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(54) Title: VALIDATION OF THERAPEUTIC T-CELLS

(57) Abstract: Provided herein are compositions and methods for monitoring the safety of immunotherapy. In particular, provided herein are inactive T-cells and uses thereof.

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VALIDATION OF THERAPEUTIC T-CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present Application claims priority to U.S. Provisional Application Serial Number 62/331,049 filed May 3, 2016, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

Provided herein are compositions and methods for monitoring the safety of immunotherapy. In particular, provided herein are inactive T-cells and uses thereof.

BACKGROUND OF THE INVENTION

When a T cell binds its target through its T-cell receptor (TCR), a series of intracellular reactions take place leading to effector functions. A variety of factors will determine the fate of the T-cell, the strength of the binding of the TCR extracellular part to the peptide-MHC complex (pMHC) being the first. If this first signal is strong enough, recruitment of signalling machinery will occur at the intracellular side of the TCR. They will, through the help of scaffolding proteins build a complex (signalosome) that will turn on a specific cascade leading to dramatic intracellular modifications (1, 2). The TCR cascade is not just simply turned on, but constitutively stimulated by a so-called tonic signal, and the balance between negative and positive regulators will determine the degree of activation (3). A very powerful negative regulator of TCR signal is a kinase, c-terminal SCR kinase (CSK) (4). The activity of this kinase is restricted to the phosphorylation of the inhibitory tyrosine of SCR family kinases (5), but it can also regulate LYP, a tyrosine phosphatase, by binding it (6). Upon TCR stimulation Lck is recruited to the vicinity of the CD3-TCR complex and phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) motifs. These domains, once phosphorylated, become anchoring points for the recruitment of the TCR-signalling machinery. CSK is believed to tune down this signalling activation by phosphorylating Lck on its inhibitory tyrosine, Tyr^{505}. This regulation is extremely important to regulate the basal level of Lck, hence the TCR signalling, even in steady state. It was elegantly demonstrated that selective inhibition of CSK was sufficient to turn on TCR intracellular signalling cascade, without extracellular stimulation (7). In addition, the same group showed that CSK played a pivotal role in the TCR orientation at the immune synapse by regulating actin remodelling (8).

The modulation of TCR signalling has been exploited in adoptive cell therapy (9). Although antigen receptor recognition should be sufficient to trigger a complete response against target cells, T-cell exhaustion or presence of inhibitory receptor such as PD-1 or CTLA-4 might hinder the potency of T-cell therapy (10). By influencing the balance in the tonic basal signal, one might increase TCR stimulation, hence improve the cytotoxic effect expected (11-15). However, although the signalling may be improved, the specificity of TCR binding intended for clinical use still remains a serious issue (16). Safe guard methods have been proposed to control and block the unexpected side effects (17, 18). Nevertheless, pre-clinical or early clinical validation of TCR selectivity and specificity still requires important innovations since the kinetics of these safe guards might be too slow to rescue patient if T-Cell activation against an unpredicted target or at an unpredicted site has occurred.

SUMMARY OF THE INVENTION

Provided herein are compositions and methods for monitoring the safety of immunotherapy. In particular, provided herein are inactive T-cells and uses thereof.

In some embodiments, the present invention provides a method of monitoring T-cell based therapy, comprising: a) administering a dummy T-cell to a subject, wherein the dummy T-cell does not function to kill cells or promote an immune response, and wherein the dummy T-cell comprises a detectable label; and b) detecting the location of the dummy T-cell in the subject. In some embodiments, the dummy T-cell is a modified therapeutic T-cell. It will be understood that the term "dummy T-cell" refers herein to a T-cell that has been engineered so that normal T-cell function has been inhibited or ablated. In some preferred embodiments, the inhibition of normal T-cell function refers to the inhibition of the ability of the T-cell to function to kill cells (i.e., inhibition of killer cell function) or to promote an immune response.

In some embodiments, the dummy T-cell overexpresses C-terminal SRC kinase (CSK). In some embodiments, the CSK is expressed in an amount sufficient to inhibit or ablate normal T-cell function as described above. In some embodiments, the dummy cell is engineered to overexpress CSK by introducing an expression vector comprising a promoter operably linked to a nucleic acid sequence encoding CSK into the dummy T-cell. In some embodiments, the nucleic acid encoding CSK encodes an amino acid sequence that has at least 80%, 90%, 95%, 97%, 98%, 99% or 100% identity to SEQ ID NO:6. Where the CSK is a variant CSK, it will be understood that the variant retains the biological activity wild type

CSK, i.e., phosphorylation of tyrosine residues located in the C-terminal end of Src-family kinases (SFKs) including, for example, SRC, HCK, FYN, LCK, LYN and YES1.

In some embodiments, the dummy T-cell is specific for a target epitope. In some embodiments, the dummy T-cell is a tumor infiltrating lymphocyte expressing a chimeric antigen receptor, or a T-cell expressing a heterologous T-cell receptor. In some embodiments, the method further comprises the step of administering a therapeutic T-cell to the subject. In some embodiments, the method further comprises the step of visualizing the location of the detectable label in subject. In some embodiments, the presence of said detectable label in a location distinct from the target location of a target epitope is indicative of non-specific binding by the dummy T-cell. In some embodiments, the dummy T-cells can be used to detect unspecific localization of the cells *in vivo* after parenteral administration. Unspecific localization may indicate that use of corresponding therapeutic T-cells would trigger unnecessary or harmful immune responses. However, if the dummy T-cells are localized at the desired site(s), as can be verified by the label, it would indicate that the immune response would be triggered at these sites. Accordingly, compositions comprising such dummy T-cells can be used in confirmation of specific localization of the cells *in vivo* after parenteral administration. The difference between a dummy T-cell and a corresponding therapeutic T-cell may be overexpression of CSK only. In some embodiments, the subject is a human subject or a non-human mammal. In some embodiments, the target site is a tumor site.

Further embodiments provide a kit or system, comprising: a) a dummy T-cell, wherein the dummy T-cell does not function to kill cells or promote an immune response, and wherein the dummy T-cell comprises a detectable label; b) a detection component for detecting the location of the dummy T-cell in the subject.

Further provided is a genetically modified human T-cell comprising a detectable label and a heterologous DNA sequence encoding a membrane bound receptor specific for a desired target epitope, wherein the genetically modified T-cell overexpresses CSK in an amount sufficient for preventing significant TCR signaling, and wherein the membrane bound receptor for the desired target epitope is expressed in an amount sufficient for specific binding of the T-cell to the desired target. In such T-cells, the membrane bound receptor for the desired target may be expressed in an amount sufficient for specific localization of the T-cell after parenteral administration and the CSK may be overexpressed in an amount sufficient for preventing an immune response. In one particular embodiment, the membrane bound receptor is specific for a cancer antigen. In another particular embodiment, the membrane bound receptor is a T-cell receptor. In some embodiments, the genetically

modified human T-cell is engineered to overexpress CSK by introducing an expression vector comprising a promoter operably linked to a nucleic acid encoding CSK into the T-cell. In some embodiments, the nucleic acid encoding CSK encodes an amino acid sequence that has at least 80%, 90%, 95%, 97%, 98%, 99% or 100% identity to SEQ ID NO:6. Where the CSK is a variant CSK, it will be understood that the variant retains the biological activity wild type CSK, i.e., phosphorylation of tyrosine residues located in the C-terminal end of Src-family kinases (SFKs) including, for example, SRC, HCK, FYN, LCK, LYN and YES1.

Further provided is a composition comprising the dummy T-cells above for use in confirmation of specific localization of the cells *in vivo* after parenteral administration, e.g. injection.

Additional embodiments are described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A-C: Overexpression of CSK potently suppresses TCR-induced phosphorylation of ERK**.** (A) The design of the vector used for overexpression of CSK. CSK_2A_GFP construct consists of a fusion of the CSK coding sequence where the STOP codon was removed and replaced by a picornavirus 2A coding sequence, followed by the GFP gene sequence. Upon transcription, one mRNA is produced and transcribed into two distinct proteins. PBMC-derived T-cells were transduced with control vector (GFP), CSK-GFP or mCSK-GFP. Incubation with αCD3 and αCD28 Abs were used to activate TCR, and signaling was detected by phospho-specific flow cytometry. (B) Histogram overlays from one representative experiment. The color code shows induced phosphorylation level, relative to unstimulated cells, using arcsinh transformation of the median fluorescence intensity. (C) αCD3/CD28-induced phosphorylation of CD3z, ZAP70 and ERK, relative to unstimulated cells. Mean ± SEM, n=4 (control, CSF) or n=3 (mCSK) of arcsinh transformed data. *$P<0.05$, paired t-test.

FIG. 2A-B: mCSK is more potent than CSK in suppressing TCR-induced distal signaling events. PBMC-derived T-cells were transduced with therapeutic TcR (TCR specific for TGFβRII frameshift peptide, termed Radium 1 TCR). The transduced T-cells were then expanded and transiently transfected with GFP, CSK-GFP or mCSK-GFP mRNA. SupT1 cells were loaded with relevant (TGFβRII peptide), or irrelevant peptide (MART1p) were used as APC1 and APC2, respectively and added to the T-cells to induce TCR

signaling. (A) One representative experiment. (B) TCR-induced phosphorylation of SLP76 and ERK, relative to unstimulated cells. Mean ± SEM, n=2 of arcsinh transformed data.

FIG. 3A-C: CSK inhibited TCR stimulation cellular consequences. (A) J76 cells were transduced with the indicated constructs and sorted. The expression of the DMF5 TCR (anti-CD3) and/or CSK_2A_GFP were analyzed by flow cytometry. (B) The same cells as in (A) were incubated with SupT1 cells expressing single chain trimer (SCT) molecules presenting MART1 peptide (SCT-MART-1p) or an irrelevant peptide (SCT-irr) for 12 hours. Supernatants were harvested and analyzed for the presence of IL-2 by ELISA. This is representative of two separate experiments. Mean± S.D., n=2. (C) T-cells isolated from PBMCs from a healthy donor were first transduced with Radium-1 TCR and then with the indicated constructs. T-cells were then incubated with APC as in (B) or left alone for 5 hours. Presence of the degranulation marker CD107a was performed to monitor T-cell stimulation. This is representative of three separate experiments. Mean± S.D., n=2.

FIG. 4A-C: TCR membrane localization is increased when CSK is overexpressed. (A) J76 cells transduced with DMF5 TCR and the indicated constructs were stained using MART-1 multimers and binding was analyzed by flow cytometry. The Geometric mean was plotted and each cell population was split in GFP positive and negative (dark green and light green, respectively) in order to compare cells from the same tube. This is single staining representative of two separate experiments. (B) The same experiment as in (A) was repeated but this time in the presence of dasatinib. (C) Sorted J76 cells expressing DMF5 TCR together with the indicated constructs were stained with CD3 antibody and the geometric means of the signal was plotted. This is representative of two separate experiments. Mean± S.E.M., n=2. *P<0.05, paired t-test.

FIG. 5A-C: Trogocytosis is not affected by CSK overexpression. A) APC (T2 cells) were loaded or not with a relevant (TGFβRIIp) or an irrelevant peptide (MART-1p, M1p) O/N prior to co-incubation with J76 cells constitutively expressing TCR-Radium1 in combination with GFP (control), CSK_2A_GFP or mCSK_2A_GFP. Presence of CD3 was then monitored on T2 cells and the Geometric mean plotted. (B) Same as in (A) but GFP^+ cells (J76) were gated and the presence of HLA-A2 detected. (C) The variation of HLA-A2 signal in T2 cells was analyzed. This is representative of two separate experiments. Mean± S.D., n=2. *P<0.05, paired t-test.

FIG. 6: TCR-dependent target cell attachment is increased by CSK. T-cells were electroporated with mRNA encoding for Radium 1 TCR and either GFP or the CSK_2A_GFP constructs. HeLa cells were used as APCs and were transfected to express

SCT presenting the TGFβ-RII peptide (SCT-TGF) or an irrelevant peptide (MART-1, SCT-M1). T-cells were incubated with HeLa cells for 15 minutes prior to live cell imaging. A total of 20 pictures of each co-culture were taken; 10 pictures before washing (data not shown) and 10 pictures after washing the plate. GFP+T-cells bound to APCs were counted per frame. The dots represent the number of counts per frame after washing. This is representative of two separate experiments. Mean ± S.D., n=10. $**P<0.02$, paired t-test.

FIG. 7: CSK_2A_GFP construct validation in T-cells. When CSK_2A_GFP was transduced into T-cells from PBMC, GFP signal correlated with CSK expression detected by intracellular staining.

FIG. 8: T-cells transduction. Radium-1 expression as detected using specific V-beta antibody and analyzed by Flow cytometry.

FIG. 9: Gating strategy used for the MART1 multimer binding study. Green are dmf5 expressing T-cells and purple are DMF5/GFP (here mCSK) double positive population.

FIG. 10: Redirected T-cells expressing CSK or mCSK are unable to kill their targets. PBMC derived T-cells were electroporated with the indicated constructs. Eight hours later they were incubated with target cells pre-loaded with Europium at an Effector to target ratio (E:T) of 50:1 and killing was determined 2 hours later. Percent target cell lysis was calculated from triplicates, Mean± S.D. The figure is representative of two separate experiments. $*P<0.05$,$**P<0.01$ paired t-test.

FIG. 11: Amino acid sequence for tyrosine-protein kinase CSK [Homo sapiens], NCBI Reference Sequence: NP_001120662.1 (SEQ ID NO:6).

DEFINITIONS

The term “variant" and "mutant" when used in reference to a polypeptide refer to an amino acid sequence that differs by one or more amino acids from another, usually related polypeptide. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties. One type of conservative amino acid substitutions refers to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; unnatural amino acids like p-aminophenylalanine, a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group

of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. More rarely, a variant may have "non-conservative" changes (e.g., replacement of a glycine with a tryptophan). Similar minor variations may also include amino acid deletions or insertions (i.e., additions), or both. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological activity may be found using computer programs well known in the art, for example, DNAStar software. Variants can be tested in functional assays. Preferred variants have less than 10%, and preferably less than 5%, and still more preferably less than 2% changes (whether substitutions, deletions, and so on). For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of lysine with alanine at position 573 is designated as "K573A" and the substitution of lysine with proline at position 573 is designated as K573P. Multiple mutations are separated by addition marks ("+") or "/", e.g., "Gly205Arg + Ser411Phe" or "G205R/S411F", representing mutations at positions 205 and 411 substituting glycine (G) with arginine (R), and serine (S) with phenylalanine (F), respectively.

