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(54) Title: AN IMMUNE CELL COMPRISING A CAR AND TRUNCATED PROTEIN

(57) Abrégé/Abstract:
The present invention relates to a cell which comprises a chimeric antigen receptor (CAR) and a signal transduction modifying protein, selected from one of the following: (i) a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM), but lacks a kinase domain; (ii) a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) but lacks a phosphatase domain; (iii) a fusion protein which comprises (a) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) or from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a heterologous domain.

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(54) Title: CELL

(57) Abstract: The present invention relates to a cell which comprises a chimeric antigen receptor (CAR) and a signal transduction modifying protein, selected from one of the following: (i) a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM), but lacks a kinase domain; (ii) a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) but lacks a phosphatase domain; (iii) a fusion protein which comprises (a) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) or from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a heterologous domain.

AN IMMUNE CELL COMPRISING A CAR AND TRUNCATED PROTEIN

FIELD OF THE INVENTION

The present invention relates to fusion proteins and truncated proteins which enable the signalling pathways which are propagated following immune cell activation to be manipulated or modulated.

BACKGROUND TO THE INVENTION

Adoptive immunotherapy with autologous T cells involves the isolation of T cells from the patient followed by their stimulation, modification and/or expansion *ex vivo* in order to generate a population of T cells which display anti-tumour specificities. Once re-infused into the patient these cells are capable of recognizing tumour-expressed antigens and mediating tumour rejection.

This approach has already been shown in a number of trials in different settings to have the potential to be a powerful, effective and long-lasting treatment for cancer. For instance, EBV-driven tumours, such as lymphoproliferative disease following solid organ transplant can be effectively treated by ex vivo expanded EBV specific T-cells.

A similar therapy for non-viral malignancies involves tumour infiltrating lymphocytes (TILs) which are isolated from resected fragments of tumour and then subjected to stimulation and expansion with autologous tumour samples. Expanded T cell cultures which show tumour reactivity can then be re-infused into the patient.

Rather than selecting and refining T cell specificities with repeated exposure to antigens, the desired anti-tumour specificity can be conferred onto the T cells through gene modification and the introduction of either a tumour-specific T cell receptor (TCR) or a chimeric antigen receptor (CAR). These cells are expanded *ex vivo* in order to produce sufficient numbers of cells to achieve meaningful clinical responses within the patient.

However, the approaches detailed above have limitations. For instance, adoptively transferred T-cells may show limited persistence and expansion *in vivo* due to insufficient signalling, lack of IL2 or differentiation. By way of further example, adoptively transferred T-cells may succumb to inhibitory stimuli within the tumour microenvironment. For example they may become exhausted, undergo activation induced cell death consequent to over activation, or may cause on-target off-tumour effects.

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Another promising approach to activating therapeutic anti-tumour immunity is the blockade of immune checkpoints. Immune checkpoints refer to the various inhibitory pathways of the immune system that are important for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in peripheral tissues.

It is known that tumours exploit certain immune-checkpoint pathways as a major mechanism of immune resistance, particularly against T cells that are specific for tumour antigens. Many of the immune checkpoints are initiated by ligand–receptor interactions, meaning that they can be blocked by antibodies or modulated by recombinant forms of ligands or receptors. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) antibodies were the first of this class of immunotherapeutics to achieve US Food and Drug Administration (FDA) approval. More recently, blockers of additional immune-checkpoint proteins, such as programmed cell death protein 1 (PD1), have been developed and shown to enhance anti-tumour immunity.

One problem with the use of immune checkpoint inhibitors is that there are a multitude of inhibitory pathways triggered by a multitude of ligand:receptor interactions. The use of an antibody or a recombinant form of the ligand/receptor will only block one such inhibitory pathway, leaving the possibility open that the tumour can compensate for the specific immune checkpoint block using other molecules.

SUMMARY OF ASPECTS OF THE INVENTION

The present inventors have developed a system for modulating and/or manipulating signal transduction pathways in immune cells, such as T cells and natural killer (NK) cells.

Intracellular signalling pathways are initiated and controlled by the reversible post-translational modification of proteins. The present inventors have determined that activating and inhibitory signalling pathways in T cells can be modulated and/or manipulated by fusion proteins or truncated proteins comprising SH2 domains from immediate T-cell signal transduction proteins. In other words, activating and inhibitory signalling pathways in T cells can be modulated and/or manipulated by fusion proteins or truncated proteins comprising SH2 domains from proteins which are capable of binding phosphorylated immunoreceptor tyrosine-based activation motifs (ITAM) or phosphorylated immunoreceptor tyrosine-based inhibition motifs (ITIM).

Thus in a first aspect, the present invention provides a cell which comprises a chimeric antigen receptor (CAR) and a signal transduction modifying protein, selected from one of the following:

(i) a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM), but lacks a kinase domain;

(ii) a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) but lacks a phosphatase domain;

(iii) a fusion protein which comprises (a) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) or from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a heterologous domain.

The signal transduction modifying protein may be a truncated protein which comprises a ZAP70 SH2 domain but lacks a ZAP70 kinase domain.

The signal transduction modifying protein may be a truncated protein which comprises an PTPN6 SH2 but lacks a PTPN6 phosphatase domain.

The signal transduction modifying protein may be a truncated protein which comprises a SHP-2 SH2 domain but lacks a SHP-2 phosphatase domain.

The signal transduction modifying protein may be a fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM); and (ii) a phosphatase domain.

The fusion protein may, for example, comprise a ZAP70 SH2 domain, a PTPN6 or an SHP-2 phosphatase domain.

The signal transduction modifying protein may be a fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a kinase domain.

The fusion protein may comprise an SH2 domain from PTPN6 or SHP-2.

The fusion protein may comprise a Zap70 kinase domain

The fusion protein may comprise an AKT or JAK kinase domain.

The signal transduction modifying protein may be a fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) or from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a heterologous signalling domain.

The fusion protein may comprise an SH2 domain from ZAP70, PTPN6 or SHP-2.

The heterologous signalling domain may be from a signalling molecule which is not usually activated by an ITAM or ITIM containing receptor.

The heterologous signalling domain may be a co-stimulatory domain. In this respect, the fusion protein may comprise a CD28, OX40 or 41BB co-stimulatory domain.

The heterologous signalling domain may be an inhibitory domain. In this respect, the inhibitory domain may be or comprise the endodomain of CD148 or CD45. Alternatively, the heterologous signalling domain is or comprises the endodomain of ICOS, CD27, BTLA, CD30, GITR or HVEM.

The signal transduction modifying protein may be a fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM); and (ii) an ITAM-containing domain.

The fusion protein may comprises a ZAP70 SH2 domain.

The ITAM-containing domain may be or comprise the endodomain of CD3-Zeta.

The signal transduction modifying protein may be a fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) an ITIM-containing domain.

The fusion protein may comprise an SH2 domain from PTPN6 or SHP-2.

The ITIM-containing domain may be or comprise the endodomain from PD1, PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 or KIR3DL3.

The signal transduction modifying protein may be a fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation

motif (ITAM) or from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a protease domain.

The fusion protein may comprise an SH2 domain from ZAP70, PTPN6 or SHP-2.

The protease domain may be or comprise Tobacco Etch Virus Protease (TeV).

The cell may also comprises a membrane-tethered transcription factor having a protease cleavage site. Cleavage at the protease cleavage site may release the transcription factor leading to increased expression of a target gene.

The target gene encodes a cytokine, for example a cytokine selected from the following group: IL-2, IL-7, IL-15 and IL-12.

In this embodiment, the chimeric antigen receptor (CAR) may be a target CAR which comprises an intracellular protease cleavage site.

The target CAR may comprise an activatory or co-stimulatory endodomain and cleavage at the protease cleavage site removes the endodomain from the target CAR.

Alternatively, the target CAR may comprise an inhibitory endodomain and cleavage at the protease cleavage site removes the inhibitory endodomain from the target CAR. The inhibitory endodomain may comprise a CD148 or CD45 endodomain.

The cell of the present invention may comprise two CARs: an activating CAR comprising an ITAM-containing endodomain; and a target CAR as defined above.

Alternatively, the cell of the present invention may comprise two CARs: an inhibitory CAR comprising an ITIM-containing endodomain; and a target CAR as defined above.

In a second aspect, the present invention provides a nucleic acid construct, which comprises:

a first nucleic acid sequence encoding a chimeric antigen receptor; and

a second nucleic acid sequence encoding a truncated protein or a fusion protein as defined in connection with the first aspect of the invention.

The nucleic acid construct may also comprise a third nucleic acid sequence encoding a membrane-tethered transcription factor as defined above.

The nucleic acid construct may also comprise a third nucleic acid sequence encoding a target CAR as defined above.

The nucleic acid construct may also comprise a fourth nucleic acid sequence encoding an activating CAR or an inhibitory CAR as defined above.

In a third aspect, the vector which comprises a nucleic acid construct according to the second aspect of the invention or first and second, and optionally third and/or fouth, nucleic acid sequences as defined above.

There is also provided a set of vectors which comprises first and second, and optionally third and/or fourth, nucleic acid sequences as defined above.

The vector or set of vectors may be retroviral or lentiviral vector(s).

In a fourth aspect, there is provided a pharmaceutical composition comprising a plurality of cells according to the first aspect of the invention.

In a fifth aspect, there is provided a pharmaceutical composition according to the fourth aspect of the invention for use in treating and/or preventing a disease.

In a sixth aspect, there is provided method for treating and/or preventing a disease, which comprises the step of administering a pharmaceutical composition according to the fourth aspect of the invention to a subject.

The method may comprise the following steps:

(i) isolation of a cell containing sample from a subject;

(ii) transduction or transfection of the cells with a nucleic acid construct according to the second aspect of the invention, a vector or set of vectors according to the third aspect of the invention; and

(iii) administering the cells from (ii) to the subject.

In a seventh aspect there is provided the use of a pharmaceutical composition according to the fourth aspect of the invention in the manufacture of a medicament for the treatment and/or prevention of a disease.

The disease may be cancer.

In an eighth aspect, there is provided a method for making a cell according to the first aspect of the invention, which comprises the step of introducing: a nucleic acid construct according to the second aspect of the invention, a vector or set of vectors according to the third aspect of the invention, into the cell.

The cell may be from a sample isolated from a subject.

In a first further aspect, the present invention also provides a fusion protein which comprises: (i) a ZAP70 or PTPN6 SH2 domain; and (ii) a heterologous domain.

The fusion protein may comprise a ZAP70 SH2 domain and an ITAM-containing domain. The ITAM-containing domain may be or comprise the endodomain of CD3-Zeta.

The fusion protein may comprise a PTPN6 SH2 domain and an ITIM-containing domain. The ITIM-containing domain may be or comprise the endodomain from PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 or KIR3DL3.

The fusion protein may comprise a PTPN6 SH2 domain and fused to a ZAP70 kinase domain.

The fusion protein may comprise a ZAP70 SH2 domain fused to a PTPN6 kinase domain.

The fusion protein may comprise: (i) a ZAP70 or PTPN6 SH2 domain; and (ii) a heterologous signalling domain.

The heterologous signalling domain may be from a signalling molecule which is not usually activated by an ITAM containing receptor. The heterologous signalling domain may be or comprise the endodomain of CD28, 41BB or OX40. The heterologous signalling domain may be or comprise the endodomain of ICOS, CD27, BTLA, CD30, GITR or HVEM.

The fusion protein may comprise: (i) a ZAP70 or PTPN6 SH2 domain; and (ii) a kinase domain.

The kinase domain may be or comprise an AKT or JAK kinase domain.

The fusion protein may comprise: (i) a ZAP70 or PTPN6 SH2 domain; and (ii) a protease domain.

The protease domain may be or comprise Tobacco Etch Virus Protease (TeV).

In a second further aspect the present invention provides a truncated protein which comprises the ZAP70 SH2 domain but lacks the ZAP70 kinase domain.

In a third further aspect the present invention provides a truncated protein which comprises the PTPN6 SH2 domain but lacks the PTPN6 kinase domain.

The present invention also provides a signalling system comprising:

(i) a receptor comprising an antigen-binding domain, a transmembrane domain and an intracellular signalling domain which comprises a CD3 zeta endodomain; and

(ii) a fusion protein according to the first further aspect of the invention which comprises a ZAP70 SH2 domain; or a truncated protein according to the second further aspect of the invention;

wherein binding of antigen to the antigen-binding domain results in binding between the CD3 zeta endodomain and the fusion/truncated protein.

The present invention also provides a signalling system comprising:

(i) a receptor comprising an antigen-binding domain, a transmembrane domain and an intracellular signalling domain which comprises a PTPN6 binding domain; and

(ii) a fusion protein according to the first further aspect of the invention which comprises a PTPN6 SH2 domain; or a truncated protein according to the third further aspect of the invention

wherein binding of antigen to the antigen-binding domain results in binding between the PTPN6 binding domain and the fusion/truncated protein.

The receptor may be a T-cell receptor (TCR) or a chimeric antigen receptor (CAR).

In a fourth further aspect the present invention provides a nucleic acid which encodes a fusion protein according to the first further aspect of the present invention or a truncated protein according to the second or third further aspects of the present invention.

In a fifth further aspect the present invention provides a nucleic acid construct which comprises a nucleic acid sequence encoding a fusion protein which comprises (i) a ZAP70 or PTPN6 SH2 domain; and (ii) a protease domain (e.g. a TeV domain) and a nucleic acid sequence encoding a membrane tethered transcription factor which comprises:

(i) a membrane tether;

(ii) a protease recognition site; and

(iii) a transcription factor.

In a sixth further aspect the present invention provides a nucleic acid construct which comprises

(a) a nucleic acid sequence encoding a fusion protein according to the first further aspect of the present invention which comprises a PTPN6 SH2 domain, or a truncated protein according to the third further aspect of the present invention; and

(b) a nucleic acid sequence encoding a receptor comprising an ITIM containing endodomain.

In a seventh further aspect the present invention provides a nucleic acid construct which comprises a nucleic acid sequence encoding a fusion protein which comprises (i) a ZAP70 or PTPN6 SH2 domain; and (ii) a protease domain (e.g. a TeV domain) and a nucleic acid sequence encoding a receptor which comprises a protease cleavage site.

In an eighth further aspect the present invention provides a nucleic acid construct which comprises:

(a) a nucleic acid sequence encoding a fusion protein which comprises (i) a PTPN6 SH2 domain; and (ii) a protease domain (e.g. a TeV domain);

(b) a nucleic acid sequence encoding a receptor which comprises a protease cleavage site; and

(c) a nucleic acid sequence encoding a receptor comprising an ITIM containing endodomain.

The receptor may be a T-cell receptor (TCR) or a chimeric antigen receptor (CAR).

Suitably, in the nucleic acid construct according to the eighth aspect of the present invention, the nucleic acid sequence (b) may be a T-cell receptor (TCR) or a chimeric antigen receptor (CAR) which comprises:

(i) a protease cleavage site between a transmembrane domain and an activating endodomain; or

(ii) an activating endodomain fused to an inhibitory endodomain via a protease cleavage site.

In a ninth further aspect the present invention provides a vector comprising a nucleic acid according to the fourth further aspect of the present invention or a nucleic acid construct according to any of fifth to the ninth further aspects of the present invention.

