stressed or necrotic cells. The particular TLRs engaged by these cells in the periphery polarize the ensuing response towards the Th1, Th2, Th17 or the Treg course, and the elucidation in recent years of the functional consequences of TLR engagement on particular cell subsets is harnessed in vaccine development. Cumulative findings ascribe a critical role for Tregs in the suppression of tumor-reactive T cells. Treg activity can be modulated by agonists of TLR2, TLR5 and TLRs, and these can be exploited in cancer immunotherapy.

There is now ample evidence indicating that distinct TLRs are also expressed by resting, activated and memory T cells. Prominent among these is TLR2, which appears to be a key TLR family member that can directly stimulate T cell survival and proliferation and boost effector functions. For example, Komai-Koma et al. (2004) demonstrated that human CD4 and CD8 T cells expressed high levels of cell surface TLR2 after treatment with anti-TCR antibodies (Abs) and IFN-α. These cells produced elevated levels of IFN-γ, IL-2 and TNF-α (all 3 known for their anti-tumor activities) in response to the TLR2 ligand bacterial lipopeptide (BLP), but not to the TLR4 ligand LPS. CD4+ CD45RO+ memory T cells constitutively expressed TLR2, produced IFN-γ and manifested elevated proliferative capacity in response to BLP. Cottalorda et al. (2006) showed that BLP stimulation of mouse CD8 T cells led to increased cell proliferation and survival, and these were associated with a sustained expression of IL-2 receptor ct-chain (IL-2Ro; or CD25) and enhanced expression of the anti-apoptotic protein Bcl-xL. Furthermore, BLP upregulated IFN-γ and granzyme B secretion, augmented the overall cytotoxic activity of antigen-activated T cells and lowered the threshold of their initial activation by APCs. Another study (Liu et al. 2006) found that BLP, together with anti-TCR Abs, induced proliferation of both CD4+ CD25+ Tregs and CD4+CD25- effector mouse T cells in the absence of...
APCs. The expanded Tregs showed a transient loss of suppressive ability, which induced the effector cells to produce IL-2. This IL-2 in turn further induced expansion of these effector cells and rendered them refractory to Treg suppression. Imanishi et al. (2007) showed that in the absence of TCR stimulation, TLR2 (triggered by 2 different ligands), but not any other TLR, directly upregulated the expression of activation markers and triggered IFN-γ production, cell proliferation and cell survival in mouse Th1 cells, and that these effects were synergistically augmented by IL-2 or IL-12 through elevated activation of MAPKs. In this study, TLR2 ligands induced IFN-γ also by CD8 T cells pre-stimulated with anti-TCR and CD28 Abs. More recently, Asprodites et al. (2008) showed that BLP augmented the anti-melanoma activity of TLR2-proficient, but not deficient, CD8 T cells from the TCR transgenic OT-1 mice.

Other TLRs that have been reported to be functionally expressed by T cells include TLR3, TLR5 and TLR7/8. TLR3 was shown to be expressed by human effector CD8 T cells (Tabiasco et al, 2006). The bacterial TLR3 agonist poly(I:C) increased IFN-γ secretion induced by TCR-dependent and -independent stimulation of these cells, but without affecting proliferation or specific cytolytic activity. In another study (Caron et al., 2005), highly purified human CD4 T cells could be directly induced by flagellin and R848, the respective ligands for TLR5 and TLR7/8 to produce IFN-γ, while no effect could be detected by poly(I:C) and LPS, the ligands for TLR3 and TLR4.

Taken together, the functional properties conferred on effector T cells by direct TLR-mediated activation may act in concert to reverse the major suppressive mechanisms operating at the tumor site and enable these cells to persist and maximize their anti-tumor activity. Such consequences may bear important implications on the efficacy of ACT.

Removal of the ligand-binding domain of most, if not all, TLRs results in constitutive signaling triggered by the truncated derivatives, referred to as constitutively active (ca), or dominant positive (DP) TLRs. This property was originally reported for Drosophila's toll (Schneider et al., 1991), then for human TLR4, the first toll homologue identified, which was shown to confer a constitutively active phenotype on transfected human monocytic and CD4 T immortalized cell lines (Medzhitov et al., 1997) and later for other human and mouse TLRs, including TLR2 (caTLR2) of both human and mouse origin (Ozinsky et al., 2000; Hasan et al., 2004). The clinical use of TLR ligands of microbial origin as immunological adjuvants to directly augment T cell effector functions either in-
vivo or ex-vivo is intriguing; however, this may produce undesirable side effects and is not favored from a regulatory point of view.

[0059] In the past several years the present inventors have been developing a new genetic platform based on a chimeric polypeptide comprising p2-microglobulin (\(\text{p2m}\)), the MHC-I invariant light chain, and a signal transduction element component, such as CD3 zeta (\(\zeta\)), as disclosed in WO 01/91698 of the same applicants. When expressed in T cells, this chimeric polypeptide will endow the cells with the capacity to transduce T cell activation signal. Thus, T cells expressing these chimeric polypeptides together with relevant antigenic peptides, are rendered functional against other specific T cells which are restricted by the modified MHC products, such as autoreactive T cells.

[0060] The inventors have also utilized the genetic platform for targeting antigenic peptides to MHC-I presentation in antigen presenting cells. In this version of the platform the selected antigenic peptides are fused to the N-terminus of \(\text{32m}\) which is anchored to the membrane as described above. WO 01/091698 teaches antigenic peptides related to an autoimmune disease and WO 03/106616, of the same applicants, teaches antigenic peptides not related to an autoimmune disease, i.e. the antigenic peptide is derived from a tumor associated, bacterial, viral, fungal or parasite antigen. US 2008/0286312 and WO 08/041231 teaches a construct encoding \(\text{32m}\) anchored to the membrane by the means of the intracellular and transmembrane regions of TLR4, which confers activation of antigen presenting cells expressing the construct. WO01091698, WO 03/106616, US 2008/0286312 and WO 08/041231 are all hereby incorporated by reference in their entirety as if fully disclosed.

[0061] Even though the roles of certain TLRs are well known in the art and attempts to use TLR ligands have been problematic as described above, the present inventors are first to suggest and implement the idea that the caTLR format administered genetically offers an ideal means for equipping effector T cells with the arsenal of functional attributes required for the improvement of current ACT protocols. However, the inventors were surprised to find that not all TLRs are capable of activating the T-cells. As shown in the Examples below, the expression of constitutively active (transmembranal and cytosolic domains) of TLR2 in caTLR2 mRNA transfected T cells does not cause induction of IFN-7 production, i.e. does not activate the T cells (see Example 2). This finding is unexpected since TLR2 is known in the art to be expressed in T cells as described above. On the other
hand, as further shown in Example 2, expression of constitutively active (transmembranal and cytosolic domains) (caTLR4) caTLR4 mRNA transfected T cells causes extraordinary activation of both CD4 and CD8 T cells. This finding is highly surprising since in the art TLR4 is not believed to be functionally expressed in T cells as evidenced by the absence of a response of T cells exposed to TLR4 ligands such as LPS as described above.

[0062] The fact that TLR4 is capable of activating Jurkat cells (Medzhitov et al., 1997), has always been considered in the art to be a specific feature of this immortalized cell line, and not as a feature shared by T cells due to the latter’s refractoriness to LPS, and has not prompted researchers to attempt expressing TLR4 in T cells. Moreover, since TLR2 and TLR4 signaling pathways differ, T cells in general would not be expected to possess all the downstream components required for TLR4 signaling. For example, TLR2-mediated signaling is solely transduced through the recruitment of the MyD88 and TIRAP adaptor proteins, which leads to the activation of the transcription factors NF-κB and AP-1. Unlike, TLR4 also harnesses the TRIF-TRAM set of adaptors, a pathway which culminates in the activation of interferon regulatory factors.

[0063] It is a remarkable advantage of the method of the present invention that rendering these cells constitutively activated merely by gene expression obviates any need in adjuvants, TLR ligands or microbial substances.

[0064] It has been found in accordance with the present invention that the expression of caTLR4 in T cells results in constitutively activated T cells (see Example 2).

[0065] It has further been found in accordance with the present invention that the expression of caTLR4 and self-aggregating CD40 in the same T cell, results in functional synergy as compared with expression in T cells of each one of these two factors alone (Examples 3 to 5).

[0066] The T cells activated by these two constitutively active molecules, a T cell costimulatory receptor of the TNFR family and the caTLR4, may thus be transfected with a single polynucleotide or vector encoding for both molecules, or it may be transfected with two separate molecules, e.g. RNA molecules, each one encoding for one of the two molecules.

[0067] Thus, in another aspect, the present invention provides a polynucleotide comprising a sequence encoding a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through its amino terminus to an
integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane, and said transmembrane oligopeptide, if existent, is linked, optionally via a bridge peptide, to a peptide tag or oligopeptide tag.

In certain embodiments, the polynucleotide encoding the constitutively active T cell costimulatory receptor of the TNFR family comprises a further sequence encoding a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through its amino terminus to an integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane, and said transmembrane oligopeptide, if existent, is linked, optionally via a bridge peptide, to a peptide tag or oligopeptide tag.