The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity". For purposes of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends in Genetics 16: 276-277), preferably version 3.0.0 or later. The optional parameters 11644.000-EP7 used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows: (Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment).

The expression "amino acid position corresponding to" a position in a reference sequence and similar expression is intended to identify the amino acid residue that in the primary or spatial structure corresponds to the particular position in the reference sequence. The skilled person will appreciate that this can be done by aligning a given sequence with the reference sequence and identifying the amino acid residue that aligns with the particular position in the reference sequence.

The expression Xnnn is intended to mean an amino acid residue X located in a position corresponding to position nnn in HSA and the expression XnnnY is intended to mean a substitution of any amino acid X located in a position corresponding to position nnn in HSA with the amino acid residue Y.

As used herein, the term "under conditions such that said subject generates an immune response" refers to any qualitative or quantitative induction, generation, and/or stimulation of an immune response (e.g., innate or acquired).

A used herein, the term "immune response" refers to a response by the immune system of a subject. For example, immune responses include, but are not limited to, a detectable alteration (e.g., increase) in Toll receptor activation, lymphokine (e.g., cytokine (e.g., Th1 or Th2 type cytokines) or chemokine) expression and/or secretion, macrophage activation, dendritic cell activation, T-cell activation (e.g., CD4+ or CD8+ T-cells), NK cell activation, and/or B cell activation (e.g., antibody generation and/or secretion). Additional examples of immune responses include binding of an immunogen (e.g., antigen (e.g., immunogenic polypeptide)) to an MHC molecule and inducing a cytotoxic T lymphocyte ("CTL") response, inducing a B cell response (e.g., antibody production), and/or T-helper lymphocyte response, and/or a delayed type hypersensitivity (DTH) response against the antigen from which the immunogenic polypeptide is derived, expansion (e.g., growth of a population of cells) of cells of the immune system (e.g., T-cells, B cells (e.g., of any stage of development (e.g., plasma cells), and increased processing and presentation of antigen by antigen presenting cells. An immune response may be to immunogens that the subject's immune system recognizes as foreign (e.g., non-self-antigens from microorganisms (e.g., pathogens), or self-antigens recognized as foreign). Thus, it is to be understood that, as used herein, "immune response" refers to any type of immune response, including, but not limited to, innate immune responses (e.g., activation of Toll receptor signaling cascade) cell-mediated immune responses (e.g., responses mediated by T-cells (e.g., antigen-specific T-cells) and non-specific cells of the immune system) and humoral immune responses (e.g., responses mediated by B cells (e.g., via generation and secretion of antibodies into the plasma, lymph, and/or tissue fluids). The term "immune response" is meant to encompass all aspects of the capability of a subject's immune system to respond to antigens and/or immunogens (e.g., both the initial response to an immunogen (e.g., a pathogen) as well as acquired (e.g., memory) responses that are a result of an adaptive immune response).

As used herein, the term "immunogen" refers to an agent (e.g., a cancer epitope) and/or portion or component thereof (e.g., a protein antigen)) that is capable of eliciting an

immune response in a subject. In some embodiments, immunogens elicit immunity against the immunogen (e.g., microorganism (e.g., pathogen or a pathogen product)).

The term "test compound" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function, or otherwise alter the physiological or cellular status of a sample. Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (*e.g.*, through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention.

The term "sample" as used herein is used in its broadest sense. As used herein, the term "sample" is used in its broadest sense. In one sense it can refer to a tissue sample. In another sense, it is meant to include a specimen or culture obtained from any source, as well as biological. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include, but are not limited to blood products, such as plasma, serum and the like. These examples are not to be construed as limiting the sample types applicable to the present invention. A sample suspected of containing a human chromosome or sequences associated with a human chromosome may comprise a cell, chromosomes isolated from a cell (*e.g.*, a spread of metaphase chromosomes), genomic DNA (in solution or bound to a solid support such as for Southern blot analysis), RNA (in solution or bound to a solid support such as for Northern blot analysis), cDNA (in solution or bound to a solid support) and the like. A sample suspected of containing a protein may comprise a cell, a portion of a tissue, an extract containing one or more proteins and the like.

DETAILED DESCRIPTION OF THE INVENTION

Provided herein are compositions and methods for monitoring the safety of immunotherapy. In particular, provided herein are inactive T-cells and uses thereof.

Cancer immunotherapy is the use of the immune system to treat cancer. Immunotherapies can be categorized as active, passive or hybrid (active and passive). These approaches exploit the fact that cancer cells often have molecules on their surface that can be detected by the immune system, known as tumor-associated antigens (TAAs); they are often proteins or other macromolecules (e.g. carbohydrates).

Active immunotherapy directs the immune system to attack tumor cells by targeting TAAs. Passive immunotherapies enhance existing anti-tumor responses and include the use of monoclonal antibodies, lymphocytes and cytokines.

Active cellular therapies usually involve the removal of immune cells from the blood or from a tumor. Those specific for the tumor are cultured and returned to the patient where they attack the tumor. Cell types that can be used in this way are natural killer cells, lymphokine-activated killer cells, cytotoxic T-cells and dendritic cells. The only US-approved cell-based therapy is Dendreon's Provenge, for the treatment of prostate cancer.

Adoptive T-cell therapy is a form of passive immunization by the transfusion of T-cells. They are found in blood and tissue and usually activate when they find foreign pathogens. Specifically they activate when the T-cell's surface receptors encounter cells that display parts of foreign proteins on their surface antigens. These can be either infected cells, or antigen presenting cells (APCs). They are found in normal tissue and in tumor tissue, where they are known as tumor infiltrating lymphocytes (TILs). They are activated by the presence of APCs such as dendritic cells that present tumor antigens. Although these cells can attack the tumor, the environment within the tumor is highly immunosuppressive, preventing immune-mediated tumor death.

T cell-based therapies have become increasingly attractive during the past decades. Technically two main methods have been exploited: 1) the isolation of patient's own T-cells derived from peripheral blood or tumor sites (known as Tumor Infiltrating Lymphocytes (TILs)). These cells are expanded ex-vivo and re-injected in the patient. 2) TILs (or any functional T-cells) are isolated from a responding patient (i.e. vaccinated patient). The T-cell Receptor (TcR) of these TILs is subsequently identified (DNA and protein sequences), cloned and expressed in T-cells from a HLA-matched patient, in order to redirect these T-cells against tumor. In addition, other targeting molecules such as the Chimeric Antigen Receptors (CARs, composed of an antibody linked to the signaling domain of the TcR) can also be used to redirect T cells against tumor.

Because adoptive transfer of redirected T cells can be highly efficient and holds promise for long-term immunoprotection, one of the remaining concerns lies in the T cells specificity. It is now well accepted that TcRs are not restricted to one peptide combined with one MHC, but can potentially recognize thousands of MHC-presented peptides. This implies that these molecules potentially can cross-react with sequence related antigens. Another important issue is the target specificity: is the antigen only expressed in tumor tissue. The methods developed to test this have been limited with the result of severe conditions of the

patients, including deaths after T-cell injection, due to unpredicted cross-reactivity or unexpected antigen distribution. Indeed, pre-clinical experiments include SCID mice curing and screening of healthy tissue in vitro. A recent review describes all the unexpected side effects observed since the first therapeutic T-cells were used (Hinrichs and Restifo. 2013 Nat. Biotech.). It shows the difficulty to mimic and predict cross-reactivity and antigen distribution.

A preferred in vivo assay is performed in the same setting as the clinical trial, but with safe version of the T-cells. The strategy described herein is to inject the same T-cells as the therapeutic ones, but with a modification preventing the cells to signal and hence to kill (CD8) or provide help (CD4), and study their tissue distribution after injection. Therefore, these cells are called "dummy T-cells". These cells, combined with a tracer, inform the clinician about the safety of a therapeutic molecule (TcR or CAR): If the T-cells are detectable only at the tumor site(s), the drug will be considered as safe. In contrast, if the T-cells are seen also at other locations (due to cross reactivity or because the antigen is expressed in unsuspected tissue) then the molecule has cross-reactivity issues.

Accordingly, provided herein are dummy T-cells for research, screening, and therapeutic uses. In some embodiments, the T-cells overexpress the C-terminal SRC kinase (CSK). The target recognition and the cell migration of these dummy T-cells is not affected by CSK overexpression. Hence, these dummy T-cells redirected with a therapeutic molecule (TcR or CAR) are labeled and used to trace the effector cells distribution when injected into the patient, without being functional (e.g., unable to kill and/or elicit an immune response). Accordingly, in the dummy T-cells, the membrane bound receptor for the desired target is expressed in an amount sufficient for specific localization of the T-cell after parenteral administration. Therefore, these dummy T-cells will serve to analyze potential side effects (mainly wrong homing) in patient in a safe manner, prior to the adoptive transfer of the fully functional redirected T-cell, i.e. the corresponding therapeutic T-cell.

In some embodiments, T-cells are engineered in vitro or ex vivo to overexpress CSK. Overexpression of CSK in a T-cell, means that the level of CSK enzyme is significantly increased compared to an unmodified T-cell. This can be achieved in many ways including modification of the native CSK-gene present in the T-cell or by introduction of a heterologous sequence encoding CSK. Overexpression of mCSK in a T-cell means that the level of mCSK enzyme is significantly increased compared to an unmodified T-cell. This can be achieved in many ways including modification of the native CSK-gene present in the T-cell or by introduction of a heterologous sequence encoding mCSK. In some embodiments, a

transgene that overexpresses CSK and a detectable label are introduced into a therapeutic T-cell.

In some embodiments, the CSK is a variant or wild-type human CSK and has at least 80%, 90%, 95%, 97%, 98%, 99% or 100% identity to SEQ ID NO:6, the reference amino acid sequence for human CSK. Where the CSK is a variant CSK, it will be understood that the variant retains the biological activity wild type CSK, i.e., phosphorylation of tyrosine residues located in the C-terminal end of Src-family kinases (SFKs) including, for example, SRC, HCK, FYN, LCK, LYN and YES1. Thus, within the scope of the invention are natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as "variants") of human CRK as defined herein. Variants, as used herein, are sequences that have a defined identity to SEQ ID NO:6, as can be measured electronically by making use of algorithms such as PILEUP and BLAST. (See, e.g., Higgins & Sharp, CABIOS 5:151 (1989); Altschul S. F., W. Gish, W. Miller, E. W. Myers, D. J. Lipman. Basic local alignment search tool. J. Mol. Biol. 1990; 215:403-10.) Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (on the worldwide web at ncbi.nlm.nih.gov/).

A "deletion" is defined here as a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent as compared to an amino acid sequence or nucleotide sequence of a parental polypeptide or nucleic acid. Within the context of a protein, a deletion can involve deletion of about two, about five, about ten, up to about twenty, up to about thirty or up to about fifty or more amino acids. A protein or a fragment thereof may contain more than one deletion.

An "insertion" or "addition" is that change in an amino acid or nucleotide sequences which has resulted in the addition of one or more amino acid or nucleotide residues, respectively, as compared to an amino acid sequence or nucleotide sequence of a parental protein. "Insertion" generally refers to addition to one or more amino acid residues within an amino acid sequence of a polypeptide, while "addition" can be an insertion or refer to amino acid residues added at an N- or C-terminus, or both termini. Within the context of a protein or a fragment thereof, an insertion or addition is usually of about one, about three, about five, about ten, up to about twenty, up to about thirty or up to about fifty or more amino acids. A protein or fragment thereof may contain more than one insertion.

A "substitution," as used herein, results from the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively as compared to an amino acid sequence or nucleotide sequence of a parental protein or a fragment thereof. It is

understood that a protein or a fragment thereof may have conservative amino acid substitutions which have substantially no effect on the protein's activity. By conservative substitutions is intended combinations such as gly, ala; val, ile, leu, met; asp, glu; asn, gln; ser, thr; lys, arg; cys, met; and phe, tyr, trp.

By means of non-limiting examples, a substitution may, for example, be a conservative substitution (as described herein) and/or an amino acid residue may be replaced by another amino acid residue that naturally occurs at the same position in CSK. Thus, any one or more substitutions, deletions or insertions, or any combination thereof, that either improve the properties of CSK or that at least do not detract too much from the desired properties or from the balance or combination of desired properties of CSK are included within the scope of the invention. A skilled person will generally be able to determine and select suitable substitutions, deletions or insertions, or suitable combinations of thereof, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may, for example, involve introducing a limited number of possible substitutions and determining their influence on the properties of the CSK and cells transformed therewith thus obtained.

The present invention is not limited to particular expression vectors for overexpressing CRK in T-cells. Exemplary vectors and expression methods are described herein. In some embodiments, CRK is expressed using any suitable vector or expression system. In some embodiments, the vectors of the present invention comprise a promoter operably linked to a nucleic acid sequence encoding a variant or wild-type CRK protein encoding by SEQ ID NO:6, or having at least 80%, 90%, 95%, 97%, 98%, 99% identify to SEQ ID NO:6.

In some embodiments, CRK is expressed via a suitable eukaryotic expression vectors (e.g., commercially available vectors). In some embodiments, CRK is expressed via a viral vector (e.g., adeno, adeno-associated, or lenti-viral vectors). Suitable vectors are introduced into T-cells and expression is induced. In some embodiments of the present invention, vectors include, but are not limited to, chromosomal, nonchromosomal and synthetic DNA sequences (e.g., derivatives of SV40, phage DNA; vectors derived from combinations of plasmids and phage DNA, retroviral vectors and viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies). It is contemplated that any vector may be used as long as it is viable and can be expressed in T-cells.

In particular, some embodiments of the present invention provide recombinant constructs comprising one or more of the sequences as broadly described above. In some

embodiments of the present invention, the constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In still other embodiments, the heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences. In preferred embodiments of the present invention, the appropriate DNA sequence is inserted into the vector using any of a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art.

Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. Such vectors include, but are not limited to, the following vectors: pWLNEO, pSV2CAT, pOG44, PXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). Any other plasmid or vector may be used as long as they are replicable and viable in the host. In some preferred embodiments of the present invention, mammalian expression vectors comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation sites, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. In other embodiments, DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

In certain embodiments of the present invention, the DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. Promoters useful in the present invention include, but are not limited to, the LTR or SV40 promoter, the phage lambda PL and PR, T3 and T7 promoters, and the cytomegalovirus (CMV) immediate early, herpes simplex virus (HSV) thymidine kinase, and mouse metallothionein-I promoters and other promoters known to control expression of gene in prokaryotic or eukaryotic cells or their viruses. In other embodiments of the present invention, recombinant expression vectors include origins of replication and selectable markers permitting transformation of the host cell (e.g., dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or tetracycline or ampicillin resistance in E. coli).