The vector may be a retroviral vector or a lentiviral vector.

In a tenth further aspect the present invention provides a cell comprising a fusion protein according to the first further aspect of the present invention or a truncated protein according to the second or third further aspects of the present invention.

In an eleventh further aspect the present invention provides a cell which comprises (a) a fusion protein according to the first further aspect of the present invention which comprises a PTPN6 SH2 domain, or a truncated protein according to the third further aspect of the present invention; and (b) a receptor comprising an ITIM containing endodomain.

The cell may be an immune cell, such as a T cell or a natural killer (NK) cell.

In a twelfth further aspect the present invention provides a cell which comprises a fusion protein which comprises (i) a ZAP70 or PTPN6 SH2 domain; and (ii) a protease domain(e.g. a TeV domain) and a receptor which comprises a protease cleavage site.

In a thirteenth further aspect the present invention provides a cell which comprises:
(a) a fusion protein which comprises (i) a PTPN6 SH2 domain; and (ii) a protease domain (e.g. a TeV domain);
(b) a receptor which comprises a protease cleavage site; and
(c) a receptor comprising an ITIM containing endodomain.

The receptor may be a T-cell receptor (TCR) or a chimeric antigen receptor (CAR).

The receptor (b) may be a T-cell receptor (TCR) or a chimeric antigen receptor (CAR) which comprises:

(i) a protease cleavage site between a transmembrane domain and an activating endodomain; or

(ii) an activating endodomain fused to an inhibitory endodomain via a protease cleavage site.

In a fourteenth further aspect the present invention provides a cell which comprises a nucleic acid according to the fourth further aspect of the present invention or a nucleic acid construct according to any of the fifth to the ninth further aspects of the present invention.

In a fifteenth further aspect the present invention provides a pharmaceutical composition comprising a plurality of cells according to any of the tenth to the fourteenth further aspects of the present invention.

In a sixteenth further aspect the present invention provides a pharmaceutical composition according to the fifteenth further aspect of the present invention for use in treating and/or preventing a disease.

In a seventeenth further aspect the present invention relates to a method for treating and/or preventing a disease, which comprises the step of administering a pharmaceutical composition according the fifteenth further aspect to a subject.

The method may comprise the following steps:

(i) isolation of a T cell or NK cell containing sample from a subject;

(ii) transduction or transfection of the T cells or NK cells with a nucleic acid according to any of the fourth to the ninth further aspects of the present invention or a vector according to the tenth further aspect of the present invention; and

(iii) administering the T cells or NK cells from (ii) to the subject.

In an eighteenth further aspect the present invention relates to the use of a pharmaceutical composition according to the fifteenth further aspect of the present invention in the manufacture of a medicament for the treatment and/or prevention of a disease.

The disease may be cancer.

In a nineteenth further aspect the present invention provides a kit which comprises a nucleic acid according to the fourth further aspect of the present invention or a nucleic acid construct according to any of the fifth to the eighth further aspects of the present invention or a vector according to the ninth further aspect of the present invention.

In a twentieth further aspect the present invention relates to a kit which comprises a cell according to any of the tenth to the fourteenth further aspects of the present invention.

In a twenty-first further aspect the present invention relates to a method for making a cell according to any of the tenth to the fourteenth further aspects of the present invention, which comprises the step of introducing: a nucleic acid sequence according to any of the fourth to the eighth further aspects of the present invention or the vector according to the ninth further aspect of the present invention, into the cell.

The cell may be from a sample isolated from a subject.

Yet further aspect of the invention are summarised in the following paragraphs:

A1. A truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) but lacks a kinase domain.

A2. A truncated protein according to paragraph A1, which comprises the ZAP70 SH2 domain but lacks the ZAP70 kinase domain.

B1. A truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) but lacks a phosphatase domain.

B2. A truncated protein according to paragraph B1, which comprises the PTPN6 SH2 domain but lacks the PTPN6 phosphatase domain.

B3. A truncated protein according to paragraph B1, which comprises the SHP-2 SH2 domain but lacks the SHP-2 phosphatase domain.

C1. A fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM); and (ii) a phosphatase domain.

C2. A fusion protein according to paragraph C1, which comprises a ZAP70 SH2 domain.

C3. A fusion protein according to paragraph C1 or C2, which comprises a PTPN6 or SHP-2 phosphatase domain.

D1. A fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a kinase domain.

D2. A fusion protein according to paragraph D1, which comprises an SH2 domain from PTPN6 or SHP-2.

D3. A fusion protein according to paragraph D1 or D2, which comprises a Zap70 kinase domain

D4. A fusion protein according to paragraph D1 or D2, which comprises an AKT or JAK kinase domain.

E1. A fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) or from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a heterologous signalling domain.

E2. A fusion protein according to paragraph E1, which comprises an SH2 domain from ZAP70, PTPN6 or SHP-2.

E3. A fusion protein according to paragraph E1 or E2, wherein the heterologous signalling domain is from a signalling molecule which is not usually activated by an ITAM or ITIM containing receptor.

E4. A fusion protein according to paragraph E1, E2 or E3, wherein the heterologous signalling domain is a co-stimulatory domain.

E5. A fusion protein according to paragraph E4 which comprises a CD28, OX40 or 41BB co-stimulatory domain.

E6. A fusion protein according to paragraph E1, E2 or E3, wherein the co-stimulatory domain is an inhibitory domain.

E7. A fusion protein according to paragraph E6, wherein the inhibitory domain comprises the endodomain of CD148 or CD45.

E8. A fusion protein according to paragraph E6, wherein the heterologous signalling domain is or comprises the endodomain of ICOS, CD27, BTLA, CD30, GITR or HVEM.

F1. A fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM); and (ii) an ITAM-containing domain.

F2. A fusion protein according to paragraph F1, which comprises a ZAP70 SH2 domain.

F3. A fusion protein according to paragraph F1 or F2, wherein the ITAM-containing domain is or comprises the endodomain of CD3-Zeta.

G1. A fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) an ITIM-containing domain.

G2. A fusion protein according to paragraph G1, which comprises an SH2 domain from PTPN6 or SHP-2.

G3. A fusion protein according to paragraph G1 or G2, wherein the ITIM-containing domain is or comprises the endodomain from PD1, PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 or KIR3DL3.

H1. A fusion protein which comprises (i) an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) or from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM); and (ii) a protease domain.

H2. A fusion protein according to paragraph H1, which comprises an SH2 domain from ZAP70, PTPN6 or SHP-2.

H3. A fusion protein according to paragraph H1 or H2, wherein the protease domain is or comprises Tobacco Etch Virus Protease (TeV).

I1. A nucleic acid sequence which encodes a truncated protein according to any of paragraphs A or B, or a fusion protein according to any of paragraphs C, D, E, F, G or H.

J1. A nucleic acid construct which comprises a nucleic acid sequence according to paragraph I and a nucleic acid sequence encoding a chimeric antigen receptor

J2. A nucleic acid construct which comprises a nucleic acid sequence according to paragraph I and a nucleic acid sequence encoding a membrane-tethered transcription factor having a protease cleavage site.

J3. A nucleic acid construct which comprises a nucleic acid sequence according to paragraph I and a nucleic acid sequence encoding a target CAR which comprises an intracellular protease cleavage site.

K1. A vector comprising a nucleic acid sequence according to paragraph I or a nucleic acid construct according to paragraphs J.

L1. A cell which comprises a truncated protein according to any of paragraphs A or B, or a fusion protein according to any of paragraphs C, D, E, F, G or H.

M1. A cell which comprises a fusion protein according to any of paragraphs H and a membrane-tethered transcription factor with a protease cleavage site.

M2. A cell according to paragraph M1, wherein cleavage at the protease cleavage site releases the transcription factor leading to increased expression of a target gene.

M3. A cell according to paragraph M2, wherein the target gene encodes a cytokine.

M4. A cell according to paragraph M3, wherein the cytokine is selected from the following group: IL-2, IL-7, IL-15 and IL-12.

M5. A cell which comprises a fusion protein according to any of paragraphs H and a target receptor (CAR) which comprises an intracellular protease cleavage site.

M6. A cell according to claim M5, wherein the target CAR comprises an activatory or co-stimulatory endodomain and cleavage at the protease cleavage site removes the endodomain from the target CAR.

M7. A cell according to claim M5, wherein the target CAR comprises an inhibitory endodomain and cleavage at the protease cleavage site removes the inhibitory endodomain from the target CAR.

M8. A cell according to paragraph M7, wherein the inhibitory endodomain comprises a CD148 or CD45 endodomain.

M9. A cell which comprises a fusion protein according to any of paragraphs H and two CARs: an activating CAR comprising an ITAM-containing endodomain; and a target CAR as defined in any of paragraphs M5 to M8.

M10. A cell which comprises a fusion protein according to any of paragraphs H and two CARs: an inhibitory CAR comprising an ITIM-containing endodomain; and a target CAR as defined in any of paragraphs M5 to M8.

The aspects of the present invention described above enable T cell signalling pathways to be modulated and altered by, for example, the mechanisms described in Table 1. Table 1 : Application of signal modulation

Type	*Mechanism*	*Application*
Blocking signal	ZAP70, SHP-2 or PTPN6 are truncated - keeping SH2 domain alone	Truncated ZAP70, SHP-2 or PTPN6 competes with wild-type full-length ZAP70, SHP-2 or PTPN6. Since this does not signal, it will inhibit activation. Applications include, for example, with ZAP70 when a very strong activation signal is deleterious, or with PTPN6 or SHP-2 when the effect of an inhibitory signal e.g. PD1/PDL1 needs to be reduced.
Crosswire signal	ZAP70 SH2 fused to PTPN6/SHP-2 phosphatase, or PTPN6/SHP-2 SH2 fused to ZAP70 kinase for instance.	In this embodiment, a ZAP70 SH2 is fused to the phosphatase from PTPN6/SHP-2, or the other way round, i.e. the PTPN6/SHP-2 SH2 domain is fused with the ZAP70 kinase domain. When the T-cell receives an inhibitory signal, it interprets it as an excitatory signal or vice versa.
Amplified signal	ZAP70 fused to further ITAM domains or PTPN6/SHP-2 fused to further ITIM domains.	A single phospho-ITAM or ITIM leads to a concatenation of ITAMs or ITIMs leading to augmented signal or

		increased sensitivity to antigen.
Bypass signal	ZAP70 SH2 or PTPN6/SHP-2 SH2 fused with e.g. CD28, 41BB endodomains or AKT kinase domain, a JAK kinase domain etc.	In this embodiment, a "non-physiological" signal can be attached to the ITAM/ITM pathway. In this way an ITAM/ITIM signal can lead to a co-stimulatory signal, or a signal such as AKT or a cytokine type signal
Transcriptional signal	ZAP70 SH2 or PTPN6/SHP-2 SH2 fused to protease domain along with co-expression of a membrane tethered transcription factor with a liberating protease cleavage site	In this embodiment, a transcriptional signal is transmitted upon immune receptor activation or inhibition. Such a signal can, for example, result in the expression of a particular cytokine upon T-cell activation or inhibition.
Castration signal	ZAP70 SH2 domain or PTPN6/SHP-2 SH2 domain fused to a protease domain; a reciprocal receptor has a protease cleavage site	In this embodiment, activation or inhibition of a receptor results in inhibition or activation of another receptor

DESCRIPTION OF THE FIGURES

Figure 1 (a) – Diagram of immediate T-cell activation pathways. T-cell receptor activation results in phosphorylation of ITAMs. Phosphorylated ITAMs are recognized by the ZAP70 SH2 domains. Upon recognition, ZAP70 is recruited to the juxta-membrane region and its kinase domain subsequently phosphorylates LAT. Phosphorylated LAT is subsequently recognized by the SH2 domains of GRAP, GRB2 and PLC-γ. (b) – Diagram of immediate T-cell inhibition pathways. Activation of an inhibitory immune-receptor such as PD1 results in phosphorylation of ITIM domains. These are recognized by the SH2 domains of PTPN6. Upon recognition, PTPN6 is recruited to the juxta-membrane region and its phosphatase domain subsequently de-phosphorylates ITAM domains inhibiting immune activation.

Figure 2 – Diagram of a blocking signal system - a) A truncated ZAP70 which does not comprise a kinase domain is over-expressed. Consequently, it competes with full-length ZAP70 for ITAMs and reduces ITAM signalling. (b) A truncated PTPN6 which does not comprise a phosphatase domain is over-expressed, competing for full-length PTPN6 reducing ITIM signalling.

Figure 3 – Diagram of a crosswire signal system: (a) ZAP70 SH2 is fused to PTPN6 phosphatase, hence acts to dampen ITAM phosphorylation; (b) PTPN6 SH2 is fused to ZAP70 kinase resulting in paradoxical activation in response to an inhibitory signal.

Figure 4 – Diagram of an amplified signal system: (a) full-length ZAP70 has a CD3 Zeta endodomain attached to its amino terminus so a cascade of ITAMs assembles. (b) full-length PTPN6 has PD1 endodomain attached to its amino terminus so a cascade of ITIMs assembles.

Figure 5 – Diagram of examples of a bypass signal system: (a) ZAP70 fused with CD28 endodomain; (b) ZAP70 fused with 41BB endodomain; (c) ZAP70 fused with AKT kinase; (d) PTPN6 SH2 domain is fused with 41BB endodomain

Figure 6 – Diagram of an illustrative transcriptional signal system: a) A ZAP-TeV fusion is co-expressed with a membrane-tethered transcription factor which can be released from the membrane by cleavage of its TeV recognition motif. This is shown co-expressed with a CD19 CAR. Hence, upon recognition of CD19 on a target cell, the T-cell becomes activated and in addition, the transcription factor becomes active. (b) An alternative system using a PTPN6-TeV fusion instead. Here the CAR consists of an ITIM-bearing endodomain. Hence, upon recognition of CD19 by the CAR, the transcription factor becomes active but this is independent of T-cell activation.

Figure 7 – Diagram of a castration signal system: two CARs are shown – one which recognizes CD19 and is activating and one which recognizes CD33 and is inhibiting - these specificities are for illustration only (a) AND NOT signal castration; here an SH2-Tev fusion protein is recruited to activated ITIM CAR upon its activation. This results in cleavage of ITAMs from an activating CAR which is constructed such that a TeV cleavage site connects the transmembrane-domain to the ITAM domain. Hence, the activating CAR is inhibited. (b) AND signal castration: Here, an SH2-Tev fusion protein is recruited to an ITIM CAR upon its activation. This results in release of a phosphatase domain from an activating CAR which is constructed so that a phosphatase is connected to its carboxy-terminus via a TeV cleavage domain. This results in release of constitutive inhibition, allowing the CAR to activate in the presence of cognate antigen.

Figure 8 – Several fusions of different SH2 domains and AKT kinase domain were constructed: ZAP-AKT, GRAP-AKT, GRB-AKT and PLC-γ.

Figure 9 - (a) Phospho-AKT staining of T-cells transduced with the different SH2/AKT fusions with and without activation with the mitogenic antibody OKT3. (b) Phospho-AKT staining of T-cells transduced with ZAP-AKT fusion, an improved ZAP-AKT fusion where ZAP and AKT are connected via a flexible linker, and a control ZAP-AKT where R190K substitution removes ability of ZAP to bind ITAMs. T-cells were either stimulated with OKT3 or not-stimulated with OKT3. The facs plots are overlaid over that of non-transduced T-cells.