The term "caTLR4" as used herein refer to any polypeptide with minor variations in its amino acid sequence as compared with transmembranal and cytosolic domains of human TLR4 (based on the sequence as set forth at GBA NM1 38554), that have ligand-independent characteristic TLR4 activity as measured for example by the activation of downstream signal pathway elements such myD88, TRIF-TRAM or NFKB.

The cytoplasmic constitutively active domain of the TLR4 molecule could conceivably be anchored by any transmembrane oligopeptide known in the art (see below). The cytoplasmic constitutively active domain of the TLR4 molecule of the chimeric protein of the present invention is linked through its amino terminus to an integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to a cell membrane. In other words, the TLR4 moiety may be linked to an oligopeptide which is buried in the lipid bilayer and does not protrude to the other side of the membrane.

As exemplified herein below, the TLR4 moiety is preferably anchored to a cell membrane by its own native transmembrane oligopeptide.

In certain embodiments, the TLR4 is a human TLR4.

In other embodiments, the bridge peptide (which is linked to the TLR4 transmembrane oligopeptide) comprises an amino acid stretch of about 5 amino acids to about 15 amino acids, preferably 13 amino acids of the membrane-proximal amino acids of human HLA-A2.

In other embodiments, the peptide tag is selected from, but is not limited to, Ha tag, a His tag or a Myc tag.
In particular, the polynucleotide encoding for the caTLR4 may comprise the following nucleotide sequences: (a) the sequence SEQ ID NO: 17 encoding for the 5' non-translated region of β2m; (b) the sequence SEQ ID NO: 18 encoding for the human β2π1 leader peptide; (c) a sequence encoding for a peptide tag for detection selected from: (i) Ha tag of SEQ ID NO: 6; (ii) Myc tag of SEQ ID NO: 7; or (iii) His tag of SEQ ID NO: 8; (d) the sequence of SEQ ID NO: 9 encoding a bridge peptide; (e) the sequence SEQ ID NO: 19 encoding for human transmembrane and cytosolic domains of TLR4; and (f) the sequence SEQ ID NO: 20 encoding for the 3' non-translated region of β2η1, or (g) polynucleotide sequences substantially identical to the sequences of (a)-(f).

The third component of our approach is based on the well documented use of Thl immunostimulatory cytokines in different ACT protocols, either during the ex-vivo activation-expansion phase, or in-vivo, as a means for enhancing the function and longevity of the transferred cells. Non-limiting examples of Thl immunostimulatory cytokines are IL-1α, IL-2, IL-7, IL-12, IL-15, IL-17, IL-18, IL-21, TNF-α, IFN-α, IFN-β and IFN-γ.

Adjuvant activities exerted by cytokines which are administered systemically are often accompanied by adverse physiological effects, which set a severe limit on the feasible treatment dose and call for alternatives application modes.

It is now widely appreciated that cells other than effector lymphocytes, and particularly Tregs residing at the tumor site and constitutively expressing high level of cytokine receptors, consume cytokines, with ample evidence for IL-2 and some for IL-15 (and IL-7). This consumption severely reduces the actual concentration of cytokines available for the effector cells and impairs treatment efficacy either in cytokine monotherapy or when cytokines are used as adjuvants in ACT. Here we propose to address this problem by expressing cytokines as integral membranal constituents of the tumor-reactive T cells. This mode of expression is expected to render these cytokines constantly available for the effector cells, presented to them either in-cis (that is, to the same cell), or in-trans, to neighboring cells in their immediate vicinity, while minimizing competition. Indeed, a limited number of reports describe the engineering and in-vivo evaluation of membrane-bound cytokines. When IL-2 was expressed as a glycoinositol phospholipid (GPI)-anchored membranal protein by the B16 mouse melanoma, but not when expressed by these cells as a soluble protein, it inhibited the in-vivo growth of the gene-modified
melanoma (Ji et al., 2002). Chakrabarti et al. (2004) demonstrated that DNA co-vaccination with plasmids encoding carcinoembryonic antigen (CEA) and transmembranal IL-12/B7.1 fusion protein was effective in-vivo against a CEA-expressing tumor. Importantly, in-vitro structural and functional analyses performed in both these studies suggested that the membranal form of these cytokines fully retains the ternary and (for IL-12, see below) the quaternary structure and the T cell-promoting activity.

Thus, it can be expected that membranal forms of other immunostimulatory cytokines would also retains the ternary and, if relevant, the quaternary structure, and the T cell-promoting activity. Below is a description of the three cytokines most widely explored in cancer immunotherapy.

IL-2. IL-2 is an essential factor for the growth, differentiation and survival of antigen-selected T’ cells. It is a 15.5 kDa protein comprising a single polypeptide chain of 133 amino acids. IL-2 is naturally produced by activated T cells and functions in an autocrine or paracrine manner. It binds to a heterotrimeric high affinity receptor comprising IL-2Ra (CD25), IL-2/15R/3 and the common cytokine-receptor γ-chain (7c). IL-2 is evidently the most widely explored cytokine in cancer immunotherapy and particularly in ACT. A most recent report of a phase I/II clinical trial in melanoma patients underscores the positive effect of IL-2 on prolonged survival of TILs following either exogenous supply or gene transduction, yet pointing to the moderate clinical response obtained in-vivo. Importantly, IL-2 substantially augments the TLR-2-mediated enhancement of the functional and proliferative status of T cells.

Competitive consumption of IL-2 by Tregs, which constitutively express high level of the full IL-2R has been suggested as one of the major suppression mechanisms executed by these cells, thus reducing the actual concentration available for effector T cells to sub-threshold level. This mechanism is likely operative also at the tumor microenvironment, depriving tumor-reactive cells of IL-2. New strategies are therefore required to reduce this competition and maintain functional concentrations of IL-2 for the effector T cells.

IL-12. IL-12 is a Th1 polarizing cytokine, driving the differentiation of CD4 Th1 and CD8 T cells and natural killer (NK) cells and is also known for its anti-angiogenic activity. It stimulates IFN-γ and TNF-α production from T and NK cells. IL-12 is a heterodimeric cytokine, consisting of two gene products: p35 and p40. It is produced mainly by activated DCs and binds to its heterodimeric receptor comprising IL-12R-β1 and
IL-12R-β2, which is expressed on T and NK cells. The anti-tumor activities of IL-12 have been explored in cancer immunotherapy, gene therapy and ACT. As noted above, IL-12 was capable of amplifying the observed influence of TLR2 ligands on T cell effector functions. The production of different forms of recombinant IL-12 or its gene delivery were facilitated by the development of single-chain derivatives of this cytokine, which retain the functional properties of the native protein (Chakrabarti et al (2004), Lode et al (1998), Lee et al, 1998).

[0083] IL-15. Like IL-2, IL-15 is a T cell (and NK cell) stimulating cytokine. It induces the generation of primary and memory antigen-specific CD8 T cells, mediates CD4 T cell help to promote longevity of CD8 cells and prevent their TRAIL-induced apoptosis. In particular, IL-15 induces telomerase activity in CD8 T cells and prolongs their proliferation. IL-15 synergizes with TLR2 in promoting proliferation of, and IFN-7 production by, human CD4 T cells. IL-15 is a key cytokine used in ACT in ex-vivo generation and propagation of tumor-reactive effector T cells. Most importantly, it was recently shown to rescue tolerant CD8 T cells for use in ACT of established tumors and reverse failed ACT. T cells engineered to express IL-15 exhibited improved anti-tumor activity and prolonged survival in-vivo (Quintarelli et al (2007), Hsu et al (2005), Klebanoff et al., 2004). It has been shown that in addition to IL-2 consumption, Tregs can bind and eliminate IL-15 (and IL-4 and IL-7). In a recent study of ACT in the mouse B16 melanoma model, this cytokine sink effect was indirectly shown to influence IL-15 (and IL-7) activity, and removal of endogenous cells capable of binding this cytokine augmented anti-tumor CD8 T cell reactivity.

[0084] To tackle the two-sided problem of systemic IL-2 toxicity and insufficient IL-2 support for cells transferred during ACT, the inventors have genetically engineered IL-2 as an integral membranal constituent of transfected cells. Membranal (mem) IL-2 was designed with a flexible spacer so as to engage its cell surface receptor in-cis (that is, on the same cell). As long as both the gene product and its endogenous IL-2 receptor are expressed at the cell surface this closed system is expected to signal constitutively, precluding IL-2 intake by tumor-resident Tregs and other cells and avoiding systemic dissemination and subsequent side effects.

[0085] It has been found in accordance with the present invention that the expression of membrane bound IL-2 in human CD8 and CD4 T cells supports their ex-vivo proliferation for at least 6 days in the total absence of exogenous IL-2 (Example 4).
It has further been found in accordance with the present invention that expression of caTLR4 and membrane bound IL-2 in the same T cell, results in functional synergy as compared with expression in T cells of each one of these two factors alone (Example 4).