In some embodiments of the present invention, transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Enhancers useful in the present invention include, but are not limited to, the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

In other embodiments, the expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. In still other embodiments of the present invention, the vector may also include appropriate sequences for amplifying expression.

In a further embodiment, the present invention provides T-cells containing the above-described constructs. In some embodiments of the present invention, following transformation of T-cells, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

In some embodiments, the present invention provides compositions, systems, and/or kits comprising the dummy T-cells described herein. In some embodiments, dummy T-cells are provided in a system with reagents and/or instruments to detect the presence of the dummy T-cells (e.g., imaging systems). In some embodiments, kits and systems further comprise one or more of instructions, controls, delivery systems, etc.

Dummy T-cells may target any antigen (e.g., cancer antigen) including but not limited to proteins, subunits, domains, motifs, and/or epitopes belonging to the following list of target antigens, which includes both soluble factors such as cytokines and membrane-bound factors, including transmembrane receptors: 17-IA, 4-1BB, 4Dc, 6-keto-PGF1a, 8-iso-PGF2a, 8-oxo-dG, A1 Adenosine Receptor, A33, ACE, ACE-2, Activin, Activin A, Activin AB, Activin B, Activin C, Activin RIA, Activin RIA ALK-2, Activin RIB ALK-4, Activin RIIA, Activin RIIB, ADAM, ADAM10, ADAM12, ADAM15, ADAM17/TACE, ADAM8, ADAM9, ADAMTS, ADAMTS4, ADAMTS5, Addressins, aFGF, ALCAM, ALK, ALK-1, ALK-7, alpha-1-antitrypsin, alpha-V/beta-1 antagonist, ANG, Ang, APAF-1, APE, APJ, APP, APRIL, AR, ARC, ART, Artemin, anti-Id, ASPARTIC, Atrial natriuretic factor, av/b3 integrin, Axl, b2M, B7-1, B7-2, B7-H, B-lymphocyte Stimulator (BlyS), BACE, BACE-1, Bad, BAFF, BAFF-R, Bag-1, BAK, Bax, BCA-1, BCAM, Bcl, BCMA, BDNF, b-ECGF, bFGF, BID, Bik, BIM, BLC, BL-CAM, BLK, BMP, BMP-2 BMP-2a, BMP-3 Osteogenin, BMP-4 BMP-2b, BMP-5, BMP-6 Vgr-1, BMP-7 (OP-1), BMP-8 (BMP-8a, OP-2), BMPR, BMPR-IA (ALK-3), BMPR-IB (ALK-6), BRK-2, RPK-1, BMPR-II (BRK-3), BMPs, b-NGF, BOK, Bombesin, Bone-derived neurotrophic factor, BPDE, BPDE-DNA, BTC, complement factor 3 (C3), C3a, C4, C5, C5a, C10, CA125, CAD-8, Calcitonin, cAMP, carcinoembryonic antigen (CEA), carcinoma-associated antigen, Cathepsin A, Cathepsin B, Cathepsin C/DPPI, Cathepsin D, Cathepsin E, Cathepsin H, Cathepsin L, Cathepsin O, Cathepsin S, Cathepsin V, Cathepsin X/Z/P, CBL, CCI, CCK2, CCL, CCL1, CCL11, CCL12, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL2, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CCL28, CCL3, CCL4, CCL5, CCL6,

CCL7, CCL8, CCL9/10, CCR, CCR1, CCR10, CCR10, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CD1, CD2, CD3, CD3E, CD4, CD5, CD6, CD7, CD8, CD10, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD27L, CD28, CD29, CD30, CD30L, CD32, CD33 (p67 proteins), CD34, CD38, CD40, CD40L, CD44, CD45, CD46, CD49a, CD52, CD54, CD55, CD56, CD61, CD64, CD66e, CD74, CD80 (B7-1), CD89, CD95, CD123, CD137, CD138, CD140a, CD146, CD147, CD148, CD152, CD164, CEACAM5, CFTR, cGMP, CINC, Clostridium botulinum toxin, Clostridium perfringens toxin, CKb8-1, CLC, CMV, CMV UL, CNTF, CNTN-1, COX, C-Ret, CRG-2, CT-1, CTACK, CTGF, CTLA-4, CX3CL1, CX3CR1, CXCL, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15, CXCL16, CXCR, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, cytokeratin tumor-associated antigen, DAN, DCC, DcR3, DC-SIGN, Decay accelerating factor, des(1-3)-IGF-I (brain IGF-1), Dhh, digoxin, DNAM-1, Dnase, Dpp, DPPIV/CD26, Dtk, ECAD, EDA, EDA-A1, EDA-A2, EDAR, EGF, EGFR (ErbB-1), EMA, EMMPRIN, ENA, endothelin receptor, Enkephalinase, eNOS, Eot, eotaxin1, EpCAM, Ephrin B2/EphB4, EPO, ERCC, E-selectin, ET-1, Factor IIa, Factor VII, Factor VIIIc, Factor IX, fibroblast activation protein (FAP), Fas, FcR1, FEN-1, Ferritin, FGF, FGF-19, FGF-2, FGF3, FGF-8, FGFR, FGFR-3, Fibrin, FL, FLIP, Flt-3, Flt-4, Follicle stimulating hormone, Fractalkine, FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, FZD10, G250, Gas 6, GCP-2, GCSF, GD2, GD3, GDF, GDF-1, GDF-3 (Vgr-2), GDF-5 (BMP-14, CDMP-1), GDF-6 (BMP-13, CDMP-2), GDF-7 (BMP-12, CDMP-3), GDF-8 (Myostatin), GDF-9, GDF-15 (MIC-1), GDNF, GDNF, GFAP, GFRa-1, GFR-alpha1, GFR-alpha2, GFR-alpha3, GITR, Glucagon, Glut 4, glycoprotein IIb/IIIa (GP IIb/IIIa), GM-CSF, gp130, gp72, GRO, Growth hormone releasing factor, Hapten (NP-cap or NIP-cap), HB-EGF, HCC, HCMV gB envelope glycoprotein, HCMV) gH envelope glycoprotein, HCMV UL, Hemopoietic growth factor (HGF), Hep B gp120, heparanase, Her2, Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4), herpes simplex virus (HSV) gB glycoprotein, HSV gD glycoprotein, HGFA, High molecular weight melanoma-associated antigen (HMW-MAA), HIV gp120, HIV IIIB gp 120 V3 loop, HLA, HLA-DR, HM1.24, HMFG PEM, HRG, Hrk, human cardiac myosin, human cytomegalovirus (HCMV), human growth hormone (HGH), HVEM, I-309, IAP, ICAM, ICAM-1, ICAM-3, ICE, ICOS, IFNg, Ig, IgA receptor, IgE, IGF, IGF binding proteins, IGF-1R, IGFBP, IGF-I, IGF-II, IL, IL-1, IL-1R, IL-2, IL-2R, IL-4, IL-4R, IL-5, IL-5R, IL-6, IL-6R, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18, IL-18R, IL-23, interferon (INF)-alpha, INF-beta, INF-gamma, Inhibin, iNOS, Insulin A-

chain, Insulin B-chain, Insulin-like growth factor 1, integrin alpha2, integrin alpha3, integrin alpha4, integrin alpha4/beta7, integrin alpha4/beta7, integrin alpha5 (alphaV), integrin alpha5/beta1, integrin alpha5/beta3, integrin alpha6, integrin beta1, integrin beta2, interferon gamma, IP-10, I-TAC, JE, Kallikrein 2, Kallikrein 5, Kallikrein 6, Kallikrein 11, Kallikrein 12, Kallikrein 14, Kallikrein 15, Kallikrein L1, Kallikrein L2, Kallikrein L3, Kallikrein L4, KC, KDR, Keratinocyte Growth Factor (KGF), laminin 5, LAMP, LAP, LAP (TGF-1), Latent TGF-1, Latent TGF-1 bp1, LBP, LDGF, LECT2, Lefty, Lewis-Y antigen, Lewis-Y related antigen, LFA-1, LFA-3, Lfo, LIF, LIGHT, lipoproteins, LIX, LKN, Lptn, L-Selectin, LT-a, LT-b, LTB4, LTBP-1, Lung surfactant, Luteinizing hormone, Lymphotoxin Beta Receptor, Mac-1, MAdCAM, MAG, MAP2, MARC, MCAM, MCAM, MCK-2, MCP, M-CSF, MDC, Mer, METALLOPROTEASES, MGDF receptor, MGMT, MHC (HLA-DR), MIF, MIG, MIP, MIP-1-alpha, MK, MMAC1, MMP, MMP-1, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-15, MMP-2, MMP-24, MMP-3, MMP-7, MMP-8, MMP-9, MPIF, Mpo, MSK, MSP, mucin (Muc1), MUC18, Muellerian-inhibitin substance, Mug, MuSK, NAIP, NAP, NCAD, N-Cadherin, NCA 90, NCAM, NCAM, Neprilysin, Neurotrophin-3, -4, or -6, Neurturin, Neuronal growth factor (NGF), NGFR, NGF-beta, nNOS, NO, NOS, Npn, NRG-3, NT, NTN, OB, OGG1, OPG, OPN, OSM, OX40L, OX40R, p150, p95, PADPr, Parathyroid hormone, PARC, PARP, PBR, PBSF, PCAD, P-Cadherin, PCNA, PDGF, PDGF, PDK-1, PECAM, PEM, PF4, PGE, PGF, PGI2, PGJ2, PIN, PLA2, placental alkaline phosphatase (PLAP), PIGF, PLP, PP14, Proinsulin, Prorelaxin, Protein C, PS, PSA, PSCA, prostate specific membrane antigen (PSMA), PTEN, PTHrp, Ptk, PTN, R51, RANK, RANKL, RANTES, RANTES, Relaxin A-chain, Relaxin B-chain, renin, respiratory syncytial virus (RSV) F, RSV Fgp, Ret, Rheumatoid factors, RLIP76, RPA2, RSK, S100, SCF/KL, SDF-1, SERINE, Serum albumin, sFRP-3, Shh, SIGIRR, SK-1, SLAM, SLPI, SMAC, SMDF, SMOH, SOD, SPARC, Stat, STEAP, STEAP-II, TACE, TACI, TAG-72 (tumor-associated glycoprotein-72), TARC, TCA-3, T-cell receptors (e.g., T-cell receptor alpha/beta), TdT, TECK, TEM1, TEM5, TEM7, TEM8, TERT, testicular PLAP-like alkaline phosphatase, TfR, TGF, TGF-alpha, TGF-beta, TGF-beta Pan Specific, TGF-beta R1 (ALK-5), TGF-beta RII, TGF-beta RIIb, TGF-beta RIII, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta4, TGF-beta5, Thrombin, Thymus Ck-1, Thyroid stimulating hormone, Tie, TIMP, TIQ, Tissue Factor, TMEFF2, Tmpo, TMPRSS2, TNF, TNF-alpha, TNF-alpha beta, TNF-beta2, TNFc, TNF-RI, TNF-RII, TNFRSF10A (TRAIL R3Apo-2, DR4), TNFRSF10B (TRAIL R2DR5, KILLER, TRICK-2A, TRICK-B), TNFRSF10C (TRAIL R3DcR1, LIT, TRID), TNFRSF10D (TRAIL R4DcR2, TRUNDD), TNFRSF11A

(RANK ODF R, TRANCE R), TNFRSF11B (OPG OCIF, TR1), TNFRSF12 (TWEAK R FN14), TNFRSF13B (TACI), TNFRSF13C (BAFF R), TNFRSF14 (HVEM ATAR, HveA, LIGHT R, TR2), TNFRSF16 (NGFR p75NTR), TNFRSF17 (BCMA), TNFRSF18 (GITR AITR), TNFRSF19 (TROY TAJ, TRADE), TNFRSF19L (RELT), TNFRSF1A (TNF R1CD120a, p55-60), TNFRSF1B (TNF RII CD120b, p75-80), TNFRSF26 (TNFRH3), TNFRSF3 (LTbR TNF RIII, TNFC R), TNFRSF4 (OX40 ACT35, TXGP1 R), TNFRSF5 (CD40 p50), TNFRSF6 (Fas Apo-1, APT1, CD95), TNFRSF6B (DcR3M68, TR6), TNFRSF7 (CD27), TNFRSF8 (CD30), TNFRSF9 (4-1 BB CD137, ILA), TNFRSF21 (DR6), TNFRSF22 (DcTRAIL R2TNFRH2), TNFRST23 (DcTRAIL R1 TNFRH1), TNFRSF25 (DR3Apo-3, LARD, TR-3, TRAMP, WSL-1), TNFSF10 (TRAIL Apo-2 Ligand, TL2), TNFSF11 (TRANCE/RANK Ligand ODF, OPG Ligand), TNFSF12 (TWEAK Apo-3 Ligand, DR3Ligand), TNFSF13 (APRIL TALL2), TNFSF13B (BAFF BLYS, TALL1, THANK, TNFSF20), TNFSF14 (LIGHT HVEM Ligand, LTg), TNFSF15 (TL1A/VEGI), TNFSF18 (GITR Ligand AITR Ligand, TL6), TNFSF1A (TNF-a Conectin, DIF, TNFSF2), TNFSF1B (TNF-b LTa, TNFSF1), TNFSF3 (LTb TNFC, p33), TNFSF4 (OX40 Ligand gp34, TXGP1), TNFSF5 (CD40 Ligand CD154, gp39, HIGM1, IMD3, TRAP), TNFSF6 (Fas Ligand Apo-1 Ligand, APT1 Ligand), TNFSF7 (CD27 Ligand CD70), TNFSF8 (CD30 Ligand CD153), TNFSF9 (4-1BB Ligand CD137 Ligand), TP-1, t-PA, Tpo, TRAIL, TRAIL R, TRAIL-R1, TRAIL-R2, TRANCE, transferring receptor, TRF, Trk, TROP-2, TSG, TSLP, tumor-associated antigen CA 125, tumor-associated antigen expressing Lewis Y related carbohydrate, TWEAK, TXB2, Ung, uPAR, uPAR-1, Urokinase, VCAM, VCAM-1, VECAD, VE-Cadherin, VE-cadherin-2, VEFGR-1 (fit-1), VEGF, VEGFR, VEGFR-3 (flt-4), VEGI, VIM, Viral antigens, VLA, VLA-1, VLA-4, VNR integrin, von Willebrands factor, WIF-1, WNT1, WNT2, WNT2B/13, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT9A, WNT9B, WNT10A, WNT10B, WNT11, WNT16, XCL1, XCL2, XCR1, XCR1, XEDAR, XIAP, XPD, and receptors for hormones and growth factors.