Figure 10 - (a) Phospho-AKT blot of T-cells activated with increasing amounts of OKT3. (b) Microscopy of ZAP-AKT or control T-cells unstimulated, stimulated with just OKT3 or stimulated with both OKT3 and IL-2. ZAP-AKT T-cells stimulated with just OKT3 resemble non-transduced T-cells stimulated with both OKT3 and IL2.

Figure 11 - (a) Implementation of direct TeV transcriptional switch. A CD19 CAR's endodomain is replaced with the TeV protease. A membrane tethered VP16/GAL4 transcription factor is also co-expressed. A Luciferase reporter detects VP16/GAL5 activity. (b) Implementation with ZAP-TeV. A standard CD19 CAR is co-expressed with ZAP-TeV fusion along with the membrane tethered transcription factor.

Figure 12 - Activity of ZAP-TeV based transcriptional switches and control expressing T-cells after exposure to CD19 negative (left), or CD19 positive (right) targets. Activity is measured by light output after adding Luciferase. In order the conditions tested are: (a) aCD19 CAR co-expressed with ZAP-TeV; (b) aCD19 CAR co-expressed with inactive (R190K); (c) aCD19 CAR co-expressed with ZAP-TeV and the membrane tethered transcription factor; (d) aCD19 CAR co-expressed with inactive (R190K) ZAP-TEV co-expressed with the membrane tethered transcription factor; (e) aCD19 CAR / TeV fusion co-expressed with the membrane-tethered transcription factor; (f) constitutively active GAL4/VP16 transcription factor.

Figure 13 - (a) Generalized architecture of a CAR: A binding domain recognizes antigen; the spacer elevates the binding domain from the cell surface; the trans-membrane domain anchors the protein to the membrane and the endodomain transmits signals. (b) to (d): Different generations and permutations of CAR endodomains: (b) initial designs transmitted ITAM signals alone through FcεR1-γ or CD3ζ endodomain, while later designs transmitted additional (c) one or (d) two co-stimulatory signals in cis.

Figure 14 - Illustrative protein sequences of the present invention

Figure 15 – PD-1 signal blockade using truncated SHP-1 (PTPN6) or truncated SHP-2
PBMC cells were cotransduced with PD1 and either CAR alone (FMC63); or a bicistronic construct containing CAR and truncated SHP-1, or CAR and truncated SHP-2. These cells were co-cultured for 48 hours with SupT1 cells transduced with CD19, PDL1 or both and IFNγ release measured by ELISA.

Figure 16 – PD-1 signal hijack using a fusion of SHP-2 SH2 domains and Zap70 kinase

PBMC cells were cotransduced with PD1 and either CAR alone (FMC63); or a bicistronic construct containing CAR and a fusion protein comprising SHP-2 SH2 domains and the ZAP70 kinase. These cells were co-cultured in a 1:1 ratio for 24 hours with SupT1 cells transduced with CD19 or PDL1. IFNγ release was measured by ELISA (A) and killing of SupT1 cells was quantified by FACS (B).

DETAILED DESCRIPTION

PROTEIN

The present invention provides a truncated protein which comprises an SH2 domain.

The present invention also provides a fusion protein comprising (i) an SH2 domain; and (ii) a heterologous domain.

The SH2 domain may be from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) or from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM).

An example of a protein which binds an ITAM is ZAP70. Examples of proteins which bind ITIMs include PTPN6 and SHP-2

The fusion protein of the invention therefore comprises an SH2 domain and at least one further domain which is not present in a wild-type protein from which the SH2 domain was derived.

SRC HOMOLOGY 2 (SH2) DOMAIN

Intracellular signalling pathways are initiated and controlled by the reversible post-translational modification of proteins including phosphorylation, ubiquitinylation and acetylation.

SH2 domains are modular protein domains that serve as adaptors and mediate protein-protein interactions by binding to phosphorylated peptides in their respective protein binding partners, often cell surface receptors. SH2 domains typically bind a phosphorylated tyrosine residue in the context of a longer peptide motif within a target protein, and SH2 domains represent the largest class of known pTyr-recognition domains

Although SH2 domains lack any intrinsic catalytic activity they are frequently coupled to independent catalytic domains and thus, in response to a specific input signal, serve to localize

these catalytic domains so particular sub-cellular locations or to the vicinity of appropriate substrates, activators or inhibitors. In addition SH2 domains can also be found linked to adaptor protein domains and so can serve in the formation of large multi-protein complexes.

ZETA-CHAIN-ASSOCIATED PROTEIN KINASE 70 (ZAP70)

ZAP70 is a protein normally expressed near the surface membrane of T cells and natural killer cells. It is part of the T cell receptor (TCR), and plays a critical role in T-cell signalling. Its molecular weight is 70 kDa, and is composed of 2 N-terminal SH2 domains and a C-terminal kinase domain. It is a member of the protein-tyrosine kinase family.

The earliest step in T cell activation is the recognition of a peptide MHC-complex on the target cell by the TCR. This initial event causes the close association of Lck kinase with the cytoplasmic tail of CD3-zeta in the TCR complex. Lck then phosphorylates tyrosine residues in the cytoplasmic tail of CD3-zeta which allows the recruitment of ZAP70. ZAP70 is an SH2 containing kinase that plays a pivotal role in T cell activation following engagement of the TCR. Tandem SH2 domains in ZAP70 bind to the phosphorylated CD3 resulting in ZAP70 being phosphorylated and activated by Lck or by other ZAP70 molecules in trans. Active ZAP70 is then able to phosphorylate downstream membrane proteins, key among them the linker of activated T cells (LAT) protein. LAT is a scaffold protein and its phosphorylation on multiple residues allows it to interact with several other SH2 domain-containing proteins including Grb2, PLC-g and Grap which recognize the phosphorylated peptides in LAT and transmit the T cell activation signal downstream ultimately resulting in a range of T cell responses. This process is summarized in Figure 1.

Human ZAP70 protein has the UniProtKB accession number P43403. This sequence is 619 amino acids in length and is shown as SEQ ID NO: 1.

ZAP70 amino acid sequence (SEQ ID NO: 1)

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYA
IAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQ
AIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYH
YLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHP
QRRIDTLNSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFG
SVRQGVYRMRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGG
PLHKFLVGKREEIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADD
SYYTARSAGKWPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMEC
PPECPPELYALMSDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

The fusion protein of the invention may comprise a ZAP70 SH2 domain. The truncated protein of the invention may comprise or consist of a ZAP70 SH2 domain. In this respect, the fusion or truncated protein may comprise or consist of the sequence shown as SEQ ID NO: 2.

ZAP70 complete SH2 domain (SEQ ID NO: 2)

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYA
IAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQ
AIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYH
YLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHP

ZAP70 has two SH2 domains at the N-terminal end of the sequence, at residues 10-102 and 163-254 of the sequence shown as SEQ ID No. 1. The truncated protein or fusion protein of the invention may therefor comprise one or both of the sequences shown as SEQ ID No. 3 and 4.

ZAP70 SH2 1 (SEQ ID NO: 3)

FFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGGKAHCG
PAELCEFYSRDPDGLPCNLRKPC

ZAP70 SH2 2 (SEQ ID NO: 4)

WYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHYLISQDKAGKYCIPEGTKFDTL
WQLVEYLKLKADGLIYCLKEAC

The fusion protein may comprise a variant of SEQ ID NO: 2, 3 or 4 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence is a SH2 domain sequence has the required properties. In other words, the variant sequence should be capable of binding to the phosphorylated tyrosine residues in the cytoplasmic tail of CD3-zeta which allow the recruitment of ZAP70.

Methods of sequence alignment are well known in the art and are accomplished using suitable alignment programs. The % sequence identity refers to the percentage of amino acid or nucleotide residues that are identical in the two sequences when they are optimally aligned. Nucleotide and protein sequence homology or identity may be determined using standard algorithms such as a BLAST program (Basic Local Alignment Search Tool at the National Center for Biotechnology Information) using default parameters, which is publicly available at http://blast.ncbi.nlm.nih.gov . Other algorithms for determining sequence identity or homology include: LALIGN (http://www.ebi.ac.uk/Tools/psa/lalign/ and http://www.ebi.ac.uk/Tools/psa/lalign/nucleotide.html), AMAS (Analysis of Multiply Aligned Sequences, at http://www.compbio.dundee.ac.uk/Software/Amas/amas.html), FASTA (http://www.ebi.ac.uk/Tools/sss/fasta/) , Clustal Omega

(http://www.ebi.ac.uk/Tools/msa/clustalo/), SIM (http://web.expasy.org/sim/), and EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html).

In certain embodiments, the fusion protein may comprise the ZAP70 SH2 domain and the ZAP70 kinase domain. For example, the fusion protein may comprise the sequence shown as SEQ ID NO: 1 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

TYROSINE-PROTEIN PHOSPHATASE NON-RECEPTOR TYPE 6 (PTPN6)

PTPN6 is also known as Src homology region 2 domain-containing phosphatase-1 (SHP-1). It is a member of the protein tyrosine phosphatase family.

The N-terminal region of PTPN6 contains two tandem SH2 domains which mediate the interaction of PTPN6 and its substrates. The C-terminal region contains a tyrosine-protein phosphatase domain.

PTPN6 is capable of binding to, and propagating signals from, a number of inhibitory immune receptors or ITIM containing receptors. Examples of such receptors include, but are not limited to, PD1, PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 and KIR3DL3.

Human PTPN6 protein has the UniProtKB accession number P29350. This sequence is 595 amino acids in length and is shown as SEQ ID NO: 5.

PTPN6 amino acid sequence (SEQ ID NO: 5)

MVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFA
TLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLS
QPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLR
QPYYATRVNAADIENRVLELNKKQESEDTAKAGFWEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNIL
PFDHSRVILQGRDSNIPGSDYINANYIKNQLLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMT
TREVEKGRNKCVPYWPEVGMQRAYGPYSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDH
GVPSEPGGVLSFLDQINQRQESLPHAGPIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQM
VRAQRSGMVQTEAQYKFIYVAIAQFIETTKKKLEVLQSQKGQESEYGNITYPPAMKNAHAKASRTSSKHK
EDVYENLHTKNKREEKVKKQRSADKEKSKGSLKRK

The fusion protein of the invention may comprise a PTPN6 SH2 domain. The truncated protein of the invention may comprise or consist of a PTPN6 SH2 domain. In this respect, the fusion or truncated protein may comprise or consist of the sequence shown as SEQ ID NO: 6.

PTPN6 SH2 complete domain (SEQ ID NO: 6)

MVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFA
TLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLS
QPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLR
QPYY

PTPN6 has two SH2 domains at the N-terminal end of the sequence, at residues 4-100 and 110-213 of the sequence shown as SEQ ID No. 5. The truncated protein or fusion protein of the invention may therefor comprise one or both of the sequences shown as SEQ ID No. 3 and 4.

PTPN6 SH2 1 (SEQ ID NO: 7)

WFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLT
ELVEYYTQQQGVLQDRDGTIIHLKYPL

PTPN6 SH2 2 (SEQ ID No. 8)

WYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVG
GLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPY

The fusion protein may comprise a variant of SEQ ID NO: 6, 7 or 8 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence is a SH2 domain sequence has the required properties. In other words, the variant sequence should be capable of binding to the phosphorylated tyrosine residues in the cytoplasmic tail of at least one of PD1, PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 or KIR3DL3 which allow the recruitment of PTPN6.

In certain embodiments, the fusion protein may comprise the PTPN6 SH2 domain and the PTPN6 phosphatase domain. For example, the fusion protein may comprise the sequence shown as SEQ ID NO: 5 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

SHP-2

SHP-2, also known as PTPN11, PTP-1D and PTP-2C is is a member of the protein tyrosine phosphatase (PTP) family. Like PTPN6, SHP-2 has a domain structure that consists of two tandem SH2 domains in its N-terminus followed by a protein tyrosine phosphatase (PTP) domain. In the inactive state, the N-terminal SH2 domain binds the PTP domain and blocks access of potential substrates to the active site. Thus, SHP-2 is auto-inhibited. Upon binding to target phospho-tyrosyl residues, the N-terminal SH2 domain is released from the PTP domain, catalytically activating the enzyme by relieving the auto-inhibition.

Human SHP-2 has the UniProtKB accession number P35235-1. This sequence is 597 amino acids in length and is shown as SEQ ID NO: 9.

SHP-2 amino acid sequence (SEQ ID NO: 9)

MTSRRWFHPNITGVEAENLLLTRGVDGSFLARPSKSNPGDFTLSVRRNGAVTHIKIQNTGDYYDLYGGEK
FATLAELVQYYMEHHGQLKEKNGDVIELKYPLNCADPTSERWFHGHLSGKEAEKLLTEKGKHGSFLVRES
QSHPGDFVLSVRTGDDKGESNDGKSKVTHVMIRCQELKYDVGGGERFDSLTDLVEHYKKNPMVETLGTVL
QLKQPLNTTRINAAEIESRVRELSKLAETTDKVKQGFWEEFETLQQQECKLLYSRKEGQRQENKNKNRYK
NILPFDHTRVVLHDGDPNEPVSDYINANIIMPEFETKCNNSKPKKSYIATQGCLQNTVNDFWRMVFQENS
RVIVMTTKEVERGKSKCVKYWPDEYALKEYGVMRVRNVKESAAHDYTLRELKLSKVGQALLQGNTERTVW
QYHFRTWPDHGVPSDPGGVLDFLEEVHHKQESIVDAGPVVVHCSAGIGRTGTFIVIDILIDIIREKGVDC
DIDVPKTIQMVRSQRSGMVQTEAQYRFIYMAVQHYIETLQRRIEEEQKSKRKGHEYTNIKYSLVDQTSGD
QSPLPPCTPTPPCAEMREDSARVYENVGLMQQQRSFR

The fusion protein of the invention may comprise a SHP-2 SH2 domain. The truncated protein of the invention may comprise or consist of a SHP-2 SH2 domain. In this respect, the fusion or truncated protein may comprise or consist of the first SH2 domain of SHP-2, for example comprising amino acids 6-102 of SEQ ID NO. 9 or the second SH2 domain of SHP-2, for example comprising amino acids 112-216 of SHP-2. The fusion or truncated protein may comprise or consist of the sequence shown as SEQ ID NO: 10, 11 or 12.