The T cells activated by these two molecules may thus be transfected with a single polynucleotide or vector encoding for both molecules, or it may be transfected with two separate molecules, e.g. RNA molecules, each one encoding for one of the two molecules. Alternatively, the T cells may be transfected with a single polynucleotide encoding a cytokine, grafted via a bridge peptide and a transmembrane oligopeptide, to the cytoplasmic domain of the self-aggregating TNFR polypeptide of the present invention, or the cytoplasmic domain of the caTLR4 of the present invention.

Thus, in one embodiment, the polynucleotide of the present invention encodes for the constitutively active T cell costimulatory receptor of the TNFR family, or the constitutively active TLR4, wherein the constitutively active T cell costimulatory receptor of the TNFR family, or the constitutively active TLR4, is linked through a transmembrane oligopeptide via a flexible bridge peptide to the carboxyl terminus of a cytokine.

The polynucleotide of the present invention may also encode for a chimeric protein comprising a cytoplasmic domain of a T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family that is linked to a heterologous polypeptide comprising at least one self-assembly domain, said chimeric peptide being linked to an integral membrane or a transmembrane oligopeptide that allows the anchorage of the chimeric polypeptide to the cell membrane, and a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic constitutively active domain of a TLR4 molecule.

Alternatively, the polynucleotide of the present invention may encode for a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through its amino terminus to an integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane, and said transmembrane oligopeptide, if existent, is linked, optionally via a bridge peptide, to a peptide tag or oligopeptide tag, and a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a self-aggregating cytoplasmic domain of a T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family.
In another embodiment, the polynucleotide of the present invention encoding for the constitutively active T cell costimulatory receptor of the TNFR family, or the constitutively active TLR4, comprises a further sequence encoding a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA.

It should be clear that a polynucleotide encoding a certain self-aggregating TNFR polypeptide may also encode for additional different self-aggregating members of the TNFR family, e.g. but not limited to, self-aggregating CD40 and self-aggregating 4-1BB. It should also be similarly clear that a polynucleotide encoding a membrane bound cytokine may also encode for additional membrane bound cytokines, e.g., but not limited to, membrane bound IL-2 and membrane bound IL-12.

In an additional aspect, the present invention is directed to a polynucleotide encoding a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA.

In certain embodiments, said cytokine is selected from IL-2, IL-12 or IL-15, and in other embodiments the cytokine is a human cytokine.

The flexible bridge peptide may comprise an amino acid stretch of about 5 amino acids to about 15 amino acids, preferably 8 amino acids of the membrane-proximal amino acids of human HLA-A2, and in certain embodiments it further comprises the 13 amino acid flexible linker Gly₅Ser(Gly₃Ser)₂ (SEQ ID NO: 21).

The cytokine molecule of the chimeric protein of the present invention is linked to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine to a cell membrane. In other words, the cytokine may be linked to an oligopeptide which is buried in the lipid bilayer and does not protrude to the other side of the membrane.

The TNFR, caTLR molecule or cytokine of the present invention could conceivably be anchored by any transmembrane oligopeptide known in the art. For
example, it may be linked through its amino terminus via a glycosylphosphatidylinositol (GPI)-anchor sequence, such as the GPI-anchor peptide of SEQ ID NO: 22, of the sequence FTLTGLLGTLVTMGLLT (from the protein DAF - complement decay-accelerating factor precursor or CD55 antigen; SWISSProt ID P08174, positions 365-381). Alternatively, the transmembrane oligopeptide may be predicted by algorithms. The sequence of a typical transmembrane peptide is well defined in the art and can easily be identified using known algorithms readily available to a person skilled in the art, such as the neural network of Pasquier and Hamodrakas (1999), which gives a perfect prediction rating of 100% by classifying all the sequences in the transmembrane class. Applied on 995 non-transmembrane protein extracted from the PDBSELECT database, the neural network predicts falsely 23 of them to be transmembrane (97.7% of correct assignment). Thus, the transmembrane stretch of amino acids could be any such stretch predicted by the above mentioned algorithm or a different algorithm with similar prediction and assignment ratings.

[0098] In cases where the cytokine is linked to a transmembrane oligopeptide, it is, in certain embodiments, the transmembrane oligopeptide of HLA-A2 and it may be linked to the cytoplasmic domain of HLA-A2. In other words, the cytokine may be linked to, and therefore anchored to the cell membrane by, the transmembrane and cytoplasmic portion of HLA-A2.

[0099] In certain embodiments, the polynucleotide encodes for a membrane bound interleukin, wherein the polynucleotide comprises the following nucleotide sequences: (a) the sequence of SEQ ID NO: 23 encoding for the transmembrane and cytoplasmic portion of HLA-A2; (b) the sequence of SEQ ID NO: 24 encoding for a bridge peptide of a 21 amino acid spacer to the cell membrane, comprising the 13 amino acid flexible linker Gly4-Ser(Gly3Ser)2 (SEQ ID NO: 21), followed by the 8 membrane-proximal amino acids of the HLA-A2 connecting peptide with an Xhol restriction site; (c) a sequence encoding for the extracellular domain of a cytokine selected from IL-2, IL-12 or IL-15, wherein: (i) the full human IL-2 and its leader peptide sequence is encoded by the sequence of SEQ ID NO: 25; (ii) the chimeric single chain derivative of human IL-12 is encoded by the sequence of SEQ ID NO: 26 encoding for the full human IL-12 p40, including the leader peptide, the sequence of SEQ ID NO: 27 encoding for the linker peptide, and the sequence of SEQ ID NO: 28 encoding for the human IL-12 p35 without the leader peptide; and (iii) chimeric human IL-15 is encoded by SEQ ID NO: 5 encoding for the leader peptide of
human j32m, and SEQ ID NO: 29 encoding for the mature peptide of human IL-15; or (d) nucleotide sequences substantially identical to the sequences of (a)-(c).

In certain embodiments, the polynucleotide encoding the chimeric protein of the present invention is an in vitro transcribed RNA molecule, and it may be polyadenylated, capped or both.

The RNA molecule of the present invention could be synthesized by any method known in the art, such as, but not limited to, transcription of an expression vector comprising the relevant DNA sequence either in a eukaryotic cell or, preferably, in vitro using purified RNA polymerase.

In yet another aspect, the present invention provides an expression vector comprising a polynucleotide as defined herein above.

In certain embodiments, the expression vector comprises a nucleic acid sequence encoding an RNA molecule in a manner which allows transcription of said nucleic acid sequence in vitro.

In still another aspect, the present invention provides a composition comprising at least one polynucleotide or the expression vector as defined herein above.

In certain embodiments, the composition comprises at least one polynucleotide, i.e. a DNA or RNA molecule, comprising a sequence encoding: (a) a chimeric protein comprising a cytoplasmic domain of T cell costimulatory receptor of the TNFR family that is linked to a heterologous polypeptide comprising at least one self-assembly domain, said chimeric peptide being linked to an integral membrane or a transmembrane oligopeptide that allows the anchorage of the chimeric polypeptide to the cell membrane; (b) a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through its amino terminus to an integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane, and said transmembrane oligopeptide, if existent, is linked, optionally via a bridge peptide, to a peptide tag or oligopeptide tag; (c) a chimeric protein comprising an extracellular domain of a cytokine linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA; (d) a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic constitutively active
domain of a TLR4 molecule; (e) a chimeric protein comprising a cytokine linked through
its carboxyl terminus via a flexible bridge peptide to a cytoplasmic domain of a self-
aggregating T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family; (f) a combination of (a) and (b); (g) a combination of (a) and (c); (h) a combination of (b) and (c); (i) a combination of (a), (b) and (c); (j) a combination of (d) and (e); (k) a combination of (a) and (d); (l) a combination of (b) and (e).

[00106] In particular, the composition comprises polynucleotides encoding for a T cell
costimulatory receptor of the TNFR family selected from CD40, CD27, 4-1 BB (CD137),
OX40 (CD134), herpesvirus entry mediator (HVEM, TNFRSF14), CD30 or
glucocorticoid-induced TNFR-related protein (GITR), or combinations thereof. Thus, it
should be clear that a composition comprising a polynucleotide encoding for a self-
aggregating TNFR polypeptide as defined in (a) to (l) may also include combinations of
polynucleotides encoding for different self-aggregating members of the TNFR family, e.g.
but not limited to, self-aggregating CD40 and self-aggregating 4-1 BB. It should also be
similarly clear that the a composition comprising a polynucleotide encoding for a
membrane bound cytokine as defined in (a) to (l) may also include combinations of
polynucleotides encoding for different cytokines, e.g., but not limited to, membrane bound
IL-2 and membrane bound IL-12.

[00107] In certain embodiments, the cytokine encoded by the polynucleotide in the
composition is selected from IL-2, IL-12 or IL-15, or combinations thereof.

[00108] In an additional aspect, the present invention relates to activated T cells
expressing at least one chimeric protein encoded by the polynucleotide or the expression
vector as defined herein above.