One skilled in the art will appreciate that the aforementioned list of targets refers not only to specific proteins and biomolecules, but the biochemical pathway or pathways that comprise them. For example, reference to CTLA-4 as a target antigen implies that the ligands and receptors that make up the T-cell co-stimulatory pathway, including CTLA-4, B7-1, B7-2, CD28, and any other undiscovered ligands or receptors that bind these proteins, are also targets. Thus, target as used herein refers not only to a specific biomolecule, but the set of

proteins that interact with said target and the members of the biochemical pathway to which said target belongs.

Experimental

Example 1

Materials and Methods

Cell lines, Media, Chemicals and Peptides

J76 (20) and SupT1 (kind gifts from M. Heemskerk, Leiden University Medical Center, and M. Pulé, University College London, UK, respectively) were maintained in RPMI (PAA, Paschung, Austria) supplemented with 10% HyClone FCS (HyClone, Logan, UT, USA) and 1% antibiotic-antimicotic (penicillin/streptomycin, p/s, PAA). T2 cells were maintained in the same medium. The packaging cells were the modified Human Embryonic Kidney cells-293, Hek-Phoenix (Hek-P) and they were grown in DMEM (PAA) with 10% FCS. HeLa cells were grown in the same medium. T-cells were grown in CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with 5% heat-inactivated human serum (Trina Bioreactives AG, Nänikon, Switzerland), 10 mM N-acetylcysteine (Mucomyst 200 mg/mL, AstraZeneca AS, London, UK), 0.01 M HEPES (Life Technologies, Norway) gentamycin 0.05 mg/mL (Garamycin, Schering-Plough Europe, Belgium). Dasatinib was from LC Laboratories (Woburn, MA, USA). The TGFbRII frameshift peptide$_{131-139}$, RLSSCVPVA was provided by Norsk Hydro ASA, (Porsgrunn, Norway). The MART-1 peptide$_{26-35}$ EAAGIGILTV was manufactured by ProImmune Ltd (Oxford, UK) and MART-1 dextramer was from Immudex (København, Denmark).

DNA Constructs:

CSK cloning was performed by amplifying cDNA from PBMC isolated from an healthy donor using the following primers: 5'-CAC CAT GTC AGC AAT ACA GGC-3' (full length construct; SEQ ID NO:1) or 3'-CAC CAT GGG CTG TGG CTG CAG CTC ACA CCC CGA AGA TGC AAT ACA GGC CG-5' (membrane targeted CSK with LCK myristoylation domain; SEQ ID NO:2) and 5'-CTCTCTTGGCTCTCAGGTGCAGCTCGTG-3' SEQ ID NO:3. The amplicon was subsequently cloned into pENTR vector (Invitrogen, Carlsbad, CA, USA) and sequence verified (Eurofins MWG Operon, Ebersberg, Germany). It was then transferred into pMP71 (retroviral vector) or pCIpA102 (mRNA synthesis construct) as described in (21). TCR

expression constructs were prepared by amplifying TCR-α and –β chains separately with specific primers and followed by a second PCR to fuse the TCR chains as a TCR-2A construct as described in (21) and in (Inderberg *et al.*, manuscript submitted) for DMF5 and Radium-1, respectively.

HLA-A2-peptide single chain trimer (SCT) expression plasmids were constructed as described in (22). Peptide coding sequence exchange to create a TGFFbR2 frameshift peptide SCT (SCT-TGFbRIIp) was performed by site direct mutagenesis using these peptides: 5'-GGC CTC GAG GCT CGT CTG TCA TCA TGC GTT CCT GTG GCT GGC TGT GGC AGC-3' SEQ ID NO:4 and 5'-GCT GCC ACA GCC AGC CAC AGG AAC GCA TGA TGA CAG ACG AGC CTC GAG GCC-3' SEQ ID NO:5.

In vitro mRNA transcription

The *in vitro* mRNA synthesis was performed essentially as previously described (23). Anti-Reverse Cap Analog (Trilink Biotechnologies Inc., San Diego, CA, USA) were used to cap the RNA. The mRNA was assessed by agarose gel electrophoresis and Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

In vitro expansion, electroporation and retroviral transduction of humanT-cells

T-cells from healthy donors were expanded using a protocol adapted for GMP production of T-cells employing Dynabeads CD3/CD28 as described in (23). In brief, PBMCs were isolated from buffy coats by density gradient centrifugation and cultured with Dynabeads (Dynabeads® *ClinExVivo*™ CD3/CD28, ThermoFischer, Oslo, Norway) at a 3:1 ratio in complete CellGro DC Medium with 100 U/mL recombinant human interleukin-2 (IL-2) (Proleukin, Prometheus Laboratories Inc., San Diego, CA, USA) for 10 days. The cells were frozen and aliquots were thawed and rested in complete medium before transfection. Expanded T-cells were washed twice and resuspended in CellGro DC medium (CellGenix GmbH) to 70×10^6 cells/mL. The mRNA was mixed with the cell suspension at 100 µg/mL, and electroporated in a 4-mm gap cuvette at 500 V and 2 ms using a BTX 830 Square Wave Electroporator (BTX Technologies Inc., Hawthorne, NY, USA). Immediately after transfection, T-cells were transferred to complete culture medium at 37°C in 5% CO_2 overnight.

Viral particles produced as described in (21) were used to transduce cells (cell line or activated T-cells) as follows: Spinoculation was performed with 1 Volume of retroviral supernatant in a 12-well or a 24-Well culture non-treated plate (Nunc A/S, Roskilde, Denmark) pre-coated with retronectin (20 µg/mL, Takara Bio. Inc., Shiga, Japan). T-cells were spinoculated twice whereas all the other cell lines were spinoculated only once. Cells

were then harvested with PBS-EDTA (0.5 mM) and grown in their medium or in X-vivo 20, 5% HS, 100 U/mL IL-2 and 2 ng/mL IL-15 for T-cells. Cells were used for experiments after 3 to 7 days.

Functional Assay, Trogocytosis and flow cytometry

For functional assays, T-cells were stimulated for 5 hours with APCs, loaded or not with the indicated peptide, at a T-cell to target ratio of 2:1 and in the presence of BD GolgiPlug and BD Golgistop at a 1/1000 dilution.

Measurement of trogocytosis was performed as follows: TCR/CSK_2_GFP or GFP expressing J76 cells were incubated with T2 cells that had been O/N loaded with the indicated peptide at saturating concentration (5 μM) at 1:1 ratio for 4 hours. Cells were then extensively washed and stained for flow analysis (see below) using specific antibodies. Trogocytosis was measured by determining the increase of signal of the transferred markers. The following antibodies were used: Vβ3- FITC (Beckman Coulter-Immunotech SAS, France), CD3-eFluor450, CD4- eFluor 450, CD4-PE-Cy7, CD8-APC, CD8-eFluor 450, CD8-PE-Cy7 (BD Biosciences, USA) and CD107a-PE-Cy5 (BD Biosciences, USA). Cells were washed in flow buffer (FB, phosphate buffered saline (PBS) with 2% human bovine serum albumin (BSA) and 0.5 μM EDTA). For dextramer and antibody staining, cells were incubated for 30 minutes at RT with the recommended dilution in FB. If fixed, cells were incubated in FB containing 1% paraformaldehyde. All antibodies were purchased from eBioscience, USA, except where noted. Cells were acquired on a BD LSR II flow cytometer and the data analyzed using FlowJo software (Treestar Inc., Ashland, OR, USA).

T-cell binding assay and microscopy

To visualize T-cell binding to cognate APC was performed as follows: 0.15×10^6 HeLa cells were seeded into Glass Bottom 6-Well Plates (MatTek Corp., MA, USA); after 12 hours, they were transfected with SCT constructs (1 μg DNA/well) and left another 24 hours. Then 0.6×10^6 T-cells were added, left for 35 minutes and cells were washed once or not with RPMI before confocal microscopy. Images were taken on an Olympus Fluoview 1000 IX-81 inverted confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan)) using 40X objective lens. HeLa cells were identified by their morphological characteristics whereas T-cells were traced by GFP expression. Ten images per condition were acquired; Image J software (Bethesda, MA, USA) was used to analyze images. The number of cells per frame was counted; plotting and statistical analysis were performed using GraphPad prism software (La Jolla, CA USA).

Results

Overexpression of CSK blocks TCR signalling

In order to develop a method for testing the safety of therapeutic T-cells, we introduced the inhibitory kinase CSK in the therapeutic T-cells, to make these unable to signal and hence stop effector functions. The negative effect of CSK on TCR stimulation was demonstrated more than two decades ago (4). It was also shown and recently confirmed that a membrane targeted CSK (mCSK) had greater inhibition potency than its cytosolic counterpart (7). We therefore built a CSK and an mCSK construct fused to GFP via a 2A ribosome skipping sequence (24) (Fig. 1A). Upon mRNA translation, the two proteins CSK (or mCSK) and GFP are released and the expressing cells can be distinguished by monitoring the presence of GFP. Indeed, the intracellular staining of CSK showed a direct relationship with GFP expression, although a small leakage could be seen (6.6% GFP-/CSK+) (Fig. 7). Therefore, we could use GFP expression to track CSK overexpressing T-cells. The impact of CSK presence on the TCR intracellular signalling cascade was then tested by activating TCR signalling in PBMC T-cells with varying amounts of anti-CD3/anti-CD28 for 2 minutes, and detected induced phosphorylation by phospho-specific flow cytometry. CSK-transduced and non-transduced T-cells could then be tracked, based on GFP expression (Fig. 1B, Fig. 7). Whereas overexpression of CSK had limited effects on early TCR signalling as measured by phosphorylation of CD3z and ZAP70, late TCR signalling was blocked as determined by lack of phosphorylation of ERK in the presence of CSK (Fig. 1B and C; GFP+ cells). Note that non-transduced GFP negative cells from control GFP and CSK-GFP transduced T-cells had identical signalling (Fig 1B).

TCR signalling in T-cells transduced with a TGFβRII-frameshift specific TcR (Radium TCR) was next tested in a more physiological setting, using antigen presenting cells (APC) loaded with relevant (TGFβRII) or irrelevant (MART1) peptide. T-cells were cultured alone or with APC cells for 5 or 15 minutes and then subjected to phospho-specific flow cytometry measurements. The presence of relevant APCs induced phosphorylation of ERK in a fraction of CD8+ and CD8- T-cells, whereas the presence of irrelevant APCs did not (Fig. 2A, B). Of the measured TCR signalling nodes (CD3z, Zap70, SLP76 and ERK), ERK became phosphorylated in the highest fraction of T-cells with 40% and 22% positive CD8T-cells 5 and 15 min post APC stimulation, respectively (Fig 2B). When the T-cells overexpressed mCSK, the APC-induced phosphorylation of ERK was almost completely blocked. The lack of blockade in a few cells could be due to that not all T-cells overexpressed

mCSK. In contrast to the potent suppressive effect of mCSK, overexpression of CSK had limited effect (Fig 2B). Together, these data show that mCSK had the ability to block TCR-induced signalling, showing that the presence of the kinase was sufficient to render the TCR signalling incompetent.

Overexpression of CSK blocks specific TCR stimulation

We next tested if CSK overexpression was sufficient to block the TCR induced IL-2 release. For this experiment we used a TCR-negative cell line, J76 (20), transduced with MART1-specific TcR DMF5 (25). These cells were super infected with CSK and $GFP^+/CD3^+$ double positive cells were sorted in order to obtain a pure population. A non-TCR transduced control population expressing CSK only was prepared and sorted (Fig. 3A). We then analyzed IL-2 release upon incubation with APC. Equal numbers of the transduced J76 cells were incubated with SupT1 cells expressing Single-Chain Trimer (SCT) fused to the MART1 peptide (MART1p) or an irrelevant peptide. As shown, despite the presence of the TCR, when CSK was co-expressed, no IL-2 was detected by ELISA (Fig. 3B) whereas the TCR alone was very efficient at specifically responding to the stimulation. These data confirmed that CSK is a powerful inhibitor of TCR signalling.

We then tested T-cells isolated from PBMC previously transduced with a TGFβRII-frameshift specific TcR (Radium 1 TCR, Inderberg et al. *submitted*). This TCR could be detected by staining with a specific anti-Vβ3 antibody (Fig. 8). We further super-infected these cells with CSK, mCSK or GFP and incubated them with APC loaded or not with a specific peptide (TGFβRIIp). T-cell stimulation was determined by detection of the degranulation marker CD107a (Fig. 3C). Similar to the J76 experiment, the presence of CSK decreased the TCR-dependent stimulation, but was unable to block it. On the other hand, the mCSK construct dramatically inhibited the T-cell activation, suggesting that T-cells are more sensitive to membrane targeting of CSK than Jurkat. These data were supported by experiments performed using mRNA electroporation of Radium-1 expressing cells with CSK or mCSK (data not shown). We can conclude that membrane targeted CSK (mCSK) overexpression had the capacity to block the specific functional activation of a TCR in a redirected T-cell population.

pMHC recognition is improved by CSK

Although the TCR localization at the membrane was not affected by the overexpression of CSK (Fig. 3A), we did not know whether this would affect TCR binding

capacity. Since no multimer was found efficient with Radium-1, we tested MART1p-MHC multimer binding using J76 expressing DMF5 TcR transduced with either GFP or the CSK_2A_GFP constructs. In this case, cells were not sorted, as we favoured the use of a mixed population in order to be able to study both the positive and the negative CSK (GFP) populations in one test tube (Fig. 9). GFP (negative control) had no impact on the staining intensity of the TcR by MART1p-MHC-multimer when compared to the GFP negative population (Fig. 4A). However, both CSK and mCSK lead to an increased staining intensity, showing that CSK proteins were not negatively affecting the TCR binding, but rather improving it. It was recently shown that the tyrosine kinase inhibitor dasatinib also had the faculty to improve the pMHC-multimer staining (26). CSK being a negative regulator of SRC family kinases, we tested if this phenotype was due to its inhibitory activity. We used the same cells as before and incubated them with or without dasatinib. As shown (Fig. 4B), GFP had again no impact on the pMHC multimer staining intensity, but, in agreement with previous data (26), the presence of dasatinib resulted in an increased multimer binding. CSK expressing cells were not sensitive to dasatinib, as no variation was observed in the GFP^+ population with or without dasatinib (Fig. 4B, dark bars for CSK), whereas the GFP^- population was still varying (Fig. 4B, light bars). These data show that the phenotype observed when CSK is overexpressed is due to its kinase activity. Nevertheless, in the presence of mCSK, the binding was increased by the kinase inhibitor, showing that the mCSK kinase activity was not the unique cause of the increase pMHC multimer binding intensity as shown.