SHP-2 first SH2 domain (SEQ ID NO: 10)

WFHPNITGVEAENLLLTRGVDGSFLARPSKSNPGDFTLSVRRNGAVTHIKIQNTGDYYDLYGGEKFATLA
ELVQYYMEHHGQLKEKNGDVIELKYPL

SHP-2 second SH2 domain (SEQ ID No. 11)

WFHGHLSGKEAEKLLTEKGKHGSFLVRESQSHPGDFVLSVRTGDDKGESNDGKSKVTHVMIRCQELKYDV
GGGERFDSLTDLVEHYKKNPMVETLGTVLQLKQPL

SHP-2 both SH2 domains (SEQ ID No. 12)

WFHPNITGVEAENLLLTRGVDGSFLARPSKSNPGDFTLSVRRNGAVTHIKIQNTGDYYDLYGGEKFATLA
ELVQYYMEHHGQLKEKNGDVIELKYPLNCADPTSERWFHGHLSGKEAEKLLTEKGKHGSFLVRESQSHPG
DFVLSVRTGDDKGESNDGKSKVTHVMIRCQELKYDVGGGERFDSLTDLVEHYKKNPMVETLGTVLQLKQP
L

The fusion protein may comprise a variant of SEQ ID NO: 10, 11 or 12 having at least 80, 85, 90, 95, 98 or 99% sequence identity, provided that the variant sequence is a SH2 domain sequence capable of binding an ITIM-containing domain. For example, the variant sequence

may be capable of binding to the phosphorylated tyrosine residues in the cytoplasmic tail of PD1, PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 or KIR3DL3.

HETEROLOGOUS DOMAIN

As used herein, the term 'heterologous domain' refers to any protein domain which is not present in:

i) wild type ZAP70 (see SEQ ID NO: 1) for fusion proteins comprising a ZAP70 SH2 domain;

ii) wild type PTPN6 (see SEQ ID NO: 5) for fusion proteins comprising a PTPN6 SH2 domain; or

iii) wild-type SHP-2 (see SEQ ID No. 9) for fusion proteins comprising a SHP-2 SH2 domain.

The heterologous domain may be or be derivable from (e.g. part of) a different protein from ZAP70, SHP-2 or PTPN6.

Alternatively the fusion protein may comprise a fusion of ZAP70 SH2 domain and a domain from PTPN6, such as the PTPN6 kinase domain. By the same token the fusion protein may comprise a fusion of PTPN6 SH2 domain and a domain from ZAP70, such as the ZAP70 kinase domain.

AMPLIFIED SIGNAL

The present invention provides a fusion protein which comprises: an SH2 domain from an ITAM-binding protein; and an ITAM-containing domain.

The present invention also provide a fusion protein which comprises: an SH2 domain from an ITIM-binding protein; and an ITIM-containing domain.

These "amplified" signalling molecules will amplify an excitatory or inhibitory signal inside an immune cell such as a T cell.

As shown in Figure 4, the presence of such molecules will lead to a concatenation of either ITAMs or ITIMs leading to an augmented activatory or inhibitory signal, respectively.

Amplification of an activatory signal is useful in situations where it is desirable to increase the sensitivity of the immune cell (such as a CAR-T cell) to antigen. This may be the case when, for example, the target antigen is expressed at low levels on the target cells.

Amplification of an inhibitory systems in situations where it is desirable to reduce or prevent T cell activation. WO2015/075469 describes a panel of "logic gate" chimeric antigen receptor pairs which, when expressed by a cell, such as a T cell, are capable of detecting a particular pattern of expression of at least two target antigens A and B). The "AND NOT gate" described in this application comprises a pair of CARs such that the T cell triggers only when antigen A but not antigen B is present on the target cell. In this AND NOT gate, one CAR (recognising antigen A) has an activating endodomain comprising and ITAM, whereas the other CAR (recognising antigen B) has an inhibitory endodomain which may comprise an ITIM. In the presence of antigen A alone, the presence of unligated inhibitory CAR is insufficient to prevent T cell activation, so activation occurs. However, in the presence of both antigens, areas of membrane form with high concentrations of both activatory and inhibitory CARs. Since both endodomains are concentrated, T-cell activation is prevented or reduced.

Amplification of the inhibitory signal using an amplified signalling molecule of the present invention could be used in an AND gate to reduce or remove any residual signalling which occurs in the presence of both antigens i.e. from incomplete inhibition of the activatory CAR by the inhibitory CAR.

ITAM-CONTAINING DOMAIN

In one embodiment, the fusion protein comprises a ZAP70 SH2 domain and an immunoreceptor tyrosine-based activation motif (ITAM)-containing domain.

A fusion of full-length ZAP70 with an ITAM containing domain results in a structure which amplifies an activating immune signal. Here, the fusion protein is recruited to a phospho-ITAM immune-receptor endodomain. ZAP70 functions normally to propagate the signal but also provides another set of ITAMs which become phosphorylated and recruit more ZAP70. This may be useful to increase signal strength and may increase sensitivity to low-density antigens, for example. In some embodiments, the fusion may include only the ZAP70 SH2 domain with an ITAM containing endodomain (e.g. the fusion does not contain a ZAP70 kinase domain). In other embodiments, the ratio of ZAP70 catalytic domains (kinase domains) with ITAMs may be varied to affect the kinetics of activation in response to dynamics of the activating receptor interactions with cognate target.

An ITAM is a conserved sequence of four amino acids that is repeated twice in the cytoplasmic tails of certain cell surface proteins of the immune system. The motif contains a tyrosine separated from a leucine or isoleucine by any two other amino acids, giving the signature YxxL/I. Two of these signatures are typically separated by between 6 and 8 amino acids in the tail of the molecule (YxxL/Ix(6-8)YxxL/I).

ITAMs are important for signal transduction in immune cells. Hence, they are found in the tails of important cell signalling molecules such as the CD3 and ζ-chains of the T cell receptor complex, the CD79-alpha and -beta chains of the B cell receptor complex, and certain Fc receptors. The tyrosine residues within these motifs become phosphorylated following interaction of the receptor molecules with their ligands and form docking sites for other proteins involved in the signalling pathways of the cell.

Several proteins are known to contain endodomains with one or more ITAM motifs. Examples of such proteins include the CD3 epsilon chain, the CD3 gamma chain and the CD3 delta chain to name a few. The ITAM motif can be easily recognized as a tyrosine separated from a leucine or isoleucine by any two other amino acids, giving the signature YxxL/I. Typically, but not always, two of these motifs are separated by between 6 and 8 amino acids in the tail of the molecule (YxxL/Ix(6-8)YxxL/I). Hence, one skilled in the art can readily find existing proteins which contain one or more ITAM to transmit an activation signal. Further, given the motif is simple and a complex secondary structure is not required, one skilled in the art can design polypeptides containing artificial ITAMs to transmit an activation signal (see WO 2000063372, which relates to synthetic signalling molecules).

The ITAM-containing domain may be or comprise a CD3-zeta endodomain. Suitably, the ITAM-containing domain may comprise the sequence shown as SEQ ID NO: 13 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity which retains the capacity to be phosphorylated and recruit ZAP70.

SEQ ID NO: 13 (CD3-zeta endodomain)

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEA
YSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

By way of example, the fusion protein may be or comprise the sequence shown as SEQ ID NO: 14, which contains a ZAP70-SH2 domain fused to a CD3-zeta endodomain.

SEQ ID NO: 14

MRRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMA
EAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRSGGGGSGGGGSGGGGSGGGGSMPDPA
AHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGGK
AHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQAIISQ
APQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHYLISQ
DKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRID
TLNSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQG
VYRMRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKF
LVGKREEIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTA
RSAGKWPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECP
PELYALMSDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

Suitably, the fusion protein may comprise the sequence shown as SEQ ID NO: 14 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

ITIM-CONTAINING DOMAIN

In one embodiment, the fusion protein comprises a PTPN6 SH2 domain and an immunoreceptor tyrosine-based inhibition motif (ITIM)-containing domain

A fusion of full-length PTPN6 with an ITIM containing domain results in a structure which amplifies an inhibitory immune signal. Here, the fusion protein is recruited to a phospho-ITIM immune-receptor endodomain. PTPN6 functions normally to propagate the signal but also provides another set of ITIMs which become phosphorylated and recruit more PTPN6. In some embodiments, the fusion may include only the PTPN6 SH2 domain with an ITIM containing endodomain (e.g. the fusion does not contain a PTPN6 phosphatase domain). In other embodiments, the ratio of PTPN6 catalytic domains (phosphatase domains) with ITIMs may be varied to affect the kinetics of activation in response to dynamics of the inhibitory receptor interactions with cognate target.

An ITIM, is a conserved sequence of amino acids (S/I/V/LxYxxI/V/L) that is found in the cytoplasmic tails of many inhibitory receptors of the immune system. After ITIM-possessing inhibitory receptors interact with their ligand, their ITIM motif becomes phosphorylated by enzymes of the Src kinases, allowing them to recruit PTPN6 via interactions between the PTPN6 SH2 domain and the phosphorylated ITIM domains.

ITIM containing endodomains include those from CD22, LAIR-1, the Killer inhibitory receptor family (KIR), LILRB1, CTLA4, PD-1, BTLA, for example.

ITIM endodomains from PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 and KIR3DL3 are shown in SEQ ID NO: 15 to 24 respectively

SEQ ID NO: 15 PDCD1 endodomain

CSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATI
VFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPL

SEQ ID NO: 16 BTLA4

KLQRRWKRTQSQQGLQENSSGQSFFVRNKKVRRAPLSEGPHSLGCYNPMMEDGISYTTLRFPEMNIPRTG
DAESSEMQRPPPDCDDTVTYSALHKRQVGDYENVIPDFPEDEGIHYSELI
QFGVGERPQAQENVDYVILKH

SEQ ID NO: 17 LILRB1

LRHRRQGKHWTSTQRKADFQHPAGAVGPEPTDRGLQWRSSPAADAQEENLYAAVKHTQPEDGVEMDTRSP
HDEDPQAVTYAEVKHSRPRREMASPPSPLSGEFLDTKDRQAEEDRQMDTEAAASEAPQDVTYAQLHSLTL
RREATEPPPSQEGPSPAVPSIYATLAIH

SEQ ID NO: 18 LAIR1

HRQNQIKQGPPRSKDEEQKPQQRPDLAVDVLERTADKATVNGLPEKDRETDTSALAAGSSQEVTYAQLDH
WALTQRTARAVSPQSTKPMAESITYAAVARH

SEQ ID NO: 19 CTLA4

FLLWILAAVSSGLFFYSFLLTAVSLSKMLKKRSPLTTGVYVKMPPTEPECEKQFQPYFIPIN

SEQ ID NO: 20 KIR2DL1

GNSRHLHVLIGTSVVIIPFAILLFFLLHRWCANKKNAVVMDQEPAGNRTVNREDSDEQDPQEVTYTQLNH
CVFTQRKITRPSQRPKTPPTDIIVYTELPNAESRSKVVSCP

SEQ ID NO: 21 KIR2DL4

GIARHLHAVIRYSVAIILFTILPFFLLHRWCSKKKENAAVMNQEPAGHRTVNREDSDEQDPQEVTYAQLD
HCIFTQRKITGPSQRSKRPSTDTSVCIELPNAEPRALSPAHEHHSQALMGSSRETTALSQTQLASSNVPA
AGI

SEQ ID NO: 22 KIR2DL5

TGIRRHLHILIGTSVAIILFIILFFFLLHCCCSNKKNAAVMDQEPAGDRTVNREDSDDQDPQEVTYAQLD
HCVFTQTKITSPSQRPKTPPTDTTMYMELPNAKPRSLSPAHKHHSQALRGSSRETTALSQNRVASSHVPA
AGI

SEQ ID NO: 23 KIR3DL1

KDPRHLHILIGTSVVIILFILLLFFLLHLWCSNKKNAAVMDQEPAGNRTANSEDSDEQDPEEVTYAQLDH
CVFTQRKITRPSQRPKTPPTDTILYTELPNAKPRSKVVSCP

SEQ ID NO: 24 KIR3DL3

KDPGNSRHLHVLIGTSVVIIPFAILLFFLLHRWCANKKNAVVMDQEPAGNRTVNREDSDEQDPQEVTYAQ
LNHCVFTQRKITRPSQRPKTPPTDTSV

The ITIM-containing domain may be or comprise a PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 or KIR3DL3 endodomain. Suitably, the ITIM-containing domain may comprise the sequence shown any of SEQ ID NO: 15 to 24 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity which retains the capacity to be phosphorylated by Src kinases and amplify an inhibitory immune signal.

By way of example, the fusion protein may be or comprise the sequence shown as SEQ ID NO: 25, which contains a PTPN6-SH2 domain fused to a PD1 endodomain.

SEQ ID NO: 25

MTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRS
AQPLRPEDGHCSWPLSGGGGSGGGGSGGGGSGGGGSMVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRK
NQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSD
PTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGG
RYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPYYATRVNAADIENRVLELNKKQESEDTAKAGF
WEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGRDSNIPGSDYINANYIKNQLLGP
DENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRNKCVPYWPEVGMQRAYGPYSVTNCGE
HDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGVPSEPGGVLSFLDQINQRQESLPHAGPIIVHCS
AGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMVQTEAQYKFIYVAIAQFIETTKKKLE
VLQSQKGQESEYGNITYPPAMKNAHAKASRTSSKHKEDVYENLHTKNKREEKVKKQRSADKEKSKGSLKR
K

Suitably, the fusion protein may comprise the sequence shown as SEQ ID NO: 25 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity

CROSSWIRE SIGNAL

The present invention provides a fusion protein which comprises: an SH2 domain from an ITAM-binding protein; and a phosphatase domain.

The present invention also provide a fusion protein which comprises: an SH2 domain from an ITIM-binding protein; and a kinase domain.

These “crosswire” signalling molecules will reverse an excitatory or inhibitory signal inside an immune cell such as a T cell. When a T-cell receives an excitatory signal, for example following recognition of a target antigen by a CAR, or MHC:peptide by a TCR, the presence of the first type of crosswire molecule will result in the cell interpreting the excitatory signal as an inhibitory signal.

Dampening down or revising T-cell activation may be useful in a variety of situations, for example, it may be used for CAR-expressing T cells where there is a high level of expression of the target antigen on the target cell. It may be used to prevent T-cell over-activation which may lead to T cell exhaustion and/or activation-induced cell death. Preventing a T-cell becoming activated too much or too quickly may also prevent or reduce pathological side effects of CAR-T cell treatment such as cytokine release syndrome (CRS).

The reverse situation is when a T-cell receives an inhibitory signal, for example following ligation of PD1, and the presence of the second type of crosswire molecule results in the cell interpreting the inhibitory signal as an excitatory signal.

Reducing or reversing T-cell inhibition will help the cell overcome the inhibitory stimuli within the hostile tumour microenvironment and should therefore increase T-cell persistence and expansion in vivo.

In one embodiment, the fusion protein comprises a PTPN6 SH2 domain and a ZAP70 kinase domain. In another embodiment the present fusion protein comprises a ZAP70 SH2 domain fused to a PTPN6 kinase domain.

In embodiments relating to a ZAP70 SH2 domain fused to the phosphatase domain from PTPN6, when the T cell receives an excitatory signal it interprets it as an inhibitory signal because the PTPN6 phosphatase domain is recruited to the activated ITAM via the ZAP70 SH2 domain.

In embodiments relating to a PTPN6 SH2 domain fused to the kinase domain from ZAP70, when the T cell receives an inhibitory signal it interprets it as an excitatory signal because the ZAP70 kinase domain is recruited to the activated ITIM via the PTPN6 domain. A fusion between PTPN6 SH2 domain and ZAP70 kinase domain will result in competition for phosphorylated ITIMs by wild-type PTPN6 blocking inhibitory signals, but in addition will transmit a paradoxical activation signal. This may have application in over-coming checkpoint blockade signals in a tumour microenvironment.