[00109] In certain embodiments the activated T cells express (a) a chimeric protein
comprising a cytoplasmic domain of T cell costimulatory receptor of the TNFR family that
is linked to a heterologous polypeptide comprising at least one self-assembly domain, said
chimeric peptide being linked to an integral membrane or a transmembrane oligopeptide
that allows the anchorage of the chimeric polypeptide to the cell membrane; (b) a chimeric
protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked
through its amino terminus to an integral membrane or transmembrane oligopeptide that
allows the anchorage of the TLR4 molecule to the cell membrane, and said transmembrane
oligopeptide, if existent, is linked, optionally via a bridge peptide, to a peptide tag or
oligopeptide tag; (c) a chimeric protein comprising an extracellular domain of a cytokine
linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA; (d) a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic constitutively active domain of a TLR4 molecule; (e) a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic domain of a self-aggregating T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family; (f) a combination of (a) and (b); (g) a combination of (a) and (c); (h) a combination of (b) and (c); (i) a combination of (a), (b) and (c); (j) a combination of (d) and (e); (k) a combination of (a) and (d); (l) a combination of (b) and (e).

[00110] In particular, the cells express polynucleotides encoding for a T cell costimulatory receptor of the TNFR family selected from CD40, CD27, 4-1BB (CD137), OX40 (CD134), herpesvirus entry mediator (HVEM, TNFRSF14), CD30 or glucocorticoid-induced TNFR-related protein (GITR), or combinations thereof. Thus, it should be clear that a cell expressing a self-aggregating TNFR polypeptide as defined in (a) to (i) may also express combinations of polynucleotides encoding for different members of the TNFR family, e.g. but not limited to, self-aggregating CD40 and self-aggregating 4-1BB. It should also be similarly clear a cell expressing a membrane bound cytokine as defined in (a) to (i) may also express combinations of polynucleotides encoding for different cytokines, e.g., but not limited to, membrane bound IL-2 and membrane bound IL-12.

[00111] In certain embodiments, the cell express a cytokine selected from IL-2, IL-12 or IL-15, or combinations thereof.

[00112] In certain embodiments, the activated T cells express a chimeric protein comprising a cytoplasmic and transmembrane polypeptide of CD40 that is linked to a yeast GCN4 leucine zipper DNA-binding motif self-assembly domain and/or a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through a TLR4 transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane.

[00113] In certain embodiments, the cytokine is linked to the transmembrane and cytoplasmic portion of HLA-A2.
The T cells used in the present invention may be autologous T cells that are naturally reactive against a tumor (tumor infiltrating lymphocytes or TILs), tumor-specific T cells isolated from peripheral blood lymphocytes (PBLs) or non-tumor-specific autologous or allogeneic cells genetically reprogrammed to express tumor-reactive TCR or chimeric TCR molecules displaying antibody-like tumor recognition capacity. For example, the polypeptide may be a chimeric T cell receptor, a T-body (T-bodies are genetically engineered T cells armed with chimeric receptors whose extracellular recognition unit is comprised of an antibody-derived recognition domain and whose intracellular region is derived from lymphocyte stimulating moiety(ies)), a natural or genetically-modified TCR (Morgan et al., 2006), or a TCR-like antibody optionally fused to a toxin, that is specific to a TAA.

Tumor infiltrating CTLs are a mixed population of effector and effector memory cells. These cells, which are highly selective to tumor antigens, are suppressed or at anergic state and do not elicit cytotoxic activity. TIL potentiation is currently a major goal of the immune research.

Thus, in certain embodiments, the activated T cells are tumor-specific T cells.

In yet an additional aspect, the present invention provides a method for treating cancer, comprising administering to a cancer patient a therapeutically effective amount of tumor specific activated T cells as defined herein above. The T cells may be transfected ex vivo, i.e. the T cells are removed from the patient, transfected with the polynucleotide in a laboratory using standard techniques and then returned to the patient; or in situ, using recent techniques that allow for the transfection of the T cells in the patient's body.

The term "treating" as used herein refers to the alleviation, reduction of progression or complete cure of the disease, or to the reduction of symptoms related to or caused by the disease.

The basic TIL-ACT protocol for the treatment of melanoma includes selected tumor-reactive TILs, employing IFN-γ secretion in response to tumor antigens as readout for specificity. TILs are expanded by vigorous TCR stimulation followed by lengthy culturing periods with IL-2, resulting in reduced survivabilit. Difficulties in generating autologous melanoma cultures, failure of cultured TILs to produce IFN-γ and exacerbation of disease during selection contribute to considerable patients' dropout and limit the widespread use of sTIL-ACT. Two laboratories have recently reported the
utilization of short-term cultured, non-selected TILs, dubbed 'young' (y)TILs, in an attempt to minimize and simplify the ex-vivo phase (Tran et al (2008), Besser et al. (2009), Besser et al, (2010), Dudley et al. (2010), Itzhaki et al., 2011). Clinical studies revealed that the use of yTILs substantially improves the proportion of treated patients and that yTIL-ACT achieves clinical response rates that are comparable to standard ACT.

[00120] Thus, in certain embodiments the TILs are autologous T cells, in particular tumor infiltrating lymphocytes such as young tumor infiltrating lymphocytes.

[00121] In one embodiment, the T cells are directed to tumor cells selected from melanoma, breast cancer; chronic myeloid leukemia; acute lymphoblastic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute myeloid leukemia, chronic lymphocytic leukemia, mature B cell neoplasms, mature T cell and natural killer cell neoplasms; liver, stomach, pancreas, intestine and kidney tumors; or bladder, glandular epithelia, lung squamous cell, gut, prostate, testis, thymus, bone marrow, and lymph nodes carcinomas.

[00122] The present invention further provides methods for preparing activated T cells comprising transfecting the T cells with the polynucleotide, the expression vector or the composition as defined herein above, whereby the polynucleotide is expressed within said T cells, thus obtaining the activated T cells.

[00123] In still another aspect, the present invention is directed to a method for propagating T cells, comprising transfecting said T cells with a polynucleotide comprising a sequence encoding a chimeric protein comprising IL-2 linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the IL-2 molecule to a cell membrane.

[00124] This method, which obviates the need for adding soluble cytokines to the T cells in order for the cells to propagate and survive, solves the two-sided problem of systemic IL-2 toxicity and insufficient IL-2 support for cells transferred during ACT.

[00125] Thus, in certain embodiments, the method does not involve contacting the cells with a soluble cytokine, i.e. the cells are grown in the absence of soluble cytokines.

[00126] The present invention also contemplates TLR4-activated T cells, wherein said T-cells are obtained by transfecting T cells with an RNA molecule including a ribonucleotide sequence encoding a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through its amino terminus to an
integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to a cell membrane, whereby the RNA molecule is expressed within said T cells, thus obtaining the TLR4-activated T cells.

[00127] The present invention further considers nucleic acid molecules that comprise DNA sequences encoding for the caTLR4, self-aggregating TNFRs and membrane bound cytokines, and combinations thereof, operably linked to a promoter. Any suitable mammalian expression vector can be used such as, but not limited to, the pCI mammalian expression vectors (Promega, Madison, WI, USA), pCDNA3 expression vectors (Invitrogen, San Diego, CA) and pBJI-Neo. The expression vector may also be a plasmid DNA in which the polynucleotide sequence is controlled by a virus, e.g. cytomegalovirus, promoter, or, most preferably, the expression vector is a recombinant viral vector such as, but not limited to, pox virus or adenovirus or adeno-associated viral vector.

[00128] Any of the techniques which are available in the art may be used to introduce the recombinant nucleic acid encoding the polypeptide into the antigen presenting cell. These techniques are collectively referred to as transfection herein and include, but are not limited to, transfection with naked or encapsulated nucleic acids, cellular fusion, protoplast fusion, viral infection, cellular endocytosis of calcium-nucleic acid microprecipitates, fusion with liposomes containing nucleic acids, and electroporation. Choice of suitable vectors for expression is well within the skill of the art. Antigen expression may be determined by any of a variety of methods known in the art, such as immunocytochemistry, ELISA, Western blotting, radioimmunoassay, or protein fingerprinting.

[00129] The activation of T cells is manifested in naive cytotoxic T-lymphocytes (CTLs) by differentiation into effector cells that can kill antigen-bearing target cells using granzymes, perforin and FAS ligand, and can rapidly produce inflammatory cytokines, such as IFNγ and TNFa upon TCR ligation and activation. Type II interferon IFN-γ, plays a key role in adaptive immunity to viral and bacterial infections and in the antitumor response. In particular, in tumor immunity, IFN-γ functions include the enhancement of antigen presentation by both MHC-I and MHC-II products, suppression of the Th1-antagonistic activities of Th2 cells and promotion of effector CD4 Th1 and CD8 T cell differentiation. IFN-γ is also known for its anti-proliferative, apoptotic and angiogenic effects, all potentially contributing to its well documented antitumor activity.
[00130] An additional cytokine which is involved in T cell activation is IL-2. This cytokine is released from T cells and bind to a specific T cell IL-2 receptor to further promote the activation and proliferation of T cells in an autocrine fashion.