We then asked if this increased pMHC-multimer binding intensity was due to an increased presence of TCR. Since J76 cells have no endogenous TCR expressed at the membrane they are CD3 negative. However, upon TcR overexpression they become CD3 positive. We therefore looked at the level of a pure population of double transduced J76 (CSK/TCR) and stained the cells with anti-CD3 antibodies (Fig. 4C). As shown, the CD3 signal intensity in the double positive population (GFP+/CD3+) was increased in the presence of CSK, confirming that CSK negatively affected TCR recycling. This increase was even more dramatic in the presence of mCSK. Taken together, these data support that CSK expressing cells have kept their specific recognitions features, but more TCR is present at the membrane probably due to lack in recycling ability.

Dummy T-cells are able to perform trogocytosis with antigen presenting cells

Membrane exchange between T-cell and APC seems to play a crucial role in target recognition (27). Knowing that CSK was able to block the TCR signaling, we tested if CSK expressing cells were still able to perform trogocytosis. It was previously shown that trogocytosis was TCR signaling dependent (28-30). To test this, we incubated J76 (TCR) cells transduced with either GFP- or mCSK/CSK_2A_GFP constructs together with different T2 target cells loaded or not with relevant or irrelevant peptides. Four hours later, the presence of transferred molecules was analyzed by flow cytometry. As shown CD3 was "traveling" from J76(TCR) to T2 cells which are CD3 negative only when the latter were incubated with the relevant peptide, confirming that trogocytosis was TCR-specific (Fig. 5A). In addition, no difference in intensity of CD3 staining was observed in T2 cells when incubated with J76 expressing or not CSK. J76(TCR), which are HLA-A2 negative, became HLA-A2 positive only when the specific peptide was presented (Fig. 5B). Although the HLA-A2 signal on T2 cells increased with peptide loading (Fig. 5C), the irrelevant peptide (MART1p) which induces HLA-A2 recruitment was not able to trigger trogocytosis. Interestingly, CSK expressing cells had a statistically significant improved membrane exchange ($p<0.03$). Taken together these data suggest that blocking the TCR at the membrane by inhibiting the signaling, did not affect trogocytosis, but rather enhanced it due to the increased amount of material accumulated on the T-cells.

Dummy T-cells trace antigen presenting cells

We have shown that CSK expressing T-cells are incapable of signaling and killing their targets, furthermore, the binding to the pMHC complex was not only maintained but even improved. In order to analyze T-cell binding to their targets, we set up a microscopy-based assay where adherent APC (HekP cells) were transfected with a SCT construct or a control SCT exposing an irrelevant peptide. T-cells electroporated with Radium-1 TCR mRNA in combination with mCSK_2A_GFP or GFP mRNA (transfection efficiency was >70% of the cell, data not shown). HeLa cells expressing single chain trimer (SCT) molecules fused to the relevant antigenic peptide (TGF-βRII, SCT-TGF) or exposing an irrelevant peptide (MART-1p, SCT-M1) were used as APCs. T-cells were incubated with SCT-HeLa cells for 35 minutes and plates were washed or not before the number of bound T-cells were counted per image. Although a tendency to stronger binding to cognate target was observed when T-cells not expressing mCSK were used, it did not reach statistical significance (Fig. 6, left graph). However, T-cell specific binding became significantly increased compared to non-specific (Fig. 6, right graph, $p=0.0029$, $n=10$) when mCSK was

expressed in the T-cells, suggesting that the same T-cells expressing equal amount of TCR can improve their attachment to target cells as there were significantly more T-cells attached to the target presenting the correct peptide. In agreement with our previous data, these results suggest that blocking TCR signaling did not negatively affect binding but rather improve it. Moreover, mCSK expressing TCR redirected T-cells could potentially be used as a safe (due to their functional inactivity) tracer to study target specificity of a potential therapeutic TCR *in vivo*.

Discussion

We have in this study shown that the presence of CSK in redirected T-cells could completely block TCR signalling although the receptor was still able to specifically recognize its cognate pMHC. We herein confirmed previous data demonstrating that constitutive targeting of CSK to the plasma membrane improves its effect (7, 31). The TCR properly and selectively recognized its target and complex mechanisms such as trogocytosis were not disrupted. Furthermore, CSK expressing T-cells attached better to their targets than non-overexpressing cells.

By exploiting a picornavirus 2A based sequence construct and link CSK to GFP, we could monitor the specific signalling of CSK expressing cells in a bulk population. This is very important for kinetics analysis of signalling, because the test and the control are present in the same tube and variations are therefore reduced (32). By the use of phosphoflow cytometry we could also detect the specific signalling of a TCR when stimulated by pMHC. This represents a serious advantage to describe precise signalling, because we can exclude non-transduced/transfected cells from the bulk population. This type of analysis has been performed only rarely with pMHC-TCR stimulation (33) and CD3/CD28 stimulation was rather commonly used. Our results led us to a precise analysis of signalling components in the presence of CSK, and confirm the central role of ERK in the TCR signalling node. Consequently, features like cytokine production or target cell killing were affected. Finally, we showed that increasing a negative TCR signal could be exploited to study redirected T-cell attachment to target. Interestingly, trogocytosis was not affected by CSK overexpression. This is surprising as this phenomenon was shown to be regulated by SCR kinases, and inhibitors of these kinases were efficient at blocking it (28, 30). However, we restricted our analysis to two proteins (TCR and MHC) and the transfer of other components might be affected by CSK overexpression.

We herein propose to use the negative effect of CSK on the TCR signalling to investigate redirected T-cell specificity in prospective studies of TCR therapy. Modification of signalling components within redirected T-cells is becoming more and more attractive, a recent report demonstrated that T-cell efficacy could be improved when intracellular signalling was modulated (11). We here show that the opposite is possible by blocking the TCR signal and thereby creating a tool for the *in vivo* investigation of possible cross-reactivity or on-target/off-tumor toxicity prior to TCR therapy. This is in agreement with what was presented in a recent report to control CD4 T-cell cytokine release in order to prevent adverse effects such as cytokine storm. Here the authors proposed to use an inducible system to control the expression of bacterial signalling components that affect TCR response (34). One could imagine the same type of constructs using CSK or mCSK as it might be favourable to use human proteins for *in vivo* validation in order to reduce potential side effects due to immunogenicity. In addition, unlike what is shown in the present paper, no information about eventual effects on TCR binding was presented when bacterial proteins were overexpressed. Although a very attractive therapy, adoptive TCR transfer has shown that precision in targeting is a crucial step. A recent example was reported on a TCR against MAGE-A3 where the TCR was cross-reactive against an unrelated peptide expressed in cardiomyocytes (35, 36). Thus prediction methods available today do not seem to fulfil the necessary requirements. Although antigenic peptides are screened against databases covering the entire proteome, allelic variations between individuals and secondary modifications such as phosphorylation (37) are difficult to detect. Furthermore, the protein expression pattern is also complicated to analyze in all tissues and in all conditions. Finally, the inherent alloreactivity of a given TCR for another MHC allele is almost impossible to predict (38, 39). The combination of these weakly predicative aspects has implications for the safety of TCR-based therapy, as the inappropriate targeting might lead to fatal outcome (16). Therefore the only valid method to certify the safety of a TCR is by injecting it into a human. Safeguard systems, such as inducible suicide genes, in which the redirected T-cells can be stopped before fatal events happen have been proposed earlier (9). However, the time of response might still represent a challenge. It is therefore becoming obvious that predictions or *in vitro* testing of off-target toxicity for a therapeutic TCR are not sufficient for safety evaluation. One could imagine alternative methods to test a TCR *in vivo*: the use of soluble TCR combined with a tracer is an attractive one, but the lack of all cellular companion proteins (co-receptors) might hinder proper detection. Another method could be the use of transient systems such as mRNA electroporation (40). Nevertheless the transient TCR

expression might not allow for detection of accumulation of the redirected T-cells at an inappropriate location. We herein postulate that an efficient way to validate a therapeutic TCR would be to exploit the same settings as the therapeutic one, but employing effector cells unable to be activated, in other words, where they would become "dummy T-cells" that can be traced *in vivo*. CSK seems to be the ideal molecule to turn off TCR signalling without affecting their binding affinity. We propose that such CSK modified T-cells could be used as tracers after labelling with tracking systems (41) to detect whether off-target/off-tumor binding occurs prior to adoptive transfer of redirected effector T-cells.

References

1. Werlen, G., and E. Palmer. 2002. The T-cell receptor signalosome: a dynamic structure with expanding complexity. *Current opinion in immunology* 14: 299-305.

2. Burkhardt, J. K., and W. A. Comrie. 2016. Action and traction: cytoskeletal control of receptor triggering at the immunological synapse. *Frontiers in immunology* 7.

3. Chen, L., and D. B. Flies. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature reviews* 13: 227-242.

4. Chow, L. M., M. Fournel, D. Davidson, and A. Veillette. 1993. Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50csk. *Nature* 365: 156-160.

5. Okada, M. 2012. Regulation of the SRC family kinases by Csk. *International journal of biological sciences* 8: 1385-1397.

6. Vang, T., W. H. Liu, L. Delacroix, S. Wu, S. Vasile, R. Dahl, L. Yang, L. Musumeci, D. Francis, J. Landskron, K. Tasken, M. L. Tremblay, B. A. Lie, R. Page, T. Mustelin, S. Rahmouni, R. C. Rickert, and L. Tautz. 2012. LYP inhibits T-cell activation when dissociated from CSK. *Nature chemical biology* 8: 437-446.

7. Schoenborn, J. R., Y. X. Tan, C. Zhang, K. M. Shokat, and A. Weiss. 2011. Feedback circuits monitor and adjust basal Lck-dependent events in T cell receptor signaling. *Science signaling* 4: ra59.

8. Tan, Y. X., B. N. Manz, T. S. Freedman, C. Zhang, K. M. Shokat, and A. Weiss. 2014. Inhibition of the kinase Csk in thymocytes reveals a requirement for actin remodeling in the initiation of full TCR signaling. *Nature immunology* 15: 186-194.

9. Wu, C. Y., L. J. Rupp, K. T. Roybal, and W. A. Lim. 2015. Synthetic biology approaches to engineer T cells. *Current opinion in immunology* 35: 123-130.

10. Wei, F., S. Zhong, Z. Ma, H. Kong, A. Medvec, R. Ahmed, G. J. Freeman, M. Krogsgaard, and J. L. Riley. 2013. Strength of PD-1 signaling differentially affects T-cell effector functions. *Proceedings of the National Academy of Sciences of the United States of America* 110: E2480-2489.

11. Palmer, D. C., G. C. Guittard, Z. Franco, J. G. Crompton, R. L. Eil, S. J. Patel, Y. Ji, N. Van Panhuys, C. A. Klebanoff, M. Sukumar, D. Clever, A. Chichura, R. Roychoudhuri, R. Varma, E. Wang, L. Gattinoni, F. M. Marincola, L. Balagopalan, L. E. Samelson, and N. P. Restifo. 2015. Cish actively silences TCR signaling in CD8+ T cells to maintain tumor tolerance. *The Journal of experimental medicine* 212: 2095-2113.

12. Daniel-Meshulam, I., M. Horovitz-Fried, and C. J. Cohen. 2013. Enhanced antitumor activity mediated by human 4-1BB-engineered T cells. *Int J Cancer* 133: 2903-2913.

13. Weichsel, R., C. Dix, L. Wooldridge, M. Clement, A. Fenton-May, A. K. Sewell, J. Zezula, E. Greiner, E. Gostick, D. A. Price, H. Einsele, and R. Seggewiss. 2008. Profound inhibition of antigen-specific T-cell effector functions by dasatinib. *Clinical cancer research : an official journal of the American Association for Cancer Research* 14: 2484-2491.

14. Okoye, I., L. Wang, K. Pallmer, K. Richter, T. Ichimura, R. Haas, J. Crouse, O. Choi, D. Heathcote, E. Lovo, C. Mauro, R. Abdi, A. Oxenius, S. Rutschmann, and P. G. Ashton-Rickardt. 2015. T cell metabolism. The protein LEM promotes CD8(+) T cell immunity through effects on mitochondrial respiration. *Science (New York, N.Y* 348: 995-1001.

15. Ebert, P. J., J. Cheung, Y. Yang, E. McNamara, R. Hong, M. Moskalenko, S. E. Gould, H. Maecker, B. A. Irving, J. M. Kim, M. Belvin, and I. Mellman. 2016. MAP Kinase Inhibition Promotes T Cell and Anti-tumor Activity in Combination with PD-L1 Checkpoint Blockade. *Immunity*.

16. Hinrichs, C. S., and N. P. Restifo. 2013. Reassessing target antigens for adoptive T-cell therapy. *Nature biotechnology* 31: 999-1008.

17. Jones, B. S., L. S. Lamb, F. Goldman, and A. Di Stasi. 2014. Improving the safety of cell therapy products by suicide gene transfer. *Frontiers in pharmacology* 5: 254.

18. Roybal, K. T., L. J. Rupp, L. Morsut, W. J. Walker, K. A. McNally, J. S. Park, and W. A. Lim. 2016. Precision Tumor Recognition by T Cells With Combinatorial Antigen-Sensing Circuits. *Cell* 164: 770-779.

19. Vang, T., H. Abrahamsen, S. Myklebust, J. Enserink, H. Prydz, T. Mustelin, M. Amarzguioui, and K. Tasken. 2004. Knockdown of C-terminal Src kinase by siRNA-mediated RNA interference augments T cell receptor signaling in mature T cells. *European journal of immunology* 34: 2191-2199.