The sequence of human ZAP70 kinase, PTPN6 phosphatase and SHP-2 phosphatase domains domains are shown as SEQ ID NO: 26, 27 and 28 respectively.

SEQ ID NO: 26 – ZAP70 kinase domain

DPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMRKKQIDVAIKVLKQGTEKADTEEMMREAQIM
HQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFLVGKREEIPVSNVAELLHQVSMGMKYLEEKNFVHR
DLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARSAGKWPLKWYAPECINFRKFSSRSDVWSYGVTMW
EALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPELYALMSDCWIYKWEDRPDFLTVEQRMRACYYSL
ASKVEGPPGSTQKAEAACA

SEQ ID NO: 27 – PTPN6 phosphatase domain

FWEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGRDSNIPGSDYINANYIKNQLLG
PDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRNKCVPYWPEVGMQRAYGPYSVTNCG
EHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGVPSEPGGVLSFLDQINQRQESLPHAGPIIVHC
SAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMVQTEAQYKFIYVAIAQFIETTKKKL

SEQ ID NO: 28 – SHP-2 phosphatase domain

WEEFETLQQQECKLLYSRKEGQRQENKNKNRYKNILPFDHTRVVLHDGDPNEPVSDYINANII
MPEFETKCNNSKPKKSYIATQGCLQNTVNDFWRMVFQENSRVIVMTTKEVERGKSKCVKYWPDEYALKE
YGVMRVRNVKESAAHDYTLRELKLSKVGQALLQGNTERTVWQYHFRTWPDHGVPSDPGGVLDFLEEVH
HKQESIMDAGPVVVHCSAGIGRTGTFIVIDILIDIIREKGVDCDIDVPKTIQMVRSQRSGMVQTEAQYRFIYM
A

The ZAP70 kinase domain, PTPN6 phosphatase domain or SHP-2 phosphatase domain may be or comprise the sequence shown as SEQ ID NO: 26, SEQ ID NO: 27 or SEQ ID NO: 28, respectively; or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity which retains the capacity to phosphorylate or dephosphorylate downstream proteins in the same manner as the wild-type kinase/phosphatase domains.

Examples of fusion protein comprising a PTPN6 SH2 domain fused to a ZAP70 kinase domain; a ZAP70 SH2 domain fused to a PTPN6 kinase domain; and a SHP-2 SH2 domain fused to a ZAP70 kinase domain are shown as SEQ ID NO: 29, SEQ ID NO: 30 and SEQ ID No. 61, respectively.

SEQ ID NO: 29 – PTPN6 SH2 domain fusion: ZAP70 kinase domain

MVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFA
TLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLS
QPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLR
QPYYSGGGGSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMRKKQIDVAIKVLKQGTEKADT
EEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFLVGKREEIPVSNVAELLHQVSMGMK
YLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARSAGKWPLKWYAPECINFRKFSSRS
DVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPELYALMSDCWIYKWEDRPDFLTVE
QRMRACYYSLASKVEGPPGSTQKAEAACA

SEQ ID NO: 30 – ZAP70 SH2 domain fusion: PTPN6 phosphatase domain

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYA
IAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQ
AIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYH
YLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHP
SGGGGSGGGGSGGGGSGGGGSFWEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGR
DSNIPGSDYINANYIKNQLLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRNKCV
PYWPEVGMQRAYGPYSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGVPSEPGGVLSF

LDQINQRQESLPHAGPIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMVQTE
AQYKFIYVAIAQFIETTKKKL

SEQ ID NO. 61 – dual SH2 domains from SHP-2 fused to ZAP70 kinase domain

WFHPNITGVEAENLLLTRGVDGSFLARPSKSNPGDFTLSVRRNGAVTHIKIQNTGDYYDLYGGEKFATLA
ELVQYYMEHHGQLKEKNGDVIELKYPLNCADPTSERWFHGHLSGKEAEKLLTEKGKHGSFLVRESQSHPG
DFVLSVRTGDDKGESNDGKSKVTHVMIRCQELKYDVGGGERFDSLTDLVEHYKKNPMVETLGTVLQLKQP
LNTTRINPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTLNSDGYTPEPARITSPDKPRPMPMDTSVYES
PYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMRKKQIDVAIKVLKQGTEKADTEEMMREA
QIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFLVGKREEIPVSNVAELLHQVSMGMKYLEEKNF
VHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARSAGKWPLKWYAPECINFRKFSSRSDVWSYGV
TMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPELYALMSDCWIYKWEDRPDFLTVEQRMRACY
YSL

The fusion protein may be or comprise the sequence shown as SEQ ID NO: 29, SEQ ID NO: 30 or SEQ ID No. 61 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

HETEROLOGOUS SIGNALLING DOMAIN

The present fusion protein may comprise (i) a ZAP70, PTPN6 or SHP-2 SH2 domain; and (ii) a heterologous signalling domain.

As used herein, the term "heterologous signalling domain" refers to a signalling domain which is not present in the wild type ZAP70, PTPN6 or SHP-2 protein. As such, where the fusion protein comprises a ZAP70 SH2 domain, it comprises a signalling domain which is not the ZAP70 kinase domain. Alternatively, where the fusion protein comprises a PTPN6 SH2 domain, it comprises a signalling domain which is not the PTPN6 phosphatase domain.

BYPASS SIGNAL

The heterologous signalling domain may be from a signalling molecule which is not usually activated by an ITAM containing receptor. In other words, the heterologous signalling domain may be from a signalling molecule which is not involved in the propagation of immunological signal 1 following the binding of antigen to the TCR. Immunological signal 1 is sufficient to trigger T-cell killing of cognate target cells but does not fully activate the T-cell to proliferate and survive.

In one embodiment of this aspect of the invention, the present invention provides a fusion protein which comprises (i) an SH2 domain from a protein which binds an ITAM; and (ii) a heterologous signalling domain.

A fusion between, for example, ZAP70 and another signaling molecule not typically activated with an ITAM containing receptor may act to bypass signal from one pathway into another. One example is co-stimulation. A fusion between ZAP70 and the endodomain of CD28 may transmit a CD28 co-stimulatory signal as well as an ITAM activatory signal. Similarly, a fusion between ZAP70 and the endodomain of 41BB or OX40 may transmit a 41BB or OX40 co-stimulatory signal. Other pathways may also be recruited, for instance a fusion between ZAP70 and AKT kinase domain may result in transmission of an AKT signal upon ITAM phosphorylation. Other examples might include Kinase domain from JAK. In this way, a T-cell may interpret a simple antigen recognition signal as transmitting a co-stimulatory or even a cytokine type signal.

Such fusion proteins may be useful, for example, in approaches where repeated *ex vivo* stimulations of T cells can result in populations which lack costimulatory surface antigens and which have limited proliferative capacity *in vivo* resulting in limited persistence and efficacy. The loss of costimulatory surface antigens leading to activation of T cells solely through the TCR has been linked to a greater degree of activation induced cell death which would negatively impact *in vivo* efficacy and persistence. The effect can be reversed by the activation of surface-expressed 4-1BB and OX40 demonstrating that costimulation can prevent activation induced cell death and can support greater expansion of tumour specific T cells.

In another embodiment of this aspect of the invention, the present invention provides a fusion protein which comprises (i) an SH2 domain from a protein which binds an ITIM; and (ii) a heterologous signalling domain.

For example, a PTPN6 SH2 domain or SHP-2 SH2 domain may be fused to a co-stimulatory endodomain so a T-cell interprets an inhibitory signal as a co-stimulatory one.

The heterologous signalling domain may be from, for example, CD28, 41BB or OX40.

CD28 provides a potent co-stimulatory signal - namely immunological signal 2, which triggers T-cell proliferation. CD28 is the receptor for CD80 (B7.1) and CD86 (B7.2) proteins.

41BB (CD137) is a type 2 transmembrane glycoprotein belonging to the TNF superfamily, expressed on activated T cells. Crosslinking of 41BB enhances T cell proliferation, IL-2 secretion survival and cytolytic activity.

OX40 (CD134) is a secondary co-stimulatory molecule, expressed after 24 to 72 hours following activation; its ligand, OX40L, is also not expressed on resting antigen presenting cells, but is following their activation. Expression of OX40 is dependent on full activation of the T cell; without CD28, expression of OX40 is delayed and of fourfold lower levels. Signalling through OX40 is required for prolonged T cell survival following initial activation and proliferation.

The CD28, 41BB and OX40 signalling domains (endodomains) are shown as SEQ ID NO: 31, 32 and 33, respectively.

SEQ ID NO: 31 – CD28 endodomain
MRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS

SEQ ID NO: 32 – 41BB endodomain
MKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

SEQ ID NO: 33 – OX40 endodomain
MRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI

The heterologous signalling domain may be or comprise the sequence shown as SEQ ID NO: 31; SEQ ID NO: 32 or SEQ ID NO: 33, respectively, or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

The heterologous signalling domain may be or comprise an inhibitory signalling domain.

For example, the inhibitory signalling domain may comprise the endodomain of CD148 or CD45. CD148 and CD45 have been shown to act naturally on the phosphorylated tyrosines up-stream of TCR signalling.

CD148 is a receptor-like protein tyrosine phosphatase which negatively regulates TCR signaling by interfering with the phosphorylation and function of PLCγ1 and LAT.

CD45 present on all hematopoetic cells, is a protein tyrosine phosphatase which is capable of regulating signal transduction and functional responses, again by phosphorylating PLC γ1.

An inhibitory signalling domain may comprise all of part of a receptor-like tyrosine phosphatase. The phospatase may interfere with the phosphorylation and/or function of elements involved in T-cell signalling, such as PLCγ1 and/or LAT.

The inhibitory signalling domain may be or comprise the endodomain of ICOS, CD27, BTLA, CD30, GITR or HVEM.

The inhibitory signalling domain may comprise the sequence shown as SEQ ID NO: 34 to 39 or a variant thereof having at least 80% sequence identity.

SEQ ID NO: 34 - ICOS endodomain

CWLTKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL

SEQ ID NO: 35 - CD27 endodomain

QRRKYRSNKGESPVEPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP

SEQ ID NO: 36 - BTLA endodomain

RRHQGKQNELSDTAGREINLVDAHLKSEQTEASTRQNSQVLLSETGIYDNDPDLCFRMQEGSEVYSNPCL
EENKPGIVYASLNHSVIGPNSRLARNVKEAPTEYASICVRS

SEQ ID NO: 37 - CD30 endodomain

HRRACRKRIRQKLHLCYPVQTSQPKLELVDSRPRRSSTQLRSGASVTEPVAEERGLMSQPLMETCHSVGA
AYLESLPLQDASPAGGPSSPRDLPEPRVSTEHTNNKIEKIYIMKADTVIVGTVKAELPEGRGLAGPAEPE
LEEELEADHTPHYPEQETEPPLGSCSDVMLSVEEEGKEDPLPTAASGK

SEQ ID NO: 38 - GITR endodomain

QLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWV

SEQ ID NO: 39 - HVEM endodomain

CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIPSFTGRSPNH

A variant sequence may have at least 80%, 85%, 90%, 95%, 98% or 99% sequence identity to SEQ ID NO: 34 to 39 provided that the sequence provides an effective intracellular signalling domain.

Suitably, the fusion protein may be or comprise any of the sequences shown as SEQ ID NOs: 40 to 45.

(SEQ ID NO: 40) - CD28 endodomain fused to amino-terminus of full-length ZAP

MRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSSGGGGSGGGGSGGGGSGGGGSMPDPAAH
LPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGGKAH
CGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQAIISQAP
QVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHYLISQDK
AGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTL
NSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVY
RMRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFLV
GKREEIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARS

AGKWPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPE
LYALMSDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

(SEQ ID NO: 41) - 41BB endodomain fused to amino-terminus of full-length ZAP

MKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELSGGGGSGGGGSGGGGSGGGGSMPDPAA
HLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGGKA
HCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQAIISQA
PQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHYLISQD
KAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDT
LNSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGV
YRMRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFL
VGKREEIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTAR
SAGKWPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPP
ELYALMSDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

(SEQ ID NO: 42) - OX40 endodomain fused to amino-terminus of full-length ZAP

MRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKISGGGGSGGGGSGGGGSGGGGSMPDPAAHLPFFY
GSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGGKAHCGPAE
LCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQAIISQAPQVEKL
IATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHYLISQDKAGKYC
IPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTLNSDGY
TPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMRKK
QIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFLVGKREE
IPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARSAGKWP
LKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPELYALM
SDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

(SEQ ID NO: 43) - CD28 endodomain fused to the amino-terminus of PTPN6 SH2 domain.

MRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSSGGGGSGGGGSGGGGSGGGGSMVRWFHR
DLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLTELVE
YYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFVL
SVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPY

(SEQ ID NO: 44) - 41BB endodomain fused to the amino-terminus of PTPN6 SH2 domain

MKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELSGGGGSGGGGSGGGGSGGGGSMVRWFH
RDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLTELV
EYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFV
LSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPY

(SEQ ID NO: 45) - OX40 endodomain fused to the amino-terminus of PTPN6 SH2 domain

MRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKISGGGGSGGGGSGGGGSGGGGSMVRWFHRDLSGL
DAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLTELVEYYTQQ
QGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFVLSVLSD
QPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPY

Suitably, the fusion protein may comprise the sequence shown as any of SEQ ID NOs: 40 to 45 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity

KINASE DOMAIN

The heterologous signalling domain may be a kinase domain. For example, the heterologous signalling domain may comprise an AKT kinase domain or a JAK kinase domain.

Akt, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration.

Following activation of the TCR, T cells secrete IL2 which supports survival and proliferation. However this secretion is transient and T cells that are activated and expanded *in vitro* become dependent on exogenous IL2 for survival. By increasing AKT phosphorylation following ITAM phosphorylation associated with TCR or CAR activation, the dependence of activated T cells on exogenous IL2 may be reduced or removed and their proliferation and survival enhanced.

The Akt kinase domain is shown as SEQ ID NO: 46.

SEQ ID NO: 46 – Akt kinase domain

AEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENR
VLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARFYGAEIVSALDYLHSEKNV
VYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMY
EMMCGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFAG
IVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDSERRPHFPQFSYSASGTA

The heterologous signalling domain may be or comprise the sequence shown as SEQ ID NO: 46, or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity provided that the sequence provides an effective kinase domain.

By way of example, the fusion protein may be or comprise the any of the sequences shown as SEQ ID NO: 47 to 49 and 62 which contain a ZAP70-SH2 domain fused directly to an Akt kinase domain, a ZAP70-SH2 domain fused to an Akt kinase domain via a linker a ZAP70 mutated to be non-functional and fused to an Akt kinase domain; and a dual SHP-2 SH2 domain fused to an Akt kinase domain, respectively.