[00131] Upon activation, effector CTLs can adopt multiple cell fates, such as short-lived effectors or memory precursors. Short-lived cells effectors can either become senescent effectors and die by apoptosis in several days, or persist into early memory as short-lived effector memory cells. Memory precursors, by contrast, are long-lived and can be differentiated into effector memory cells or central memory cells. Although the exhausted cell is depicted as sharing a common effector cell precursor with other cell fates, it is currently unclear at what point during effector cell differentiation this fate branches. In addition to the phenotypes of the activated T cells already mentioned, it is expected that the TLR-activated CD4 T cells are capable of conferring immune memory to activated CD8 T cells, i.e. the constitutive activation of both CD4 and CD8 T cells by the means of the method of the present invention not only produces armed killer T cells but may also produce memory T cells that are capable of sustaining a prolonged attack on the target cells.

[00132] The marker used herein to measure T cell activation is mainly the measurement of the level of secreted IFN-γ; however, the level of other markers such as, but not limited to, IL-2, TNFa, granzyme B, perforin and FAS ligand secretion and transcription factor NF-κB activity, or combination of markers, for T cell maturation could also be assessed. It should be clear to a person skilled in the art that a CD8 T cell that secretes high levels of IFN-γ is an activated, fully mature CD8 T cell, i.e. a cytotoxic T cell (also known as TC, CTL, T-Killer cell, cytolytic T cell, or killer T cell) which is capable of recognizing and killing a specific antigen-bearing cell without further reinforcement or licensing from antigen presenting cells and/or CD4 T cells. It should be equally clear that a CD4 T cell that secretes high levels of IFN-γ is a fully mature CD4 Th1-cell (also known as effector Th1 cell or Th1 cell) which is capable of exerting all Th1 helper functions associated with the cellular immune response. Accordingly, the phrase "preparing TLR-activated T cells" is used herein to describe the production of T cells exhibiting the hallmarks of a mature phenotype, for example secreting high levels of IFN-γ. An indication that the IFN-γ is high is that it is 10-fold, 100-fold or 1000-fold higher than the level secreted by naive T cells, as shown in Example 2 herein below.
The activated T cells of the present invention are preferably prepared by transfecting T cells with an RNA molecule encoding constitutively active factors and/or membrane cytokines. In contrast, most ACT procedures which require the genetic reprogramming of human T cells utilize either retroviral or lentiviral vectors. However, the clinical implementation of such vectors entails complicated and lengthy procedures, while the number and size of genes that can be introduced is restricted. Most importantly, the use of these vectors in the clinic raises serious safety concerns which further limit their wide application. Unlike, the transfection of mRNA usually achieves very high yield while avoiding most of the problems associated with DNA, because: mRNA should only gain entry into the cell cytoplasm where it is ready for translation, thus requiring milder transfection conditions; the introduced RNA is similar in composition to the endogenous mRNA and does not carry any inherent danger signals; pre-defined mixtures of mRNA can be easily prepared and introduced simultaneously, rendering this approach highly versatile; and, mRNA offers maximal safety.

Indeed, the use of mRNA as the genetic vehicle of choice for therapeutic purposes has attracted much attention in the past several years. In particular, the ability to confer tumor specificity on polyclonal T cell populations through mRNA electroporation is beginning to gain momentum.

Perhaps the most significant pitfall of mRNA (and DNA) transfection is the transient nature of expression. Indeed, mRNA may not be the ideal vehicle for conferring durable tumor specificity through the transfer of anti-tumor TCR or T-body genes. Nonetheless, this consideration is irrelevant to other ACT approaches which utilize T cells that are naturally reactive against the tumor (TILs, membrane-capturing, allogeneic and T cells activated by tumor or tumor antigens ex-vivo). In contrast, modification of the functional status of T cells (and other immune cells) is usually accomplished in the course of a natural immune response following a transient, rather than a constitutive, stimulus. This commonly leads to phenotypic changes which are irreversible in the time frame of the effector phase, and can thus be ideally provided by mRNA transfection. Importantly, transient expression of immunostimulatory molecules obviates the risk of cell transformation resulting from uncontrolled growth signaling.

The term peptide as used herein refers to a short amino acid stretch of between 2 and about 10 amino acids and the term oligopeptide as herein refers to a short amino acid stretch of between 2 and about 20 amino acids.
The term "about" as used herein refers to a maximum deviation of ± 10% from the value preceded by this term.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article, unless context clearly indicates otherwise. By way of example, "an element" means one element or more than one element.

The invention will now be illustrated by the following non-limiting examples:

**EXAMPLES**

(i) *RNA preparation and transfection of DCs and APC clones*. Genes of interest were cloned into the multiple cloning site of a special vector designed for in-vitro production of mRNA {pEGM4Z/GFP/A64}, following removal of the GFP gene. This vector, described in detail in Boczkowski et al., 2000. Cancer Res 60:1028-1034, is based on the vector pGEM4Z (Promega, Madison, WI). Messenger RNA was prepared from cloned templates using the AmpliCap-Max T7 High Yield Massange Marker Kit (Epicenter) following plasmid linearization with Sptel. Immature and mature DCs were harvested and washed two times in cold PBS, then resuspended in cold Opti-MEM at 2-3 x10⁶ cells/100 µl and were mixed in 2 mm gap cuvette (Bio-Rad) along with 5-10 µg of in-vitro transcribed mRNA. The mixture was then subjected to appropriate conditions in a square wave electroporator (BTX-ECM 830, San Diego, CA).

(ii) *FACS analysis*. Cells were stained with indicated antibodies according to standard procedures and were subjected to flow cytometry analysis. 10⁶ cells were washed with FACS buffer - phosphate-buffered saline (PBS) containing 0.09% sodium azide and 0.5 % Bovine serum albumin (BSA) incubated for 30 minutes on ice with 10 µg/ml of conjugated Abs or the same concentration of a control (Isotype) antibody or no antibody at 100 µl FACS buffer. Cells were washed and resuspended in PBS and analyzed by a LSR II (BD Biosciences, Mountain View, CA). Statistical analysis was performed with the FCS express software (De Novo software, Los Angeles, CA). Quantitative analysis of cell surface antigens was performed with eBioscience Ab (eBioscience, San Diego, CA) according to the manufacturer’s instructions.
Example 1. GFP is highly expressed in human CD8 T cells following mRNA transfection.

[00142] In a series of experiments, using enhanced GFP (EGFP) mRNA, we have optimized the conditions for efficient mRNA transfection of human T cells, reaching unprecedented yields. In one representative experiment, we separated an initial PBL population obtained from a healthy donor into CD4 and CD8 subsets, CD8 T cells were separated by positive selection, grown for an additional 24 hours in the presence of 0.5 \( \mu g/ml \) each of anti-CD3 and anti-CD28 mAbs and subjected to electroporation with 5 \( \mu g \) mRNA using a square-wave pulse at 400 V, 0.95 msec in 0.1 ml in 2 mm cuvettes. Fig 1, showing a representative experiment shows that the mean transfection efficiency is 90±10%. No differences in transfection efficiencies between CD8 and CD4 T cells, or between PBLs and TILs, were observed (data not shown).

Example 2. Expression via mRNA transfection of caTLR4, but not caTLR2, potently activates human CD4 and CD8 T cells to secrete IFN-\( \gamma \).

[00143] The materials, cells and methods used, are all described in detail in US 2008/0286312 and WO 08/041231, hereby incorporated by reference in its entirety as if fully disclosed herein. Accordingly, the transmembrane (tm) and cytoplasmic (cyt) portion of both mouse and human TLR2 and TLR4 were engrafted as the ‘anchor’ segment in the \( \delta \)2m modality, depicted in Fig. 2 at the gene level as the Xhol-NotI fragment. The respective DNA stretches were amplified by RT-PCR performed on RNA from RAW264.7 cells using specific oligonucleotide primers, and were provided with suitable restriction sites for cloning. In all experiments described in the examples we used human \( \delta \)2m (h\( \delta \)2m).

[00144] In several experiments we performed with human T cells grown from PBLs of healthy donors or with TILs from melanoma patients we could detect no significant induction of IFN-7 production, neither by our GFP-TLR2 mRNA, nor by the TLR2 ligand BLP (Pam3CysSK4, or Pam3 in short - not shown). We therefore decided to include in these experiments mRNA encoding caTLR4, which has been highly functional in our previous experiments with human dendritic cells (DCs). Ex-vivo propagation, separation and mRNA transfection of CD4 and CD8 T cells cultured from PBLs of a healthy donor were performed exactly as described in Example 1. Cells were separated by CD4 magnetic
beads (BD IMag anti-human CD4 particles) positive selection, grown for additional 24 hours in the presence of 0.5 \( \mu g/ml \) each anti-CD3 and anti-CD28 mAbs and subjected to electroporation with 5 \( \mu g \) mRNA using a square-wave pulse at 400 V, 0.95 msec in 0.1 ml in 2 mm cuvettes. Control cells were either non-treated (NT), incubated in the presence of LPS or Pam3 (1 \( \mu g/ml \) each) or transfected with EGFP-encoding mRNA as a negative control. Secretion of IFN-\( \gamma \) the growth medium was monitored by ELISA (R&D systems Human IFN-gamma DuoSet Economy Pack) 24 h post-transfection.