20. Heemskerk, M. H., M. Hoogeboom, R. A. de Paus, M. G. Kester, M. A. van der Hoorn, E. Goulmy, R. Willemze, and J. H. Falkenburg. 2003. Redirection of antileukemic reactivity of peripheral T lymphocytes using gene transfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved alpha joining region. *Blood* 102: 3530-3540.

21. Walchli, S., G. A. Loset, S. Kumari, J. N. Johansen, W. Yang, I. Sandlie, and J. Olweus. 2011. A practical approach to T-cell receptor cloning and expression. *PloS one* 6: e27930.

22. Walseng, E., S. Walchli, L. E. Fallang, W. Yang, A. Vefferstad, A. Areffard, and J. Olweus. 2015. Soluble T-cell receptors produced in human cells for targeted delivery. *PloS one* 10: e0119559.

23. Almasbak, H., E. Rian, H. J. Hoel, M. Pule, S. Walchli, G. Kvalheim, G. Gaudernack, and A. M. Rasmussen. 2011. Transiently redirected T cells for adoptive transfer. *Cytotherapy* 13: 629-640.

24. de Felipe, P., G. A. Luke, L. E. Hughes, D. Gani, C. Halpin, and M. D. Ryan. 2006. E unum pluribus: multiple proteins from a self-processing polyprotein. *Trends in biotechnology* 24: 68-75.

25. Johnson, L. A., B. Heemskerk, D. J. Powell, Jr., C. J. Cohen, R. A. Morgan, M. E. Dudley, P. F. Robbins, and S. A. Rosenberg. 2006. Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. *J Immunol* 177: 6548-6559.

26. Lissina, A., K. Ladell, A. Skowera, M. Clement, E. Edwards, R. Seggewiss, H. A. van den Berg, E. Gostick, K. Gallagher, E. Jones, J. J. Melenhorst, A. J. Godkin, M. Peakman, D. A. Price, A. K. Sewell, and L. Wooldridge. 2009. Protein kinase inhibitors substantially improve the physical detection of T-cells with peptide-MHC tetramers. *Journal of immunological methods* 340: 11-24.

27. Davis, D. M. 2007. Intercellular transfer of cell-surface proteins is common and can affect many stages of an immune response. *Nature reviews* 7: 238-243.

28. Hudrisier, D., J. Riond, H. Mazarguil, J. E. Gairin, and E. Joly. 2001. Cutting edge: CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner. *J Immunol* 166: 3645-3649.

29. Osborne, D. G., and S. A. Wetzel. 2012. Trogocytosis results in sustained intracellular signaling in CD4(+) T cells. *J Immunol* 189: 4728-4739.

30. Aucher, A., E. Magdeleine, E. Joly, and D. Hudrisier. 2008. Capture of plasma membrane fragments from target cells by trogocytosis requires signaling in T cells but not in B cells. *Blood* 111: 5621-5628.

31. Torgersen, K. M., T. Vang, H. Abrahamsen, S. Yaqub, V. Horejsi, B. Schraven, B. Rolstad, T. Mustelin, and K. Tasken. 2001. Release from tonic inhibition of T cell activation through transient displacement of C-terminal Src kinase (Csk) from lipid rafts. *The Journal of biological chemistry* 276: 29313-29318.

32. Irish, J. M., N. Kotecha, and G. P. Nolan. 2006. Mapping normal and cancer cell signalling networks: towards single-cell proteomics. *Nature reviews. Cancer* 6: 146-155.

33. Wolchinsky, R., M. Hod-Marco, K. Oved, S. S. Shen-Orr, S. C. Bendall, G. P. Nolan, and Y. Reiter. 2014. Antigen-dependent integration of opposing proximal TCR-signaling cascades determines the functional fate of T lymphocytes. *J Immunol* 192: 2109-2119.

34. Wei, P., W. W. Wong, J. S. Park, E. E. Corcoran, S. G. Peisajovich, J. J. Onuffer, A. Weiss, and W. A. Lim. 2012. Bacterial virulence proteins as tools to rewire kinase pathways in yeast and immune cells. *Nature* 488: 384-388.

35. Linette, G. P., E. A. Stadtmauer, M. V. Maus, A. P. Rapoport, B. L. Levine, L. Emery, L. Litzky, A. Bagg, B. M. Carreno, P. J. Cimino, G. K. Binder-Scholl, D. P. Smethurst, A. B. Gerry, N. J. Pumphrey, A. D. Bennett, J. E. Brewer, J. Dukes, J. Harper, H. K. Tayton-Martin, B. K. Jakobsen, N. J. Hassan, M. Kalos, and C. H. June. 2013. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood* 122: 863-871.

36. Cameron, B. J., A. B. Gerry, J. Dukes, J. V. Harper, V. Kannan, F. C. Bianchi, F. Grand, J. E. Brewer, M. Gupta, G. Plesa, G. Bossi, A. Vuidepot, A. S. Powlesland, A. Legg, K. J. Adams, A. D. Bennett, N. J. Pumphrey, D. D. Williams, G. Binder-Scholl, I. Kulikovskaya, B. L. Levine, J. L. Riley, A. Varela-Rohena, E. A. Stadtmauer, A. P. Rapoport, G. P. Linette, C. H. June, N. J. Hassan, M. Kalos, and B. K. Jakobsen. 2013. Identification of a Titin-derived HLA-A1-presented peptide as a cross-reactive target for engineered MAGE A3-directed T cells. *Science translational medicine* 5: 197ra103.

37. Depontieu, F. R., J. Qian, A. L. Zarling, T. L. McMiller, T. M. Salay, A. Norris, A. M. English, J. Shabanowitz, V. H. Engelhard, D. F. Hunt, and S. L. Topalian. 2009. Identification of tumor-associated, MHC class II-restricted phosphopeptides as targets for immunotherapy. *Proceedings of the National Academy of Sciences of the United States of America* 106: 12073-12078.

38. Piepenbrink, K. H., S. J. Blevins, D. R. Scott, and B. M. Baker. 2013. The basis for limited specificity and MHC restriction in a T cell receptor interface. *Nature communications* 4: 1948.

39. Sewell, A. K. 2012. Why must T cells be cross-reactive? *Nature reviews* 12: 669-677.

40. Almasbak, H., M. Lundby, and A. M. Rasmussen. 2010. Non-MHC-dependent redirected T cells against tumor cells. *Methods in molecular biology* 629: 453-493.

41. Liu, Z., and Z. Li. 2014. Molecular imaging in tracking tumor-specific cytotoxic T lymphocytes (CTLs). *Theranostics* 4: 990-1001.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.

CLAIMS

1. A method of monitoring T-cell based therapy, comprising:

a) administering a dummy T-cell to a subject, wherein said dummy T-cell comprises a detectable label and does not function to kill cells or promote an immune response;

b) detecting the location of said dummy T-cell in said subject.

2. The method of claim 1, wherein said dummy T-cell is a modified therapeutic T-cell.

3. The method of claim 1 or 2, wherein said dummy T-cell overexpresses C-terminal SRC kinase (CSK).

4. The method of any one of claims 1 to 3, wherein said dummy T-cell is specific for a target epitope.

5. The method of any one of claims 1 to 4, wherein said dummy T-cell is a tumor infiltrating lymphocyte expressing a chimeric antigen receptor, or a T-cell expressing a heterologous T-cell receptor.

6. The method of any one of claims 1 to 5, further comprising the step of administering a therapeutic T-cell to said subject.

7. The method of any one of claims 1 to 6, further comprising the step of visualizing the location of said detectable label in said subject.

8. The method of claim 7, wherein the presence of said detectable label in a location distinct from the target location of a target epitope is indicative of non-specific binding by said dummy T-cell.

9. The method of any one of claims 1 to 8, wherein said subject is a human subject or a non-human mammal.

10. The method of claim 8, wherein said target site is a tumor site.

11. A kit or system, comprising:

a) a dummy T-cell, wherein said dummy T-cell comprises a detectable label and does not function to kill cells or promote an immune response;

b) a detection component for detecting the location of said dummy T-cell in said subject.

12. The kit or system of claim 11, wherein said dummy T-cell is a modified therapeutic T-cell.

13. The kit or system of claim 11 or 12, wherein said dummy T-cell overexpresses C-terminal SRC kinase (CSK).

14. The kit or system of any one of claims 11 to 13, wherein said dummy T-cell is specific for a target epitope.

15. The kit or system of any one of claims 11 to 14, wherein said dummy T-cell is a tumor infiltrating lymphocyte expressing a chimeric antigen receptor, or a T-cell expressing a heterologous T-cell receptor.

16. A genetically modified human T-cell comprising a detectable label and a heterologous DNA sequence encoding a membrane bound receptor specific for a desired target epitope, wherein the genetically modified T-cell overexpresses CSK in an amount sufficient for preventing significant TCR signaling, and wherein the membrane bound receptor for the desired target epitope is expressed in an amount sufficient for specific binding of the T-cell to the desired target.

17. T-cell according to claim 16, wherein the CSK is membrane targeted.

18. T-cell according to any one of claim 16 or 17, wherein the membrane bound receptor for the desired target is expressed in an amount sufficient for specific localization of the T-cell after parenteral administration.

19. T-cell according to any one of claim 16 to 18, wherein the CSK is overexpressed in an amount sufficient for preventing an immune response.

20. T-cell according to any one of claim 16 to 19, wherein the membrane bound receptor is specific for a cancer antigen.

21. T-cell according to any one of claim 16 to 20, wherein the membrane bound receptor is a T-cell receptor.

22. A composition comprising T-cells according to any one of claim 16 to 21 for use in confirmation of specific localization of the cells *in vivo* after parenteral administration.

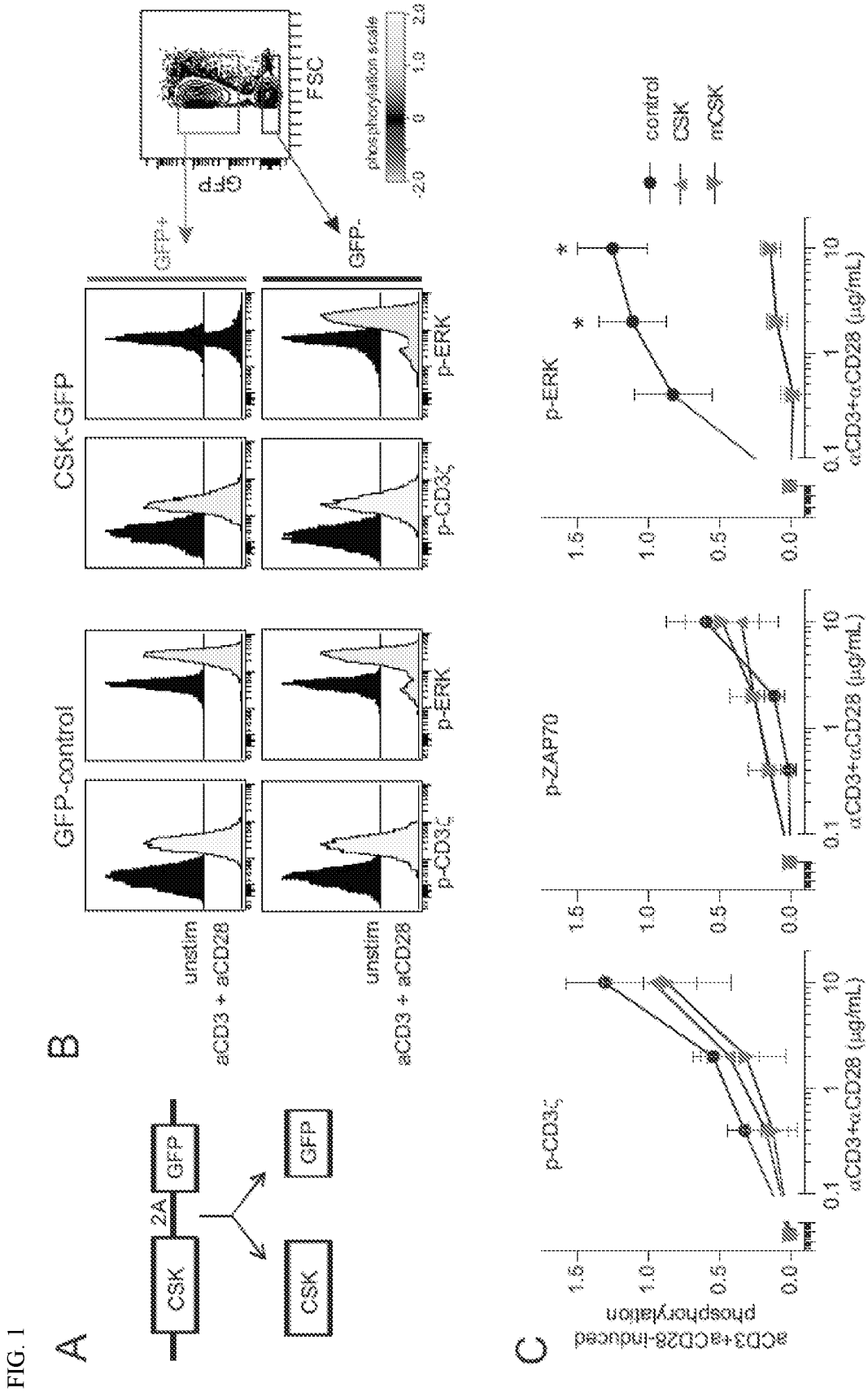
A
CSK
2A
GFP
CSK
GFP
B
GFP-control
CSK-GFP
unstim
aCD3 + aCD28
p-CD3ζ
p-ERK
GFP+
GFP-
GFP
FSC
phosphorylation scale
-2.0
0
1.0
2.0
C
aCD3+aCD28-induced phosphorylation
p-CD3ζ
p-ZAP70
p-ERK
αCD3+αCD28 (μg/mL)
0.1
1
10
0.0
0.5
1.0
1.5
control
CSK
mCSK
*