SEQ ID NO: 47 - ZAP70-SH2 domain fused directly to an Akt kinase domain

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYA
IAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQ
AIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYH
YLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHP
AEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENR
VLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARFYGAEIVSALDYLHSEKNV
VYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMY

EMMCGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFAG
IVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDSERRPHFPQFSYSASGTA

SEQ ID NO: 48 - ZAP70-SH2 domain fused to an Akt kinase domain via a linker

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYA
IAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQ
AIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYH
YLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHP
SGGGGSGGGGSGGGGSGGGGSAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMK
ILKKEVIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDR
ARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPE
VLEDNDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPK
QRLGGGSEDAKEIMQHRFFAGIVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSME
CVDSERRPHFPQFSYSASGTA

SEQ ID NO: 49 - ZAP70 mutated to be non-functional and fused to an Akt kinase domain

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYA
IAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQ
AIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLKPRKEQGTYALSLIYGKTVYH
YLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHP
AEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENR
VLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARFYGAEIVSALDYLHSEKNV
VYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMY
EMMCGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFAG
IVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDSERRPHFPQFSYSASGTA

SEQ ID No. 62 - dual SHP-2 SH2 domain fused to an Akt kinase domain

WFHPNITGVEAENLLLTRGVDGSFLARPSKSNPGDFTLSVRRNGAVTHIKIQNTGDYYDLYGGEKFATLA
ELVQYYMEHHGQLKEKNGDVIELKYPLNCADPTSERWFHGHLSGKEAEKLLTEKGKHGSFLVRESQSHPG
DFVLSVRTGDDKGESNDGKSKVTHVMIRCQELKYDVGGGERFDSLTDLVEHYKKNPMVETLGTVLQLKQP
LNTTRINAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVA
HTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARFYGAEIVSALDY
LHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWW
GLGVVMYEMMCGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIM
QHRFFAGIVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDSERRPHFPQFS
YSASGTA

The fusion protein may comprise the sequence shown as any of SEQ ID NO: 47 to 49 or 62 or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

Janus kinase (JAK) is a family of intracellular, nonreceptor tyrosine kinases that transduce cytokine-mediated signals via the JAK-STAT pathway. The four JAK family members are: Janus kinase 1 (JAK1); Janus kinase 2 (JAK2); Janus kinase 3 (JAK3); and Tyrosine kinase 2 (TYK2).

SEQ ID NO: 50 – Kinase containing domain of JAK2

RNEDLIFNESLGQGTFTKIFKGVRREVGDYGQLHETEVLLKVLDKAHRNYSESFFEAASMMSKLSHKHLV
LNYGVCVCGDENILVQEFVKFGSLDTYLKKNKNCINILWKLEVAKQLAWAMHFLEENTLIHGNVCAKNIL
LIREEDRKTGNPPFIKLSDPGISITVLPKDILQERIPWVPPECIENPKNLNLATDKWSFGTTLWEICSGG

DKPLSALDSQRKLQFYEDRHQLPAPKWAELANLINNCMDYEPDFRPSFRAIIRDLNSLFTPDYELLTEND
MLPNMRIGALGFSGAFEDRDPTQFEERHLKFLQQLGKGNFGSVEMCRYDPLQDNTGEVVAVKKLQHSTEE
HLRDFEREIEILKSLQHDNIVKYKGVCYSAGRRNLKLIMEYLPYGSLRDYLQKHKERIDHIKLLQYTSQI
CKGMEYLGTKRYIHRDLATRNILVENENRVKIGDFGLTKVLPQDKEYYKVKEPGESPIFWYAPESLTESK
FSVASDVWSFGVVLYELFTYIEKSKSPPAEFMRMIGNDKQGQMIVFHLIELLKNNGRLPRPDGCPDEIYM
IMTECWNNNVNQRPSFRDLALRVDQIRDNM

PROTEASE DOMAIN

The present invention also provides a fusion protein which comprises (i) an SH2 domain from a protein which binds an ITAM or ITIM-containing protein and (ii) a protease domain.

The protease domain may be any protease which is capable of cleaving at a specific recognition sequence. As such the protease domain may be any protease which enables the separation of a single target polypeptide into two distinct polypeptides via cleavage at a specific target sequence.

The protease domain may be a Tobacco Etch Virus (TeV) protease domain.

TeV protease is a highly sequence-specific cysteine protease which is chymotrypsin-like proteases. It is very specific for its target cleavage site and is therefore frequently used for the controlled cleavage of fusion proteins both in vitro and in vivo. The consensus TeV cleavage site is ENLYFQ\S (where '\' denotes the cleaved peptide bond). Mammalian cells, such as human cells, do not express endogenous TeV protease.

Accordingly, the TeV cleavage recognition site is shown as SEQ ID NO: 51.

SEQ ID NO: 51 – Tev cleavage site
ENLYFQS

The TeV protease domain is shown as SEQ ID NO: 52.

SEQ ID NO: 52

SLFKGPRDYNPISSTICHLTNESDGHTTSLYGIGFGPFIITNKHLFRRNNGTLLVQSLHGVFKVKNTTTL
QQHLIDGRDMIIIRMPKDFPPFPQKLKFREPQREERICLVTTNFQTKSMSSMVSDTSCTFPSSDGIFWKH
WIQTKDGQCGSPLVSTRDGFIVGIHSASNFTNTNNYFTSVPKNFMELLTNQEAQQWVSGWRLNADSVLWG
GHKVFMSKPEEPFQPVKEATQLMNELVYSQ

Accordingly, the protease domain may be or comprise the sequence shown as SEQ ID NO: 52, or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity provided that the sequence provides an effective protease function.

By way of example, the fusion protein may be or comprise the sequence shown as SEQ ID NO: 53 or 54, which contains a ZAP70-SH2 domain fused to a TEV protease sequence or a PTPN6-SH2 domain fused to a TEV protease sequence; respectively.

SEQ ID NO: 53

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYA
IAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQ
AIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYH
YLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHP
SGGGGSGGGGSGGGGSGGGGSSLFKGPRDYNPISSTICHLTNESDGHTTSLYGIGFGPFIITNKHLFRRN
NGTLLVQSLHGVFKVKNTTTLQQHLIDGRDMIIIRMPKDFPPFPQKLKFREPQREERICLVTTNFQTKSM
SSMVSDTSCTFPSSDGIFWKHWIQTKDGQCGSPLVSTRDGFIVGIHSASNFTNTNNYFTSVPKNFMELLT
NQEAQQWVSGWRLNADSVLWGGHKVFMSKPEEPFQPVKEATQLMNELVYSQ

SEQ ID NO: 54

MVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFA
TLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLS
QPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLR
QPYYSGGGGSSLFKGPRDYNPISSTICHLTNESDGHTTSLYGIGFGPFIITNKHLFRRNNGTLLVQSLHG
VFKVKNTTTLQQHLIDGRDMIIIRMPKDFPPFPQKLKFREPQREERICLVTTNFQTKSMSSMVSDTSCTF
PSSDGIFWKHWIQTKDGQCGSPLVSTRDGFIVGIHSASNFTNTNNYFTSVPKNFMELLTNQEAQQWVSGW
RLNADSVLWGGHKVFMSKPEEPFQPVKEATQLMNELVYSQ

The fusion protein may comprise the sequence shown as SEQ ID NO: 53 or 54; or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

The SH2 domain and heterologous domain of the fusion protein may be separated by a linker in order to spatially separate the SH2 domain and the heterologous domain.

TRANSCRIPTIONAL SIGNAL

A fusion protein which comprises a protease, as described in the previous section, may be co-expressed in a cell with a membrane-tethered protein having a protease cleavage site. Cleavage of the membrane-tethered protein at the protease site will release the membrane-distal part of the protein.

The membrane tethered protein may, for example, be a membrane-tethered transcription factor. When cleavage occurs, the transcription is released from its tether and free to transit to the nucleus.

A fusion between ZAP70 SH2 or PTPN6 SH2 domain and a protease domain will result in membrane-proximal recruitment of the protease following ITAM or ITIM phosphorylation, respectively.

Phosphorylation of ITAM or ITIM domains results in recruitment of the ZAP70 SH2 or PTPN6 SH2 fused with the protease domain, respectively, to the membrane-proximal area. This results in the transcription factor being cleaved from its tether and transferred to the nucleus. This may have many applications: for example upon activation the T-cell may be programmed to express transcription factors which act to prevent the T-cell from differentiating. For instance, upon activation the T-cell may be programmed to express a cytokine such as IL2, IL7 or IL15 which may act to stimulate proliferation and survival of the T-cell, or IL12 which may convert a hostile tumour microenvironment to one which more favours immune rejection of a tumour.

In particular, there is provided a cell which co-expresses:

(i) a fusion protein comprising an SH2 domain from a protein which binds a phosphorylated ITAM; and

(ii) a membrane tethered transcription factor

wherein the transcription factor, when released from the membrane tether, increases the expression of IL2, Il7 and/or IL15 in the cell.

There is also provided a cell which co-expresses:

(i) a fusion protein comprising an SH2 domain from a protein which binds a phosphorylated ITIM; and

(ii) a membrane tethered transcription factor

wherein the transcription factor, when released from the membrane tether, increases the expression of IL12 in the cell.

Protease recognition site

The protease recognition site may be any amino acid sequence which enables the protease domain of the fusion protein to specifically cleave the membrane tethered transcription factor between the membrane tether and the transcription factor. For example, in one embodiment

the protease domain is a TeV protease domain and the protease recognition site is a TeV protease recognition site.

Membrane tether

The membrane tether may be any sequence, signal or domain which is capable of localising the transcription factor and protease recognition site proximal to a membrane. For example, the membrane tether may be a myrsitylation signal or a transmembrane domain.

Suitably, a transmembrane domain may be any protein structure which is thermodynamically stable in a membrane. This is typically an alpha helix comprising of several hydrophobic residues. The transmembrane domain of any transmembrane protein can be used to supply the transmembrane portion. The presence and span of a transmembrane domain of a protein can be determined by those skilled in the art using the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Further, given that the transmembrane domain of a protein is a relatively simple structure, i.e a polypeptide sequence predicted to form a hydrophobic alpha helix of sufficient length to span the membrane, an artificially designed TM domain may also be used (US 7052906 B1 describes synthetic transmembrane components).

The transmembrane domain may be derived from CD28, which gives good stability.

Transcription factor

The transcription factor may be any transcription factor chosen to stimulate a desired response following phosphorylation of the relevant ITAM or ITIM motifs.

The transcripton factor can be natural or artificial. Artificial transcription factors may be derived from, for example, TALENs, zinc-finger assemblies or CrispR/CAS9, the latter co-expressed with a guide mRNA.

Preferably, the transcription factor will contain a nuclear localization signal to aid its transportation to the nuclease following cleavage by the protease domain.

By way of example, nucleic acid sequence (ii) (which encodes a protein comprising a membrane tethered transcription factor which comprises: (i) a membrane tether; (ii) a protease recognition site; and (iii) a transcription factor) may encode a protein which consists of or comprises the sequence shown as SEQ ID NO: 55, which contains a RQR8 domain; a CD4-

Endotox1 transmembrane domain, a TEV protease recognition site and a VP16-GAL4 transcription factor.

SEQ ID NO: 55

MGTSLLCWMALCLLGADHADACPYSNPSLCSGGGGSELPTQGTFSNVSTNVSPAKPTTTACPYSNPSLCS
GGGGSPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDMALIVLGGVAGLLLFIGLGIFFC
VRCRHRRRQAERMAQIKRVVSEKKTAQAPHRFQKTCSPISGGGGSENLYFQMPKKKRKVAPPTDVSLGDE
LHLDGEDVAMAHADALDDFDLDMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGGSGG
GSMQILVASDATMKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVE
SRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNVNKDAVTDRLASVETDMPLTLRQHRIS
ATSSSEESSNKGQRQLTV

Suitably, the protein may comprise the sequence shown as SEQ ID NO: 55; or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity

RECEPTOR

The present invention further provides a nucleic acid construct which comprises (a) a nucleic acid sequence encoding a fusion protein according to the first aspect of the present invention which comprises a PTPN6 SH2 domain, or a truncated protein according to the third aspect of the present invention; and (b) a nucleic acid sequence encoding a receptor comprising an ITIM containing endodomain.

CASTRATION SIGNAL

A fusion protein which comprises a protease, as described above, may be co-expressed in a cell with a target receptor which comprises an intracellular protease cleavage site. Cleavage of the target receptor at the protease site will release an intracellular, membrane-distal part of the target receptor.

The target receptor may, for example, be a T-cell receptor (TCR), or a chimeric antigen receptor (CAR).

The receptor may comprise an activatory or co-stimulatory endodomain positioned at the end of the intracellular part of the protein. Cleavage at the protease cleavage site then removes the activatory or co-stimulatory endodomain from the target CAR, reducing or preventing target receptor-mediated T cell activation.

Alternatively, the target receptor may comprise an inhibitory endodomain positioned at the end of the intracellular part of the protein. Cleavage at the protease cleavage site then removes the inhibitory endodomain from the target CAR, "switching-on" the potential for target receptor mediated T cell activation.

The inhibitory endodomain may, for example, comprise a CD148 or CD45 endodomain or an ITIM-containing endodomain from a protein such a PD1, PDCD1, BTLA4, LILRB1, LAIR1, CTLA4, KIR2DL1, KIR2DL4, KIR2DL5, KIR3DL1 or KIR3DL3.

By way of example, the target receptor may comprise the sequence shown as SEQ ID NO: 56, which contains a CAR against CD33 containing an ITIM endodomain from PD-1.

SEQ ID NO: 56

MAVPTQVLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLIYD
TNRLADGVPSRFSGSGSGTQYTLTISSLQPEDFATYYCQHYKNYPLTFGQGTKLEIKRSGGGGSGGGGSG
GGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMHWIRQAPGKGLEWVSSISLNGGSTY
YRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYCAAQDAYTGGYFDYWGQGTLVTVSSMDPATTTKP
VLRTPSPVHPTGTSQPQRPEDCRPRGSVKGTGLDFACDIYVGVVGGLLGSLVLLVWVLAVICSRAARGTI
GARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADG
PRSAQPLRPEDGHCSWPL

Suitably, the protein may comprise the sequence shown as SEQ ID NO: 56; or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

Where the receptor comprises a protease cleavage site between a transmembrane domain and an activating endodomain, the castration signal fusion protein may be used to inhibit the receptor. For instance, a first CAR might be constructed whereby its endodomain is separated from the transmembrane domain by a protease cleavage site. A second CAR recognizing a different antigen might comprise of an ITIM containing endodomain. Recognition of the cognate antigen of the second receptor would result in recruitment of the castration signal fusion protein to the membrane and subsequent cleavage at the protease recognition site. Such cleavage would separate the activating endodomain from the first receptor and prevent activation and signal propagation from said receptor.

This would result in an "AND NOT" type logic gate where a sustained signal would be transmitted only if the first CAR was activated in isolation (i.e. when the first CAR bound its cognate antigen but the second CAR did not bind its cognate antigen). Such 'logic gates' may be useful, for example, because it is relatively rare for the presence (or absence) of a single antigen to effectively describe a cancer, which can lead to a lack of specificity. Targeting antigen expression on normal cells leads to on-target, off-tumour toxicity. In some cancers, a

tumour is best defined by presence of one antigen (typically a tissue-specific antigen) and the absence of another antigen which is present on normal cells. For example, acute myeloid leukaemia (AML) cells express CD33. Normal stem cells express CD33 but also express CD34, while AML cells are typically CD34 negative. Targeting CD33 alone to treat AML is associated with significant toxicity as it depletes normal stem cells. However, specifically targeting cells which are CD33 positive but not CD34 positive would avoid this considerable off-target toxicity.