Results of the first experiment in which we examined caTLR4 are shown in Fig. 3 and reveal extraordinary activation of both CD4 and CD8 T cells, but no activation, or marginal, at best, with caTLR2 or either TLR4 or TLR2 ligands (LPS and Pam3, respectively).

We do not have a clear idea why caTLR2 (or the TLR2 ligand Pam3) failed to activate T cells in our experiment. Yet, our results suggest that although human T cells express little TLR4, all the associated signaling components are fully functional and allow caTLR4 to trigger robust activation.

The caTLR4 constructs we used in the last experiment encode an HLA-A2-binding tumor-associated peptide linked to \( \beta 2m \), while the donor was HLA-A2+. To rule out any possible activation by the peptide, we performed an additional experiment in which we compared the dominant positive effect of the peptide-P2m-TLR4 to that of a peptide-less construct (p2m-TLR4 only), on CD4 and CD8 T cells from an HLA-A2+ as well as an HLA-A2- donor, propagated as described in Example 1, using either 5 \( \mu g \) (as before) or as little as 1 \( \mu g \) mRNA (Figs. 4A-B). Secretion of IFN-\( \gamma \) to the growth medium was monitored by ELISA two days post-transfection (maximal readout of this assay was 1000 pg/ml). This experiment showed that:

1. The linked peptide has no significant effect on the activation status of the transfected T cells;
2. caTLR4 is active in CD4 and CD8 T cells of both donors; and
3. Transfection with 1 \( \mu g \) mRNA suffices to exert maximal, or close to maximal, effect.

This latter finding is surprising and attests to the remarkable potency of caTLR4 in this context, which also bears important implications on practical aspects of the potential use of this procedure in ACT in the treatment of cancer.
To test the ability of caTLR4 to potentiate melanoma specific lymphocytes, we transfected human melanoma derived primary TILs and CTL clones with caTLR4 according to the experimental details described in Example 2. In short, a Melan-A27-35/HLA-A2-specific CTL clone, 1C9, isolated from a human melanoma patient by tetramer sorting, were thawed and incubated in culture medium and 6000 u/ml IL-2 was added every 2 days (for the method of tetramer sorting, see for example Ogg and McMichael (1998) Current Opinion in Immunology, 10: 393-396). Electroporation was preformed 5 days post thawing. TIL 122, isolated from a human melanoma tumor, were thawed and incubated for 2 days in culture medium containing 6000 u/ml IL-2. At day 3, CD4 positive magnetic beads separation was preformed (BD IMag anti-human CD4 particles). After separation the cells were incubated in 6 ml culture medium and 6000 u/ml IL-2 was added every 2 days. Electroporation was preformed 7 days post thawing. After the electroporation the cells were incubated in 12 well-plates in 3 ml culture medium in the absence of IL-2. 24 h later the supernatant was collected and IFN-7 was measured using R&D systems' Human IFN-gamma DuoSet Economy Pack.

Table 1. IFN-γ production by melanoma derived CTL clone TIL 1C9, anti-melan A

<table>
<thead>
<tr>
<th>TLR-ligand</th>
<th>Non</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone/Treatment</td>
<td>541 B2m-</td>
<td>540 GFP</td>
</tr>
<tr>
<td>IFN-γ, pg/ml</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Average</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. IFN-γ production by TIL 122

<table>
<thead>
<tr>
<th>T-cell type</th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone/Treatment</td>
<td>541</td>
<td>931</td>
</tr>
<tr>
<td>IFN-γ, pg/ml</td>
<td>312</td>
<td>&lt;1000</td>
</tr>
<tr>
<td>Average</td>
<td>265</td>
<td>276</td>
</tr>
</tbody>
</table>

Two examples of these experiments are summarized in Tables 1 and 2, which shows IFNγ production by 1C9 and TIL 122 cells, respectively. The results clearly
demonstrate that caTLR4, but not TLR ligands, electroporation or irrelevant RNA triggered \( \text{IFN}\gamma \) production in anti-rumor specific lymphocytes.

[00151] It is known that in CTLs, IL-2Ra (CD25) and CD69 elevation is linked to the effector phenotype of CTLs and was reported as an activation marker following CTL incubation with the TLR2 ligand, Pam3.

[00152] To test the effect of caTLR4 on these markers, The MelanA specific CTL clone 1C9, or the primary TIL 431-4, was transfected with caTLR4 or irrelevant RNAs (GFP). As seen in Figs 4A-B, caTLR4 induced remarkable elevation of both CD25 (1C9; Fig. 5A) and CD69 (431-4, Fig 5B). Two other markers, NKG2D, an activating receptor that is expressed by T cells, as well as by natural killer (NK) cells and macrophages, and CD137, a lymphocyte activation marker also known as 4-1BB, were not elevated by the transfection with caTLR4.

[00153] This shows that constitutively TLR4-activated tumor-specific lymphocytes not only secreted IFN-\( \gamma \), but also up-regulate effector phenotype markers, which indicate that this simple genetic approach provides an effective means for equipping effector T cells with the arsenal of functional attributes required for the improvement of current ACT protocols, without the need for adjuvants, TLR ligands or microbial substances.

Example 3. Generation of constitutively active oligomeric CD40 (caCD40) based on the yeast leucine zipper motif GCN4,

In order to gain full CD8 T cell priming capacity, DCs must complete their maturation and secrete IL-12. Acquisition of this capacity requires initial triggering of the DC via TLRs (or other innate pathogen-recognition receptors), followed by licensing signals mediated by the engagement of trimeric CD40 ligand (CD40L) at the surface of activated Th1 cell with the DC CD40 receptor. We have previously demonstrated that the mere expression of the CD40 signaling domain in the context of \( \beta 2 \)m could induce some activation of the NF-\( \kappa B \) signaling pathway.

[00154] As mentioned above, full CD40-mediated DC activation requires oligomerization of CD40. This can be achieved by soluble CD40L, anti-CD40 antibodies and or cross-linking of genetically engineered CD40. These approaches cannot be incorporated into our genetic platform. To overcome this obstacle, we have generated a new set of genetic constructs harboring the GCN4 yeast transcriptional activator, which
contains a leucine zipper motif that induces homophilic interactions. We have used the /32m-CD40 genetic construct as a template, and incorporated the GCN4 binding motifs in 3 configurations encoding dimers, trimers and tetramers, based on Harbury et al, (1993; 1995) (see Figs. 6A-C) using the primers for PCR-mediated cloning having the following sequences: For the dimers, primers 1133 and 1134 of SEQ ID NO: 30 and 31, respectively; for the trimers, primers 1135 and 1136 of SEQ ID NO: 32 and 33, respectively; and for the tetramers, primers 1137 and 1138 of SEQ ID NOs: 34 and 35, respectively. In addition, the primers 1128 and 1129 having the sequences of SEQ ID NOs: 36 and 37, respectively, were used.

[00155] The basic scheme of the genetic design and the expected configurations of both peptide-32m-GCN4-CD40 and His-GCN4-CD40 are illustrated in Figs. 6A-C.

Example 4. Generation of membrane bound cytokines and characterization of their activity in transtransfected T cells alone or in combination with caTLR4 or caCD40.

[00156] To tackle the two-sided problem of systemic IL-2 toxicity and insufficient IL-2 support for the transferred cells we have genetically engineered IL-2 as an integral membranal constituent of transfected cells. We also constructed chimeric genes encoding membranal single chain IL-12 and IL-15.

[00157] These chimeric genes comprises the sequence of SEQ ID NO: 23 encoding for the transmembrane and cytoplasmic portion of HLA-A2 and the sequence of SEQ ID NO: 24 encoding for a bridge peptide; and

[00158] for IL-2: the full human IL-2 and its leader peptide sequence is encoded by the sequence of SEQ ID NO: 25;

[00159] for IL-12: the chimeric single chain derivative of human IL-12 is encoded by the sequence of SEQ ID NO: 26 encoding for the full human IL-12 p40, including the leader peptide, the sequence of SEQ ID NO: 27 encoding for the linker peptide, and the sequence of SEQ ID NO: 28 encoding for the human IL-12 p35 without the leader peptide (see Fig. 7); and

[00160] for IL-15: chimeric human IL-15 is encoded by SEQ ID NO: 5 encoding for the leader peptide of human β2m, and SEQ ID NO: 29 encoding for the mature peptide of
human IL-15. The person skilled in the art can easily introduce these sequences into a vector for in vitro RNA transcription using generally accepted techniques known in the art.

Membranal (mem) IL-2, IL-12 and IL-15 are designed with a flexible spacer so as to engage its cell surface receptor in-cis (that is, on the same cell). To test this modality, CD8 T cells, from PBLs of a healthy donor, were grown for 4 days in OKT3 and IL-2 and separated to CD8 and CD4 T cells (OKT3 (Janssen-Cilag, or collected from a hybndoma available in our lab) is a mouse monoclonal that binds to and blocks function of T lymphocytes responsible for antigen recognition) Thereafter, the cells were electroporated with memIL-2 and irrelevant mRNA(s) or treated as indicated (Fig. 8).