FIG. 1

FIG. 2

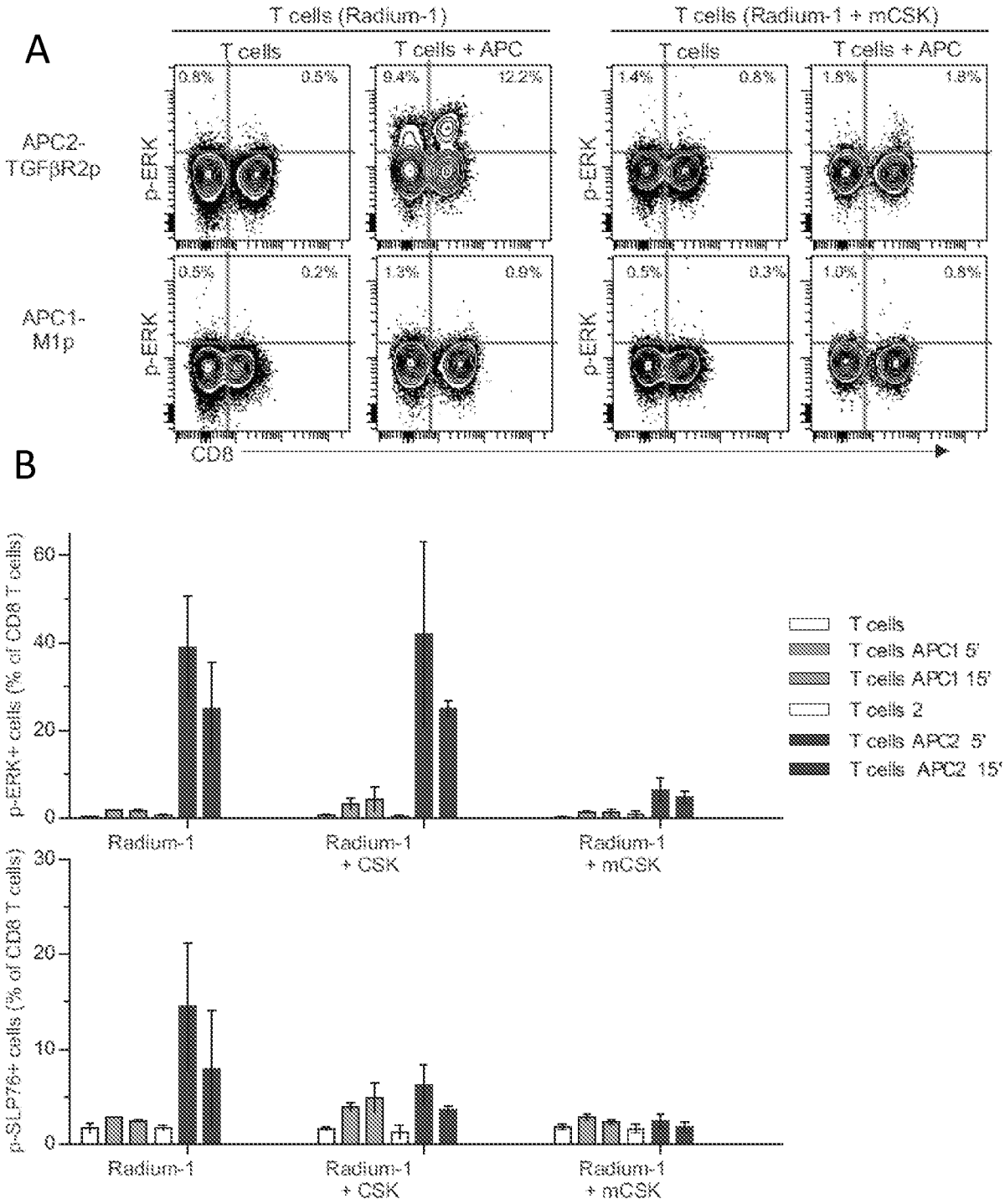
A
T cells (Radium-1)
T cells (Radium-1 + mCSK)
T cells
T cells + APC
APC2-TGFβR2p
APC1-M1p
p-ERK
CD8
0.8%
0.5%
9.4%
12.2%
1.4%
0.8%
1.8%
1.8%
0.5%
0.2%
1.3%
0.9%
0.5%
0.3%
1.0%
0.8%
B
p-ERK+ cells (% of CD8 T cells)
p-SLP76+ cells (% of CD8 T cells)
Radium-1
Radium-1 + CSK
Radium-1 + mCSK
T cells
T cells APC1 5'
T cells APC1 15'
T cells 2
T cells APC2 5'
T cells APC2 15'

FIG. 3

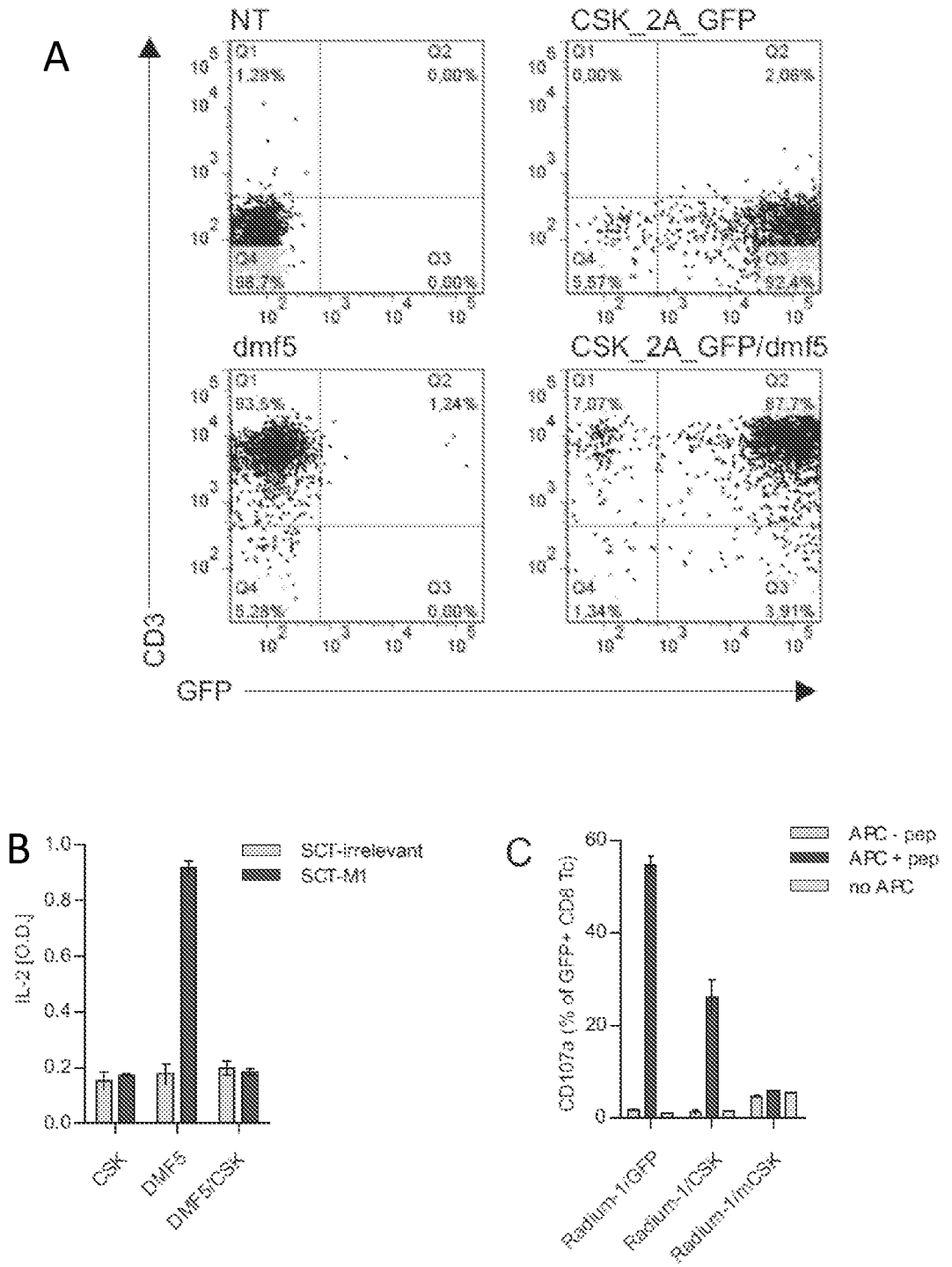
A
NT
CSK_2A_GFP
dmf5
CSK_2A_GFP/dmf5
CD3
GFP
B
SCT-irrelevant
SCT-M1
IL-2 [O.D.]
CSK
DMF5
DMF5/CSK
C
APC - pep
APC + pep
no APC
CD107a (% of GFP+ CD8 Tc)
Radium-1/GFP
Radium-1/CSK
Radium-1/mCSK

FIG. 4

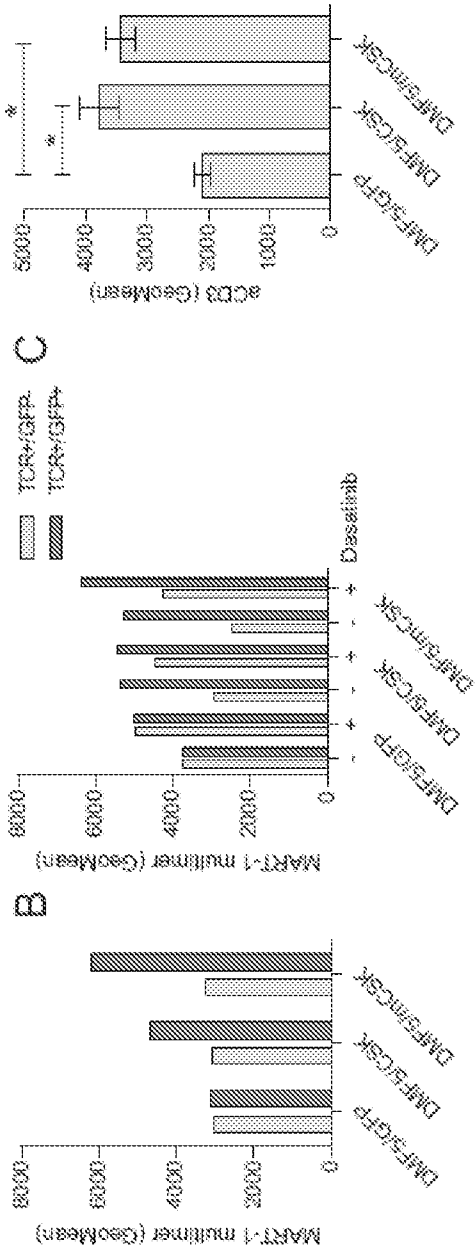
A
MART-1 multimer (GeoMean)
8000
6000
4000
2000
0
DMF5.GFP
DMF5.IL2CSK
DMF5.mTORCSK
B
MART-1 multimer (GeoMean)
8000
6000
4000
2000
0
TCR+/GFP-
TCR+/GFP+
Dasatinib
+
-
DMF5.GFP
DMF5.IL2CSK
DMF5.mTORCSK
C
aCD3 (GeoMean)
5000
4000
3000
2000
1000
0
*
*
DMF5.GFP
DMF5.IL2CSK
DMF5.mTORCSK

FIG. 5

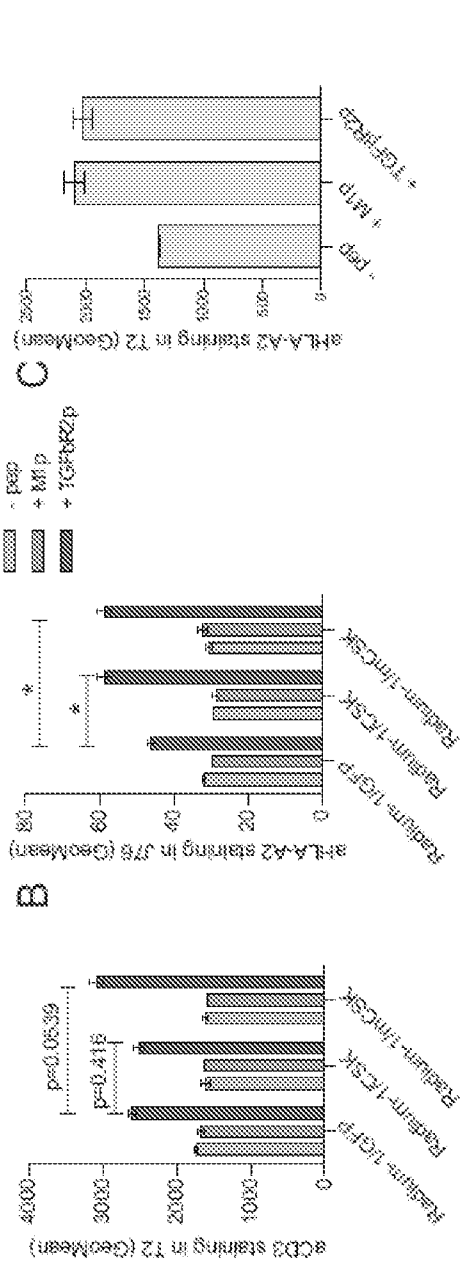
A
aCD3 staining in T2 (GeoMean)
4000
3000
2000
1000
0
p=0.0539
p=0.416
Raddum-1.GFP
Raddum-1.IL2CSK
Raddum-1.mTORCSK
B
anti-A-A2 staining in JY6 (GeoMean)
80
60
40
20
0
*
*
Raddum-1.GFP
Raddum-1.IL2CSK
Raddum-1.mTORCSK
C
anti-A-A2 staining in T2 (GeoMean)
2500
2000
1500
1000
500
0
- pep
+ MM p
+ YGFRKRZp