Potential pairs of antigens for such an 'AND NOT' gate are shown in Table 2.

TABLE 2

Disease	**TAA**	**Normal cell which expresses TAA**	**Antigen expressed by normal cell but not cancer cell**
AML	CD33	stem cells	CD34
Myeloma	BCMA	Dendritic cells	CD1c
B-CLL	CD160	Natural Killer cells	CD56
Prostate cancer	PSMA	Neural Tissue	NCAM
Bowel cancer	A33	Normal bowel epithelium	HLA class I

By way of example, the receptor which comprises a protease cleavage site between a transmembrane domain and an activating endodomain may be the sequence shown as SEQ ID NO: 57, which contains a CAR against CD19 with a cleavable CD3-zeta endodomain.

SEQ ID NO: 57

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIY
HTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITKAGGGGSGGGGS
GGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYN
SALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRP
PTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRCRHR
RRQAERMAQIKRVVSEKKTAQAPHRFQKTCSPISGGGGSENLYFQMRRVKFSRSADAPAYQQGQNQLYNE
LNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGL
STATKDTYDALHMQALPPR

Suitably, the receptor may comprise the sequence shown as SEQ ID NO: 57; or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

Where the receptor comprises an activating endodomain fused to an inhibitory endodomain via a protease cleavage site, a castration signal fusion protein can be used to activate artificial signalling domains. For instance, a first CAR might be constructed whereby its endodomain

comprises an activating endodomain fused to an inhibitory endodomain via a protease cleavage site. A second CAR recognizing a different antigen might comprise of an ITIM containing endodomain. Recognition of the cognate antigen of the second receptor would result in recruitment of the castration signal fusion protein to the membrane and subsequent cleavage of inhibitory endodomain from the activating endodomain of the first receptor. Cleavage, and thus separation, of the inhibitory domain from the activating domain would allow activation of the first CAR following antigen binding and hence activation of signalling via the first receptor.

This would result in an "AND" type CAR logic gate where productive signalling would occur only if both the first and second receptors were activated. Such 'logic gates' are useful, for example, because most cancers cannot be differentiated from normal tissues on the basis of a single antigen. Hence, considerable "on-target off-tumour" toxicity occurs whereby normal tissues are damaged by the therapy. For some cancers, targeting the presence of two cancer antigens may be more selective and therefore effective than targeting one. For example, B-chronic lymphocytic leukaemia (B-CLL) is a common leukaemia which is currently treated by targeting CD19. This treats the lymphoma but also depletes the entire B-cell compartment such that the treatment has a considerable toxic effect. B-CLL has an unusual phenotype in that CD5 and CD19 are co-expressed. By targeting only cells which express CD5 and CD19, it would be possible to considerably reduce on-target off-tumour toxicity.

Potential pairs of antigens for such an 'AND' logic gate are shown in Table 3.

Table 3

Cancer Type	*Antigens*
Chronic Lymphocytic Leukaemia	CD5, CD19
Neuroblastoma	ALK, GD2
Glioma	EGFR, Vimentin
Multiple myeloma	BCMA, CD138
Renal Cell Carcinoma	Carbonic anhydrase IX, G250
T-ALL	CD2, N-Cadherin
Prostate Cancer	PSMA, hepsin (or others)

By way of example, the receptor which comprises an activating endodomain fused to an inhibitory endodomain via a protease cleavage site may be the sequence shown as SEQ ID NO: 58, which contains a CAR against CD19 with a CD3-zeta endodomain and a cleavable CD148 endodomain.

SEQ ID NO: 58

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIY
HTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITKAGGGGSGGGGS
GGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYN
SALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRP
PTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRRVKF
SRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI
GMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRENLYFQMAVFGCIFGALVIVTVGGFIFWRKKR
KDAKNNEVSFSQIKPKKSKLIRVENFEAYFKKQQADSNCGFAEEYEDLKLVGISQPKYAAELAENRGKNR
YNNVLPYDISRVKLSVQTHSTDDYINANYMPGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAIIMLTK
CVEQGRTKCEEYWPSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHPLRQFHFTSWPDHGVPD
TTDLLINFRYLVRDYMKQSPPESPILVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYDLRMHRPL
MVQTEDQYVFLNQCVLDIVRSQKDSKVDLIYQNTTAMTIYENLAPVTTFGKTNGYIA

Suitably, the receptor may comprise the sequence shown as SEQ ID NO: 58; or a variant thereof having at least 80, 85, 90, 95, 98 or 99% sequence identity.

BLOCKING SIGNAL

The present invention provides a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) but lacks a kinase domain

For example, the truncated protein may comprise the ZAP70 SH2 domain but lack the ZAP70 kinase domain. In other words, the present invention provides a truncated protein which: (i) comprises the sequence shown as SEQ ID NO: 2 but does not comprise the sequence shown as SEQ ID NO: 26.

Over-expression of the ZAP70 SH2 domain results in competition with full-length / wild-type ZAP70. Since the truncated ZAP70 cannot propagate signals, signal transmission is reduced in proportion to the ratio between wild-type ZAP70 and the truncated protein. This may be useful to reduce strength of T-cell activation for instance to prevent T-cell over-activation which can result in T-cell exhaustion, activation induced cell death and in a clinical setting can result in cytokine storms.

The present invention also provide a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) but lacks a phosphatase domain

For example, the truncated protein may comprise the PTPN6 SH2 domain but lack the PTPN6 phosphatase domain. In other words, the present invention provides a truncated protein which:

(i) comprises the sequence shown as SEQ ID NO: 6 but does not comprise the sequence shown as SEQ ID NO: 27.

In this case, ITIM signalling can be reduced in proportion to the ratio between wild-type PTPN6 and the truncated protein. This may be useful to reduce inhibitory signals such as PD1 signalling. This may have application when T-cells are targeting a tumour which over-expresses PDL1 (or similar inhibitory receptors) to evade immune rejection.

The use of a blocking signal or a cross-wire signal as described above, offers a significant advantage over traditional immune checkpoint blockade approaches which typically block a single ligand/receptor interaction, such as PD-L1/PD1, with an antibody. As explained above, the inhibitory immune receptor class contains many members with redundancies and expression patterns which fluctuate with T-cell state. The use of an antibody or a recombinant ligand/receptor may effectively block one inhibitory receptor, but will not affect inhibitory signals transmitted from the rest. Genomic editing of individual inhibitory receptors (Menger et al, Cancer Res. 2016 Apr 15;76(8):2087-93) has a similar limitation. Strategies of fusions between individual inhibitory receptors and co-stimulatory domains also suffer from similar limitations (Liu et al, Cancer Res. 2016 Mar 15;76(6):1578-90).

The method of the present invention will block (and depending on the strategy re-interpret) inhibitory signals transmitted via an ITIM. Hence an entire class of inhibitory signals are modulated. A list of inhibitory receptors which signal through ITIMs is provided in Table II of Odorizzi and Wherry (2012) J. Immunol. 188:2957-2965. They include: PD1, BTLA, 2B4, CTLA-4, GP49B, Lair-1, Pir-B, PECAM-1, CD22, Siglec 7, Siglec 9, KLRG1, ILT2, CD94-NKG2A and CD5.

NUCLEIC ACID

In one aspect the present invention provides a nucleic acid which encodes a fusion protein or a truncated protein according to the present invention.

As used herein, the terms “polynucleotide”, “nucleotide”, and “nucleic acid” are intended to be synonymous with each other.

It will be understood by a skilled person that numerous different polynucleotides and nucleic acids can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make

nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides described here to reflect the codon usage of any particular host organism in which the polypeptides are to be expressed.

Nucleic acids according to the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the use as described herein, it is to be understood that the polynucleotides may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of interest.

The terms "variant", "homologue" or "derivative" in relation to a nucleotide sequence include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence.

NUCLEIC ACID CONSTRUCT

In one aspect the present invention provides a nucleic acid construct which co-expresses a truncated protein or fusion protein of the present invention with another protein. The nucleic acid construct may comprise: a nucleic acid sequence encoding a truncated protein or a fusion protein of the present invention; and a nucleic acid encoding another protein.

The present invention provides a nucleic acid construct which co-expresses a truncated protein or fusion protein of the present invention with a chimeric antigen receptor. The nucleic acid construct may comprise: (i) a nucleic acid sequence encoding a truncated protein or a fusion protein of the present invention; and (ii) a nucleic acid encoding a chimeric antigen receptor.

The chimeric antigen receptor (CAR) may be an activatory CAR comprising an ITAM-containing endodomain, such as CD3 zeta. The CAR may be an inhibitory CAR comprising a "ligation-off" endodomain, as described in WO2015/075469 which may comprise all or part of the endodomain from a receptor-like tyrosine phosphatase, such as CD148 or CD45. The CAR may be an inhibitory CAR comprising a "ligation-on" endodomain, as described in WO2015/075470 which may comprise an ITIM domain.

The fusion proteins and truncated proteins of the invention may be used together with a cell expressing a "logic gate" combination of two or more CARs. An OR gate comprises two activatory CARs as described in WO2015/075468. An AND gate comprises an activatory CAR and a "ligation off" inhibitory CAR, as described in WO2015/075469. An AND not comprises an activatory CAR and a "ligation on" inhibitory CAR, as described in WO2015/075470.

Thus the present invention provides a nucleic acid construct which comprises:

(i) a nucleic acid sequence encoding a truncated protein or fusion protein of the invention;

(ii) a first chimeric antigen receptor (CAR); and

(iii) a second chimeric antigen receptor.

With reference to the transcription signal aspect of the invention, there is provided a nucleic acid construct which comprises (i) a nucleic acid sequence encoding a fusion protein comprising a SH2 domain; and a protease domain; and (ii) a nucleic acid sequence encoding a membrane tethered transcription factor which comprises: a membrane tether; a protease recognition site; and a transcription factor.

With reference to the castration signal aspect of the invention, there is provided a nucleic acid construct which comprises (i) a nucleic acid sequence encoding a fusion protein which comprises an SH2 domain and a protease domain (e.g. a TeV domain); and (ii) a nucleic acid sequence encoding a receptor which comprises a protease cleavage site.

For example, the present invention provides a nucleic acid construct which comprises: (a) a nucleic acid sequence encoding a fusion protein which comprises (i) PTPN6 SH2 domain; and (ii) a protease domain (e.g. a TeV domain); (b) a nucleic acid sequence encoding a receptor which comprises a protease cleavage site; and (c) a nucleic acid sequence encoding a receptor comprising an ITIM containing endodomain.

The receptor may be a T-cell receptor (TCR) or a chimeric antigen receptor (CAR).

Suitably, the protein encoded by nucleic acid sequence (b) may be a T-cell receptor (TCR) or a chimeric antigen receptor (CAR) which comprises: (i) a protease cleavage site between a transmembrane domain and an activating endodomain; or (ii) an activating endodomain fused to an inhibitory endodomain via a protease cleavage site.

Where the nucleic acid construct of the invention, produces discrete polypeptides, such when it coexpresses a fusion protein of the invention and a CAR, it may also comprise a nucleic acid sequence enabling expression of both proteins. For example, it may comprise a sequence encoding a cleavage site between the two nucleic acid sequences. The cleavage site may be self-cleaving, such that when the nascent polypeptide is produced, it is immediately cleaved into the two proteins without the need for any external cleavage activity.

Various self-cleaving sites are known, including the Foot-and-Mouth disease virus (FMDV) 2a self-cleaving peptide, which has the sequence shown:

SEQ ID NO: 59
RAEGRGSLLTCGDVEENPGP

or

SEQ ID NO: 60
QCTNYALLKLAGDVESNPGP

The co-expressing sequence may be an internal ribosome entry sequence (IRES). The co-expressing sequence may be an internal promoter.

CHIMERIC ANTIGEN RECEPTOR (CAR)

CARs, which are shown schematically in Figure 13, are chimeric type I trans-membrane proteins which connect an extracellular antigen-recognizing domain (binder) to an intracellular signalling domain (endodomain). The binder is typically a single-chain variable fragment (scFv) derived from a monoclonal antibody (mAb), but it can be based on other formats which comprise an antibody-like antigen binding site. A spacer domain is usually necessary to isolate the binder from the membrane and to allow it a suitable orientation. A common spacer domain used is the Fc of IgG1. More compact spacers can suffice e.g. the stalk from CD8α and even just the IgG1 hinge alone, depending on the antigen. A trans-membrane domain anchors the protein in the cell membrane and connects the spacer to the endodomain.

Early CAR designs had endodomains derived from the intracellular parts of either the γ chain of the FcεR1 or CD3ζ. Consequently, these first generation receptors transmitted immunological signal 1, which was sufficient to trigger T-cell killing of cognate target cells but failed to fully activate the T-cell to proliferate and survive. To overcome this limitation, compound endodomains have been constructed: fusion of the intracellular part of a T-cell co-stimulatory

molecule to that of CD3ζ results in second generation receptors which can transmit an activating and co-stimulatory signal simultaneously after antigen recognition. The co-stimulatory domain most commonly used is that of CD28. This supplies the most potent co-stimulatory signal - namely immunological signal 2, which triggers T-cell proliferation. Some receptors have also been described which include TNF receptor family endodomains, such as the closely related OX40 and 41BB which transmit survival signals. Even more potent third generation CARs have now been described which have endodomains capable of transmitting activation, proliferation and survival signals.

CAR-encoding nucleic acids may be transferred to T cells using, for example, retroviral vectors. Lentiviral vectors may be employed. In this way, a large number of cancer-specific T cells can be generated for adoptive cell transfer. When the CAR binds the target-antigen, this results in the transmission of an activating signal to the T-cell it is expressed on. Thus the CAR directs the specificity and cytotoxicity of the T cell towards tumour cells expressing the targeted antigen.

CARs typically therefore comprise: (i) an antigen-binding domain; (ii) a spacer; (iii) a transmembrane domain; and (iii) an intracellular domain which comprises or associates with a signalling domain.

ANTIGEN BINDING DOMAIN

The antigen binding domain is the portion of the CAR which recognizes antigen. Numerous antigen-binding domains are known in the art, including those based on the antigen binding site of an antibody, antibody mimetics, and T-cell receptors. For example, the antigen-binding domain may comprise: a single-chain variable fragment (scFv) derived from a monoclonal antibody; a natural ligand of the target antigen; a peptide with sufficient affinity for the target; a single domain antibody; an artificial single binder such as a Darpin (designed ankyrin repeat protein); or a single-chain derived from a T-cell receptor.

The antigen binding domain may comprise a domain which is not based on the antigen binding site of an antibody. For example the antigen binding domain may comprise a domain based on a protein/peptide which is a soluble ligand for a tumour cell surface receptor (e.g. a soluble peptide such as a cytokine or a chemokine); or an extracellular domain of a membrane anchored ligand or a receptor for which the binding pair counterpart is expressed on the tumour cell.

The antigen binding domain may be based on a natural ligand of the antigen.

The antigen binding domain may comprise an affinity peptide from a combinatorial library or a de novo designed affinity protein/peptide.