Cell proliferation was measured by carboxyfluorescein succinimidyl ester (CFSE) dilution assay. CFSE is an effective and popular means to monitor lymphocyte division. CFSE covalently labels long-lived intracellular molecules with the fluorescent dye, carboxyfluorescein. Thus, when a CFSE-labeled cell divides, its progeny are endowed with half the number of carboxyfluorescein-tagged molecules and thus each cell division can be assessed by measuring the corresponding decrease in cell fluorescence via flow cytometry.

As can be seen in Fig. 8, the experiment demonstrated the ability of memIL-2 to support T cell proliferation for at least 6 days, and the ability of the combined expression of memIL-2 with caTLR4 to further increase the propagation rate in the total absence of soluble IL-2.

Next we tested the combined effects of memIL-2, caCD40 and caTLR4 on CTLs. To this end, melanoma TILs were thawed and grown in complete medium in the presence of 2,000 U/ml IL-2. Four days post-thawing cells were electroporated with 10 μg of each mRNA. 24 hours post-transfection, growth medium was collected for IFN-γ ELISA. Fig 9 shows that memIL-2, memIL-12 and caCD40 work synergistically with caTLR4, as can be seen by the tremendous increase in secreted IFN-γ. This figure also show that caCD40 and to a lesser extent, caTLR4, are capable of activating the TILs alone.

M427 is a primary melanoma cells that are recognized by its autologous TIL-425. Although these cells recognize its target, they are considered exhausted and exhibit very low killing activity. To test whether memIL-2, caCD40 and caTLR4 can restore its functional activity, TIL-425 were thawed and cultured with 3000 u/ml IL-2 for 4 days. The cells were grown for an additional 3 days to wash out the direct mRNA effect (measured by IFN-γ production). Thereafter 1X10⁵ cells were incubated at 1/1 ratio with
its targets melanoma cells for 5 hrs. The M171 melanoma cells served as a negative control. Functional activity was measured by IFN-γ production and degranulation (as measured by expression of CD107). As shown in Fig. 10A, transfection of the cells with the 3 constructs restored its functional activity, while irrelevant RNA had no effect (Fig. 10B). Also in this experimental setting, there is clear synergy between the caTLR4 and caCD40.

Example 5. ACT using tumor-specific T cells expressing membranal cytokines, caTNFR or caTLR4, or their combinations.

Evaluation of the anti-tumor activity of gene-modified TILs in nude mice. Nude mice are used for comparative evaluation of T cell tumoricidal activity in-vivo, using a transplantable human melanoma. For this purpose we utilize the human melanoma cell line 624mel. Briefly, male CDlnu/nu mice are injected s.c. in the upper back with 1x10⁶ 624mel melanoma cells admixed 1:1 with Matrigel. Five days later mice are divided into groups of ten and each mouse is injected in the tumor area or IV with 1x10⁶ 624mel-reactive CD8 T cells cloned from anti-melanoma TILs. Prior to injection, all T cells from each group (>10x10⁶) will be electroporated in a single cuvette with the respective in-vitro transcribed mRNA under investigation, or irrelevant mRNA as control and then divided for cell transfer. Additional control groups receive none-modified T cells or no T cells. Mice are injected with 1,000 units IL-2 into the tumor site twice daily for 5 days and survival is monitored daily.

The in-vitro transcribed mRNA tested in this experiment encode for caTLR4, caCD40, caCD27, ca4-IBB (CD137), caOX40 (CD134), caHVEM, caCD30 and caGITR as well as memIL-2, memIL-12 and memIL-15. Also T cells transfected with combinations thereof will be used. First, caTLR4, caCD40 and mem-IL2 expressing T cells will be examined, and later other combinations.
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CLAIMS

1. A polynucleotide comprising a sequence encoding a chimeric protein comprising a cytoplasmic domain of a T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family that is linked to a heterologous polypeptide comprising at least one self-assembly domain, said chimeric peptide being linked to an integral membrane or a transmembrane oligopeptide that allows the anchorage of the chimeric polypeptide to the cell membrane.

2. The polynucleotide according to 1, wherein said T cell costimulatory receptor of the TNFR family is selected from CD40, CD27, 4-1BB (CD137), OX40 (CD134), herpesvirus entry mediator (HVEM, TNFRSF14), CD30 or glucocorticoid-induced TNFR-related protein (GITR).

3. The polynucleotide according to claim 2, wherein said T cell costimulatory receptor of the TNFR family is a human T cell costimulatory receptor of the TNFR family.

4. The polynucleotide according to claim 1, wherein said self-assembly domain is a yeast GCN4 leucine zipper DNA-binding motif.

5. The polynucleotide according to claim 4, wherein said yeast GCN4 leucine zipper DNA-binding motif is of SEQ ID NO: 2 forming homodimers, SEQ ID NO: 3 forming homotrimeres or SEQ ID NO: 4 forming homotetramers.

6. The polynucleotide according to claim 1, wherein said transmembrane oligopeptide is the transmembrane oligopeptide of CD40.

7. The polynucleotide according to claim 1, wherein said transmembrane oligopeptide is linked, optionally via a bridge peptide comprises an amino acid stretch of about 5 amino acids to about 15 amino acids, preferably 13 amino acids of the membrane-proximal amino acids of human HLA-A2, to a peptide tag or oligopeptide tag.

8. The polynucleotide according to claim 7, wherein said peptide tag is selected from a Ha tag, a His tag or a Myc tag.

9. The polynucleotide according to claim 8, wherein said polynucleotide comprises the following nucleotide sequences:
a. the sequence of SEQ ID NO: 5 encoding for the leader peptide of the human \( \beta_2 \)m gene;

b. a sequence encoding for a peptide tag for detection selected from:
   i. Ha tag of SEQ ID NO: 6;
   ii. Myc tag of SEQ ID NO: 7; or
   iii. His tag of SEQ ID NO: 8;

c. the sequence of SEQ ID NO: 9 encoding a bridge peptide;

d. the sequence of SEQ ID NO: 10 encoding for the transmembrane domain of human CD40;

e. the sequence of SEQ ID NO: 11 encoding for a GCN4-derived homooligomerizing domain forming homotrimers; and

f. a sequence encoding for the cytosolic signaling domain of a T cell costimulatory receptor of the TNFR family selected from:
   (iv) human CD40 of SEQ ID NO: 12;
   (v) human 4-IBB of SEQ ID NO: 13;
   (vi) human OX40 of SEQ ID NO: 14;
   (vii) human CD27 of SEQ ID NO: 15; or
   (viii) human GITR of SEQ ID NO: 16; or

g. nucleotide sequences substantially identical to the sequences of (a)-(f).

10. The polynucleotide according to claim 9, comprising said sequence of (iv)
    encoding for the cytosolic signaling domain of human CD40.

11. The polynucleotide according to claim 1, comprising a further sequence encoding a
    chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4
    molecule linked through its amino terminus to an integral membrane or transmembrane
    oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane, and
    said transmembrane oligopeptide, if existent, is linked, optionally via a bridge peptide, to a
    peptide tag or oligopeptide tag.

12. A polynucleotide comprising a sequence encoding a chimeric protein comprising a
    cytoplasmic constitutively active domain of a TLR4 molecule linked through its amino
    terminus to an integral membrane or transmembrane oligopeptide that allows the
    anchorage of the TLR4 molecule to the cell membrane, and said transmembrane
oligopeptide, if existent, is linked, optionally via a bridge peptide, to a peptide tag or oligopeptide tag.

13. The polynucleotide according to claim 11 or 12, wherein said transmembrane oligopeptide is the transmembrane oligopeptide of TLR4.

14. The polynucleotide according to claim 13, wherein said TLR4 is human TLR4.

15. The polynucleotide according to claim 13, wherein said bridge peptide comprises an amino acid stretch of about 5 amino acids to about 15 amino acids, preferably 13 amino acids of the membrane-proximal amino acids of human HLA-A2.

16. The polynucleotide according to claim 13, wherein said peptide tag is selected from a Ha tag, a His tag or a Myc tag.

17. The polynucleotide according to any one of claims 11 to 16, wherein said polynucleotide comprises the following nucleotide sequences:

a. the sequence SEQ ID NO: 17 encoding for the 5' non-translated region of β2m;

b. the sequence SEQ ID NO: 18 encoding for the human β2m leader peptide;

c. a sequence encoding for a peptide tag for detection selected from:
   i. Ha tag of SEQ ID NO: 6;
   ii. Myc tag of SEQ ID NO: 7; or
   iii. His tag of SEQ ID NO: 8;

d. the sequence of SEQ ID NO: 9 encoding a bridge peptide;

e. the sequence SEQ ID NO: 19 encoding for human transmembrane and cytosolic domains of TLR4; and

f. the sequence SEQ ID NO: 20 encoding for the 3' non-translated region of β2m, or

g. polynucleotide sequences substantially identical to the sequences of (a)-(f).