FIG. 6

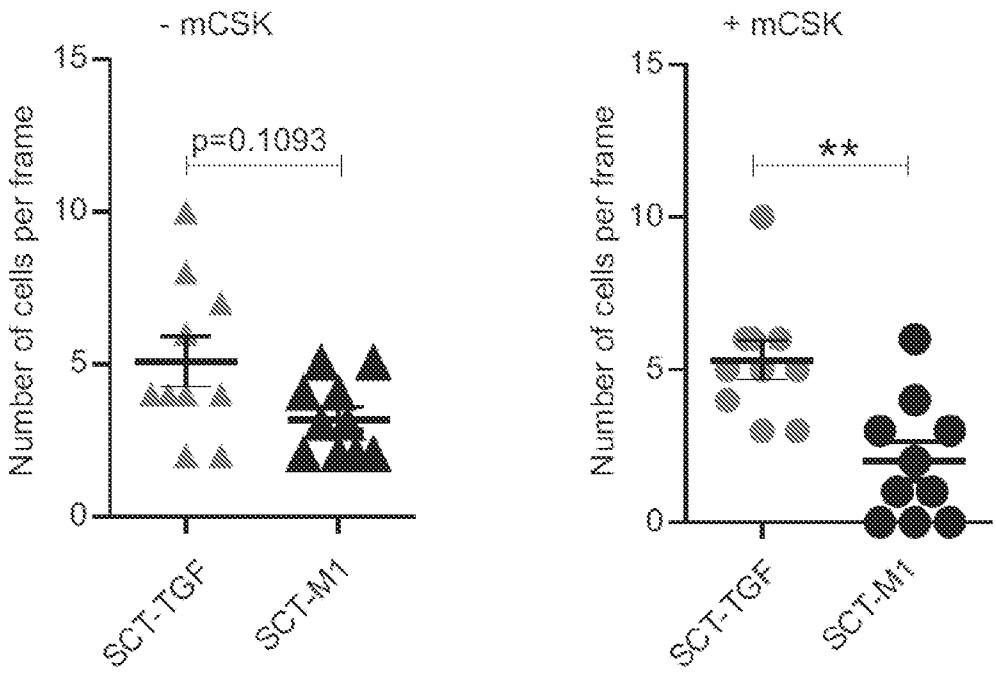
- mCSK
+ mCSK
Number of cells per frame
15
10
5
0
p=0.1093
**
SCT-TGF
SCT-M1

FIG. 7

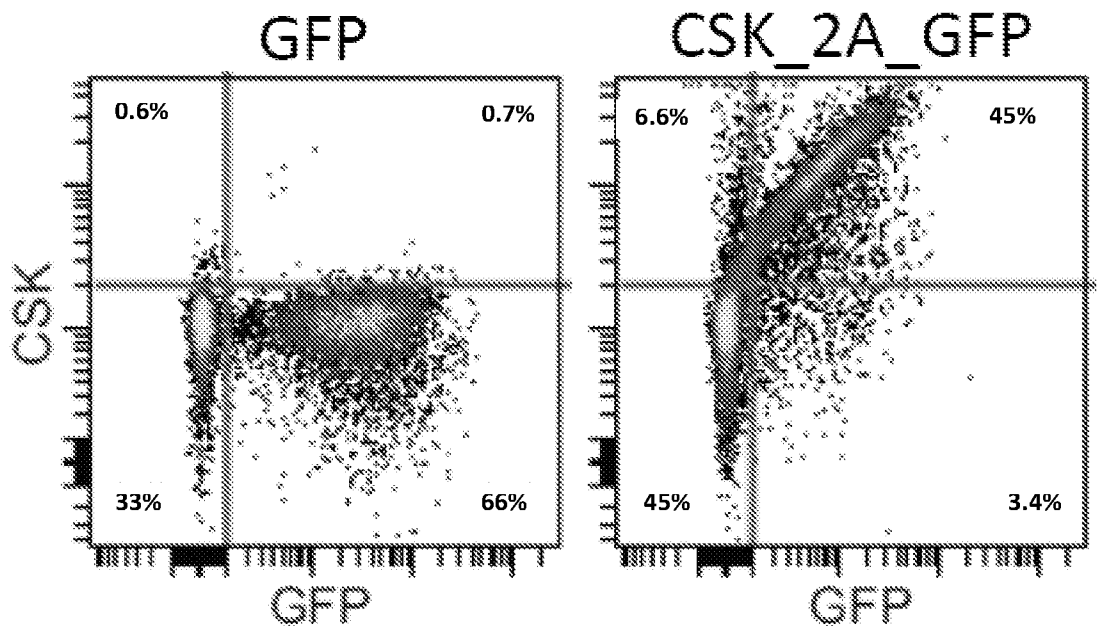
GFP
CSK_2A_GFP
CSK
GFP
0.6%
0.7%
33%
66%
6.6%
45%
45%
3.4%

FIG. 8

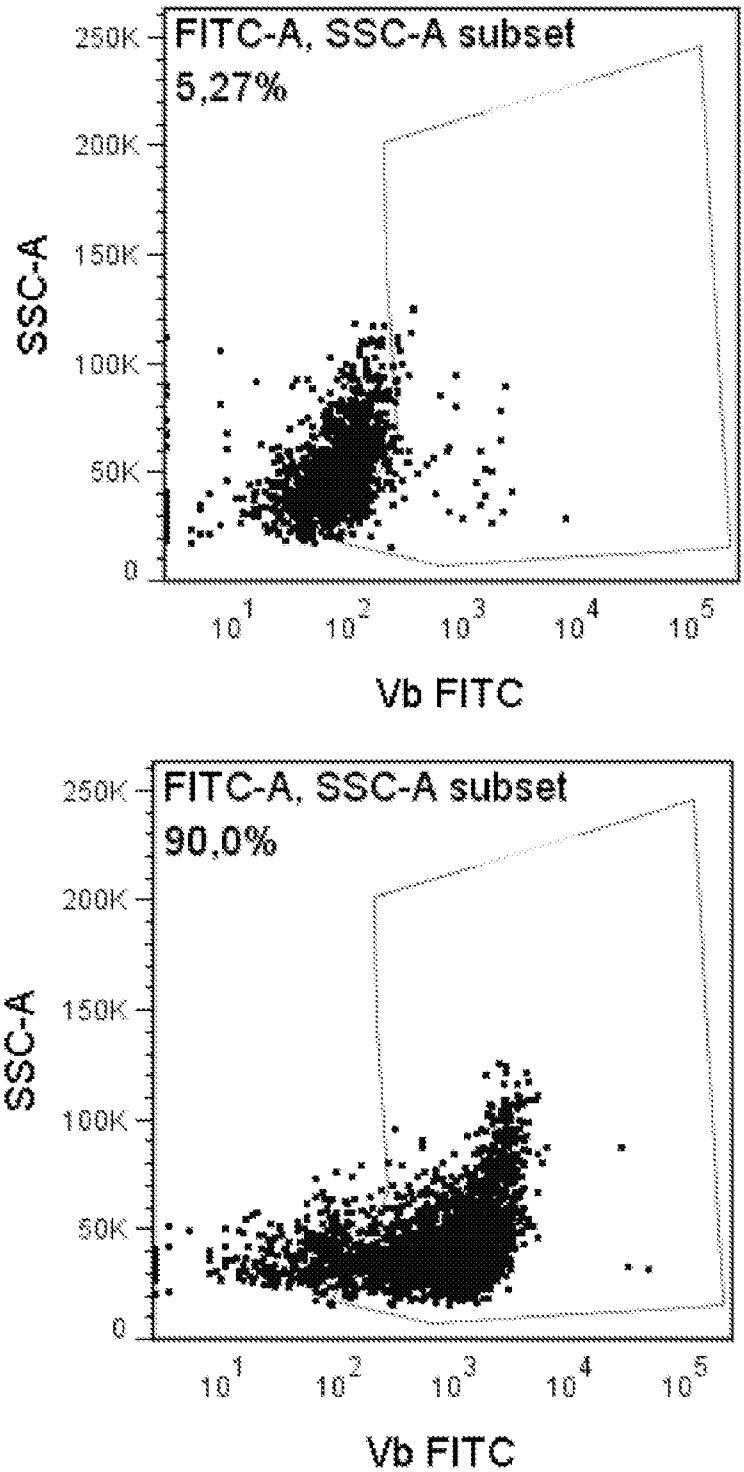
FITC-A, SSC-A subset
5,27%
250K
200K
150K
100K
50K
0
SSC-A
10^1
10^2
10^3
10^4
10^5
Vb FITC
FITC-A, SSC-A subset
90,0%
250K
200K
150K
100K
50K
0
SSC-A
10^1
10^2
10^3
10^4
10^5
Vb FITC

FIG. 9

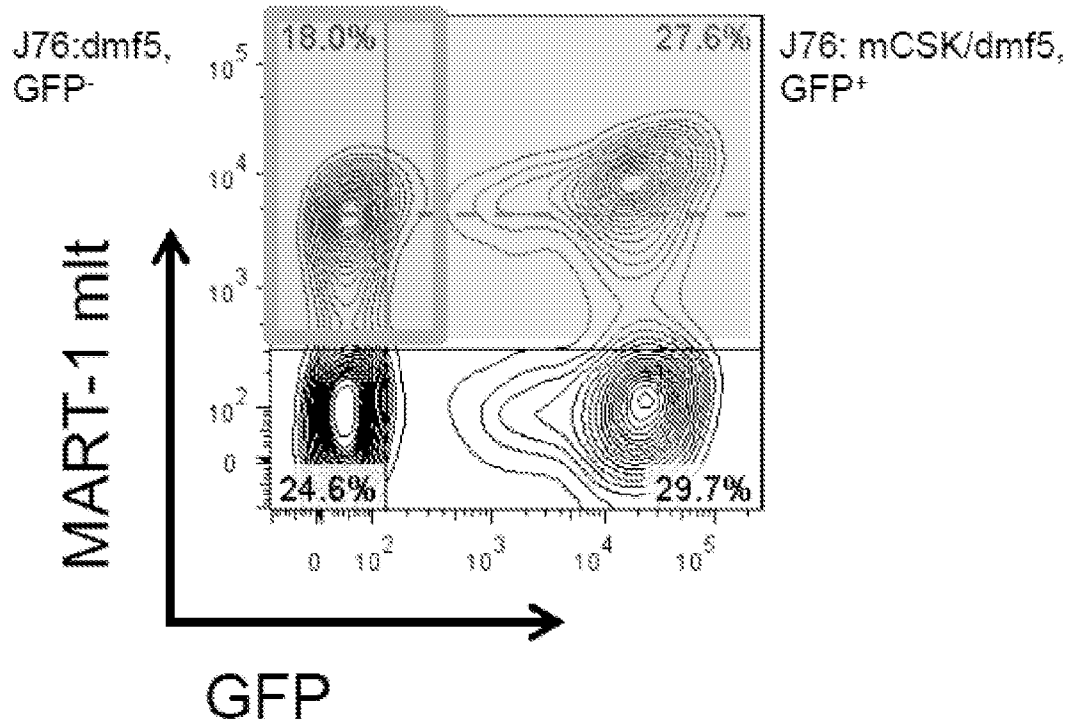
J76:dmf5, GFP-
J76: mCSK/dmf5, GFP+
18.0%
27.6%
24.6%
29.7%
MART-1 mlt
GFP

FIG. 10

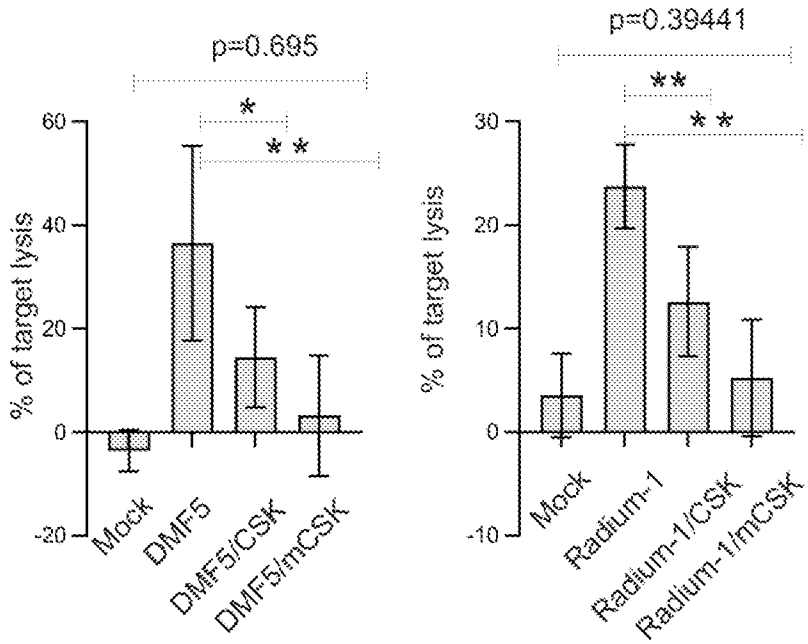
p=0.695
p=0.39441
% of target lysis
Mock
DMF5
DMF5/CSK
DMF5/mCSK
Radium-1
Radium-1/CSK
Radium-1/mCSK

FIG. 11

tyrosine-protein kinase CSK [Homo sapiens]

NCBI Reference Sequence: NP_001120662.1

SEQ ID NO:6

```
  1 msaiqaawps gteciakynf hgtaeqdlpf ckgdvltiva vtkdpnwyka knkvgregii
 61 panyvqkreg vkagtklslm pwfhgkitre qaerllyppe tglflvrest nypgdytlcv
121 scdgkvehyr imyhasklsi deevyfenlm qlvehytsda dglctrlikp kvmegtvaaq
181 defyrsgwal nmkelkllqt igkgefgdvm lgdyrgnkva vkcikndata qaflaeasvm
241 tqlrhsnlvq llgviveekg glyivteyma kgslvdylrs rgrsvlggdc llkfsldvce
301 ameylegnnf vhrdlaarnv lvsednvakv sdfgltkeas stqdtgklpv kwtapealre
361 kkfstksdvw sfgillweiy sfgrvpypri plkdvvprve kgykmdapdg cppavyevmk
421 ncwhldaamr psflqlreql ehikthelhl
```

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2017/000563

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/17 A61N1/39 G01N33/50
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHOW L M ET AL: "Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50csk.", NATURE 09 SEP 1993, vol. 365, no. 6442, 9 September 1993 (1993-09-09), pages 156-160, XP002772225, ISSN: 0028-0836 ----- -/--	1-22

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 2017	01/08/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bochelen, Damien

4

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2017/000563

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KJETIL TASKEN: "Negative regulation of T-cell receptor activation by the cAMP-PKA-Csk signalling pathway in T-cell lipid rafts", FRONTIERS IN BIOSCIENCE, vol. 11, no. 1, 1 January 2006 (2006-01-01), page 2929, XP055391629, US ISSN: 1093-9946, DOI: 10.2741/2022 the whole document -----	1-22
A	WO 2014/184744 A1 (CELLECTIS [FR]) 20 November 2014 (2014-11-20) the whole document -----	1-22

4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2017/000563

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014184744 A1	20-11-2014	AU 2014266833 A1	24-12-2015
		CA 2912375 A1	20-11-2014
		CN 105378067 A	02-03-2016
		EP 2997133 A1	23-03-2016
		JP 2016524464 A	18-08-2016
		KR 20160030103 A	16-03-2016
		RU 2015153241 A	21-06-2017
		WO 2014184744 A1	20-11-2014