18. The polynucleotide according to any one of claims 1 to 18, wherein said transmembrane oligopeptide is linked via a flexible bridge peptide to the carboxyl terminus of a cytokine.
19. The polynucleotide according to any one of claims 1 to 18, comprising a further sequence encoding a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA,

20. The polynucleotide according to claim 1, comprising a further sequence encoding a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic constitutively active domain of a TLR4 molecule.

21. The polynucleotide according to claim 12, comprising a further sequence encoding a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic domain of a T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family.

22. A polynucleotide comprising a sequence encoding a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA.

23. The polynucleotide according to any one of claims 18 to 22, wherein said cytokine is selected from IL-2, IL-12 or IL-15.

24. The polynucleotide according to claim 23, wherein said cytokine is a human cytokine.

25. The polynucleotide according to claim 23, wherein said bridge peptide comprises an amino acid stretch of about 5 amino acids to about 15 amino acids, preferably 8 amino acids of the membrane-proximal amino acids of human HLA-A2.

26. The polynucleotide according to claim 25, wherein said bridge peptide further comprises the 13 amino acid flexible linker Gly$_4$Ser(Gly$_3$Ser)$_2$ (SEQ ID NO: 21).
27. The polynucleotide according to claim 23, wherein said transmembrane oligopeptide, if existent, is the transmembrane oligopeptide of HLA-A2 and is linked to the cytoplasmic domain of HLA-A2.

28. The polynucleotide according to claim 24, wherein said polynucleotide comprises the following nucleotide sequences:

   a. the sequence of SEQ ID NO: 23 encoding for the transmembrane and cytoplasmic portion of HLA-A2;
   b. the sequence of SEQ ID NO: 24 encoding for a bridge peptide;
   c. a sequence encoding for a cytokine selected from IL-2, IL-12 or IL-15, wherein:
      i. the full human IL-2 and its leader peptide sequence is encoded by the sequence of SEQ ID NO: 25;
      ii. the chimeric single chain derivative of human IL-12 is encoded by the sequence of SEQ ID NO: 26 encoding for the full human IL-12 p40, including the leader peptide, the sequence of SEQ ID NO: 27 encoding for the linker peptide, and the sequence of SEQ ID NO: 28 encoding for the human IL-12 p35 without the leader peptide; and
      iii. chimeric human IL-15 is encoded by SEQ ID NO: 5 encoding for the leader peptide of human \( \beta \)-m, and SEQ ID NO: 29 encoding for the mature peptide of human IL-15; or
   d. nucleotide sequences substantially identical to the sequences of (a)-(c).

29. The polynucleotide according to any one of claims 1 to 28, which is an RNA molecule.

30. An expression vector comprising a polynucleotide according to any one of claims 1 to 28.

31. The expression vector according to claim 30, comprising a nucleic acid sequence encoding an RNA molecule in a manner which allows transcription of said nucleic acid sequence in vitro.
32. A composition comprising at least one polynucleotide according to any one of claims 1 to 29 or the expression vector according to claim 30.

33. The composition according to claim 32, comprising a polynucleotide comprising a sequence encoding:

a. a chimeric protein comprising a cytoplasmic domain of a T cell costimulatory receptor of the TNFR family that is linked to a heterologous polypeptide comprising at least one self-assembly domain, said chimeric peptide being linked to an integral membrane or a transmembrane oligopeptide that allows the anchorage of the chimeric polypeptide to the cell membrane;

b. a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through its amino terminus to an integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane, and said transmembrane oligopeptide if existent, is linked, optionally via a bridge peptide, to a peptide tag or oligopeptide tag;

c. a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA;

d. a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic constitutively active domain of a TLR4 molecule;

e. a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a
cytoplasmic domain of a T cell costimulatory receptor of the
tumor necrosis factor receptor (TNFR) family;
f. a combination of (a) and (b);
g. a combination of (a) and (c);
h. a combination of (b) and (c);
i. a combination of (a), (b) and (c);
j. a combination of (d) and (e);
k. a combination of (a) and (d);
l. a combination of (b) and (e).

34. The composition according to claim 33, wherein said T cell costimulatory receptor
of the TNFR family is selected from CD40, CD27, 4-IBB (CD137), OX40 (CD134),
herpesvirus entry mediator (HVEM, TNFRSF14), CD30 or glucocorticoid-induced TNFR-
related protein (GITR).

35. The composition according to claim 33, wherein said cytokine is selected from IL-2, IL-12 or IL-15.

36. The composition according to claim 35, wherein said cytokine is linked to the
transmembrane and cytoplasmic portion of HLA-A2.

37. Activated T cells expressing at least one chimeric protein encoded by the
polynucleotide according to any one of claims 1 to 29 or the expression vector according to
claim 30.

38. The activated T cells according to claim 37, expressing

a. a chimeric protein comprising a cytoplasmic domain of a T
cell costimulatory receptor of the TNFR family that is linked
to a heterologous polypeptide comprising at least one self-
assembly domain, said chimeric peptide being linked to an
integral membrane or a transmembrane oligopeptide that
allows the anchorage of the chimeric polypeptide to the cell
membrane;
b. a chimeric protein comprising a cytoplasmic constitutively
active domain of a TLR4 molecule linked through its amino
terminus to an integral membrane or transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane, and said transmembrane oligopeptide, if existent, is linked, optionally via a bridge peptide, to a peptide tag or oligopeptide tag;

c. a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the cytokine molecule to a cell membrane, with the proviso that said chimeric protein does not comprise IL-2 linked to a glycoinositol phospholipid, IL-12 linked to the costimulatory molecule CD80 (B7.1) or IL-15 linked to IL-15 RA;

d. a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic constitutively active domain of a TLR4 molecule;

e. a chimeric protein comprising a cytokine linked through its carboxyl terminus via a flexible bridge peptide to a cytoplasmic domain of a T cell costimulatory receptor of the tumor necrosis factor receptor (TNFR) family;

f. a combination of (a) and (b);

g. a combination of (a) and (c);

h. a combination of (b) and (c);

i. a combination of (a), (b) and (c);

j. a combination of (d) and (e);

k. a combination of (d) and (c);

l. a combination of (b) and (e).

39. The activated T cells according to claim 38, wherein said T cell costimulatory receptor of the TNFR family is selected from CD40, CD27, 4-IBB (CD137), OX40 (CD134), herpesvirus entry mediator (HVEM, TNFRSF14), CD30 or glucocorticoid-induced TNFR-related protein (GITR), or combinations thereof.
40. The activated T cells according to claim 38, wherein said cytokine is selected from IL-2, IL-12 or IL-15, or combinations thereof.

41. The activated T cells according to claim 38, expressing a chimeric protein comprising a cytoplasmic and transmembrane polypeptide of CD40 that is linked to a yeast GCN4 leucine zipper DNA-binding motif self-assembly domain.

42. The activated T cells according to claim 38, expressing a chimeric protein comprising a cytoplasmic constitutively active domain of a TLR4 molecule linked through a TLR4 transmembrane oligopeptide that allows the anchorage of the TLR4 molecule to the cell membrane.

43. The activated T cells according to claim 38, wherein said cytokine is linked to the transmembrane and cytoplasmic portion of HLA-A2.

44. The activated T cells according to any one of claim 37 to 43, that are tumor-specific T cells.

45. A method for treating cancer, comprising administering to a cancer patient a therapeutically effective amount of activated T cells according to claim 44.

46. The method according to claim 45, wherein said T cells are autologous T cells.

47. The method according to claim 46, wherein said T cells are tumor infiltrating lymphocytes, preferably young tumor infiltrating lymphocytes.

48. The method according to any one of claims 45 to 47, wherein said T cells are directed to tumor cells selected from melanoma, breast cancer; chronic myeloid leukemia; acute lymphoblastic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, acute myeloid leukemia, chronic lymphocytic leukemia, mature B cell neoplasms, mature T cell and natural killer cell neoplasms; liver, stomach, pancreas, intestine and kidney tumors; or bladder, glandular epithelia, lung squamous cell, gut, prostate, testis, thymus, bone marrow, and lymph nodes carcinomas.

49. A method for preparing activated T cells comprising transfecting said T cells with a polynucleotide according to any one of claims 1 to 29, the expression vector according to
claim 30 or the composition according to any one of claims 32 to 36, whereby the polynucleotide is expressed within said T cells, thus obtaining the activated T cells.

50. A method for propagating T cells, comprising transfecting said T cells with a polynucleotide comprising a sequence encoding a chimeric protein comprising IL-2 linked through its carboxyl terminus via a flexible bridge peptide to an integral membrane or transmembrane oligopeptide that allows the anchorage of the IL-2 molecule to a cell membrane.

51. The method according to claim 50, wherein said method does not involve contacting the cells with a soluble cytokine.
Fig. 1

Fig. 2A

Fig. 2B

SUBSTITUTE SHEET (RULE 26)
Fig. 3

Fig. 4A

Fig. 4B

SUBSTITUTE SHEET (RULE 26)
### Fig. 10A

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### Fig. 10B

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