SPACER DOMAIN

CARs comprise a spacer sequence to connect the antigen-binding domain with the transmembrane domain and spatially separate the antigen-binding domain from the endodomain. A flexible spacer allows the antigen-binding domain to orient in different directions to facilitate binding.

In aspects of the present invention which require two CARs, the first and second CARs may comprise different spacer molecules. For example, the spacer sequence may, for example, comprise an IgG1 Fc region, an IgG1 hinge or a human CD8 stalk or the mouse CD8 stalk. The spacer may alternatively comprise an alternative linker sequence which has similar length and/or domain spacing properties as an IgG1 Fc region, an IgG1 hinge or a CD8 stalk. A human IgG1 spacer may be altered to remove Fc binding motifs.

All the spacer domains mentioned above form homodimers. However the mechanism is not limited to using homodimeric receptors and should work with monomeric receptors as long as the spacer is sufficiently rigid. An example of such a spacer is CD2 or truncated CD22.

Since CARs are typically homodimers (see Figure 13a), cross-pairing may result in a heterodimeric chimeric antigen receptor. This is undesirable for various reasons, for example: (1) the epitope may not be at the same "level" on the target cell so that a cross-paired CAR may only be able to bind to one antigen; (2) the VH and VL from the two different scFv could swap over and either fail to recognize target or worse recognize an unexpected and unpredicted antigen. For the “AND” and “AND NOT” gates described above, the spacer of the first CAR may be sufficiently different from the spacer of the second CAR in order to avoid cross-pairing but sufficiently similar to co-localise. Pairs of orthologous spacer sequences may be employed. Examples are murine and human CD8 stalks, or alternatively spacer domains which are monomeric – for instance the ectodomain of CD2.

Examples of spacer pairs which co-localise are shown in the following Table:

Stimulatory CAR spacer	Inhibitory CAR spacer
Human-CD8aSTK	Mouse CD8aSTK
Human-CD28STK	Mouse CD8aSTK

Human IgG Hinge	Human CD3z ectodomain
Human-CD8aSTK	Mouse CD28STK
Human-CD28STK	Mouse CD28STK
Human-IgG-Hinge-CH2CH3	Human-IgM-Hinge-CH2CH3CD4

TRANSMEMBRANE DOMAIN

The transmembrane domain is the sequence of the CAR that spans the membrane.

A transmembrane domain may be any protein structure which is thermodynamically stable in a membrane. This is typically an alpha helix comprising of several hydrophobic residues. The transmembrane domain of any transmembrane protein can be used to supply the transmembrane portion of the invention. The presence and span of a transmembrane domain of a protein can be determined by those skilled in the art using the TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Further, given that the transmembrane domain of a protein is a relatively simple structure, i.e a polypeptide sequence predicted to form a hydrophobic alpha helix of sufficient length to span the membrane, an artificially designed TM domain may also be used (US 7052906 B1 describes synthetic transmembrane components).

The transmembrane domain may be derived from CD28, which gives good receptor stability.

ACTIVATING ENDODOMAIN

The endodomain is the signal-transmission portion of the CAR. It may be part of or associate with the intracellular domain of the CAR. After antigen recognition, receptors cluster, native CD45 and CD148 are excluded from the synapse and a signal is transmitted to the cell. The most commonly used endodomain component is that of CD3-zeta which contains 3 ITAMs. This transmits an activation signal to the T cell after antigen is bound. CD3-zeta may not provide a fully competent activation signal and additional co-stimulatory signaling may be needed. For example, chimeric CD28 and OX40 can be used with CD3-Zeta to transmit a proliferative / survival signal, or all three can be used together.

Where a CAR comprises an activating endodomain, it may comprise the CD3-Zeta endodomain alone, the CD3-Zeta endodomain with that of either CD28 or OX40 or the CD28 endodomain and OX40 and CD3-Zeta endodomain.

Any endodomain which contains an ITAM motif can act as an activation endodomain in this invention. Suitable endodomains which contain an ITAM motif are described herein.

INHIBITORY DOMAIN

In embodiments referred to above as the "AND" gate, the first CAR may comprise an activating endodomain fused to an inhibitory endodomain via a protease cleavage site. As such the inhibitory endodomain inhibits T-cell activation by the first CAR in the absence of activation of the second CAR. Upon activation of the second CAR, the ITIM in the endodomain of the second CAR is phosphorylated and the PTPN6/protease domain fusion protein is recruited to the membrane. This results in cleavage of the first CAR between the activating endodomain and inhibitory endodomain, thus enabling T-cell activation.

The inhibitory endodomains may comprise any sequence which inhibits T-cell signalling by the activating CAR when it is in the same endodomain.

The inhibitory endodomain may be or comprise a tyrosine phosphatase, such as a receptor-like tyrosine phosphatase. An inhibitory endodomain may be or comprise any tyrosine phosphatase that is capable of inhibiting the TCR signalling when co-localised with the activating endodomain of the CAR. An inhibitory endodomain may be or comprise any tyrosine phosphatase with a sufficiently fast catalytic rate for phosphorylated ITAMs that is capable of inhibiting the TCR signalling when co-localised with the activating endodomain of the CAR.

VECTOR

The present invention also provides a vector, or kit of vectors which comprises one or more nucleic acid sequence(s) or construct(s) according to the present invention. Such a vector may be used to introduce the nucleic acid sequence(s) or construct(s) into a host cell so that it expresses the proteins encoded by the nucleic acid sequence or construct.

The vector may, for example, be a plasmid or a viral vector, such as a retroviral vector or a lentiviral vector, or a transposon based vector or synthetic mRNA.

The vector may be capable of transfecting or transducing a T cell.

CELL

The present invention also relates to an immune cell comprising the fusion protein, truncated protein, nucleic acid and/or nucleic acid construct of the present invention.

The cell may be a cytolytic immune cell.

Cytolytic immune cells can be T cells or T lymphocytes which are a type of lymphocyte that play a central role in cell-mediated immunity. They can be distinguished from other lymphocytes, such as B cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface. There are various types of T cell, as summarised below.

Helper T helper cells (TH cells) assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. TH cells express CD4 on their surface. TH cells become activated when they are presented with peptide antigens by MHC class II molecules on the surface of antigen presenting cells (APCs). These cells can differentiate into one of several subtypes, including TH1, TH2, TH3, TH17, Th9, or TFH, which secrete different cytokines to facilitate different types of immune responses.

Cytolytic T cells (TC cells, or CTLs) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. CTLs express the CD8 at their surface. These cells recognize their targets by binding to antigen associated with MHC class I, which is present on the surface of all nucleated cells. Through IL-10, adenosine and other molecules secreted by regulatory T cells, the CD8+ cells can be inactivated to an anergic state, which prevent autoimmune diseases such as experimental autoimmune encephalomyelitis.

Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise three subtypes: central memory T cells (TCM cells) and two types of effector memory T cells (TEM cells and TEMRA cells). Memory cells may be either CD4+ or CD8+. Memory T cells typically express the cell surface protein CD45RO.

Regulatory T cells (Treg cells), formerly known as suppressor T cells, are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus.

Two major classes of CD4+ Treg cells have been described — naturally occurring Treg cells and adaptive Treg cells.

Naturally occurring Treg cells (also known as CD4+CD25+FoxP3+ Treg cells) arise in the thymus and have been linked to interactions between developing T cells with both myeloid (CD11c+) and plasmacytoid (CD123+) dendritic cells that have been activated with TSLP. Naturally occurring Treg cells can be distinguished from other T cells by the presence of an intracellular molecule called FoxP3. Mutations of the FOXP3 gene can prevent regulatory T cell development, causing the fatal autoimmune disease IPEX.

Adaptive Treg cells (also known as Tr1 cells or Th3 cells) may originate during a normal immune response.

Natural Killer Cells (or NK cells) are a type of cytolytic cell which form part of the innate immune system. NK cells provide rapid responses to innate signals from virally infected cells in an MHC independent manner.

NK cells (belonging to the group of innate lymphoid cells) are defined as large granular lymphocytes (LGL) and constitute the third kind of cells differentiated from the common lymphoid progenitor generating B and T lymphocytes. NK cells are known to differentiate and mature in the bone marrow, lymph node, spleen, tonsils and thymus where they then enter into the circulation.

The cells of the invention may be any of the cell types mentioned above.

T or NK cells expressing the molecules of the invention may either be created *ex vivo* either from a patient's own peripheral blood (1st party), or in the setting of a haematopoietic stem cell transplant from donor peripheral blood (2nd party), or peripheral blood from an unconnected donor (3rd party).

Alternatively, T or NK cells expressing the molecules of the invention may be derived from *ex vivo* differentiation of inducible progenitor cells or embryonic progenitor cells to T cells. Alternatively, an immortalized T-cell line which retains its lytic function and could act as a therapeutic may be used.

In all these embodiments, cells are generated by introducing DNA or RNA coding for the receptor component and signalling component by one of many means including transduction with a viral vector, transfection with DNA or RNA.

The cell of the invention may be an *ex vivo* T or NK cell from a subject. The T or NK cell may be from a peripheral blood mononuclear cell (PBMC) sample. T or NK cells may be activated and/or expanded prior to being transduced with nucleic acid of the invention, for example by treatment with an anti-CD3 monoclonal antibody.

The T or NK cell of the invention may be made by:

(i) isolation of a T or NK cell-containing sample from a subject or other sources listed above; and

(ii) transduction or transfection of the T or NK cells with one or more a nucleic acid sequence(s) according to the invention.

The T or NK cells may then by purified, for example, selected on the basis of expression of the antigen-binding domain of the antigen-binding polypeptide.

The present invention also provides a cell which comprises a fusion protein or a truncated protein of the invention and a chimeric antigen receptor (CAR).

The chimeric antigen receptor (CAR) may be an activatory CAR comprising an ITAM-containing endodomain, such as CD3 zeta. The CAR may be an inhibitory CAR comprising a "ligation-off" endodomain, as described in WO2015/075469 which may comprise all or part of the endodomain from a receptor-like tyrosine phosphatase, such as CD148 or CD45. The CAR may be an inhibitory CAR comprising a "ligation-on" endodomain, as described in WO2015/075470 which may comprise an ITIM domain.

The fusion proteins and truncated proteins of the invention may be used together with a cell expressing a "logic gate" combination of two or more CARs. An OR gate comprises two activatory CARs as described in WO2015/075468. An AND gate comprises an activatory CAR and a "ligation off" inhibitory CAR, as described in WO2015/075469. An AND not comprises an activatory CAR and a "ligation on" inhibitory CAR, as described in WO2015/075470.

Thus the present invention provides a cell which comprises:

(i) a nucleic acid sequence encoding a truncated protein or fusion protein of the invention;

(ii) a first chimeric antigen receptor (CAR); and

(iii) a second chimeric antigen receptor.

With reference to the transcription signal aspect of the invention, there is provided a cell which comprises (i) a fusion protein comprising an SH2 domain and a protease; and (ii) a membrane tethered transcription factor which comprises: a membrane tether, a protease recognition site; and a transcription factor.

With reference to the castration signal aspect of the invention there is provided a cell which comprises (i) a fusion protein comprising an SH2 domain and a protease; and (ii) receptor which comprises a protease cleavage site.

The receptor may, for example be a T-cell receptor (TCR) or a chimeric antigen receptor (CAR) which comprises: (i) a protease cleavage site between a transmembrane domain and an activating endodomain; or (ii) an activating endodomain fused to an inhibitory endodomain via a protease cleavage site.

COMPOSITION

The present invention also relates to a pharmaceutical composition containing a plurality of cells of the invention. The pharmaceutical composition may additionally comprise a pharmaceutically acceptable carrier, diluent or excipient. The pharmaceutical composition may optionally comprise one or more further pharmaceutically active polypeptides and/or compounds. Such a formulation may, for example, be in a form suitable for intravenous infusion.

METHOD OF TREATMENT

The cells of the present invention may be capable of killing target cells, such as cancer cells.

The cells of the present invention may be used for the treatment of an infection, such as a viral infection.

The cells of the invention may also be used for the control of pathogenic immune responses, for example in autoimmune diseases, allergies and graft-vs-host rejection.

The cells of the invention may be used for the treatment of a cancerous disease, such as bladder cancer, breast cancer, colon cancer, endometrial cancer, kidney cancer (renal cell), leukemia, lung cancer, melanoma, non-Hodgkin lymphoma, pancreatic cancer, prostate cancer and thyroid cancer.

The cells of the invention may be used to treat: cancers of the oral cavity and pharynx which includes cancer of the tongue, mouth and pharynx; cancers of the digestive system which includes oesophageal, gastric and colorectal cancers; cancers of the liver and biliary tree which includes hepatocellular carcinomas and cholangiocarcinomas; cancers of the respiratory system which includes bronchogenic cancers and cancers of the larynx; cancers of bone and joints which includes osteosarcoma; cancers of the skin which includes melanoma; breast cancer; cancers of the genital tract which include uterine, ovarian and cervical cancer in women, prostate and testicular cancer in men; cancers of the renal tract which include renal cell carcinoma and transitional cell carcinomas of the utterers or bladder; brain cancers including gliomas, glioblastoma multiforme and medullobastomas; cancers of the endocrine system including thyroid cancer, adrenal carcinoma and cancers associated with multiple endocrine neoplasm syndromes; lymphomas including Hodgkin's lymphoma and non-Hodgkin lymphoma; Multiple Myeloma and plasmacytomas; leukaemias both acute and chronic, myeloid or lymphoid; and cancers of other and unspecified sites including neuroblastoma.

Treatment with the cells of the invention may help prevent the escape or release of tumour cells which often occurs with standard approaches.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1 – Subjugation of the T cell activation pathway to augmented or non-physiological signals

A number of SH2 domains involved in early T cell signal activation were tested to determine whether T cell activation signals could be subjugated or “hijacked”, such that when a T cell was activated the signal could be modulated or re-transmitted.

The inventors generated several chimeric AKT constructs by linking the kinase domain of AKT to SH2 domains from Zap70, Grap, Grb2 and PLCγ (Figure 8).

In non-transduced (NT) T cells, very low levels of phosphorylation of the endogenous AKT were detectable following treatment with OKT3 to induce cross-linking and activation of the TCR

(Figure 10b:top panel). However, in cells expression the Zap-AKT construct, significant levels of phospho-AKT were observed (Figure 10b:bottom panel).

Linker for activation of T cell (LAT) is a downstream target of ZAP70 and is bound by several SH2-containing proteins, such as Grb2, Grap and PLCγ. It was anticipated that the SH2 domains from each of these LAT-binders would also allow the activation signal from CD3-zeta to be hijacked. However, this was not the case.

No TCR-dependent phosphorylation of the AKT kinase domain was observed above levels observed for NT T cells when the AKT kinase domain was linked to the SH2 domains from Grb2, Grap or PLCγ (Figure 9a).

This demonstrates that this system of T cell signalling hijacking specifically requires the tandem SH2 domain from a very early T cell signalling molecule, such as Zap70 or Tyrosine-protein phosphatase non-receptor type 6 (PTPN6).

Example 2 – Transcriptional Control

The TeV protease was fused to the Zap70 SH2 domain. A membrane-bound transcription factor was also generated as follows: RQR8 was cloned in frame with the VP16/GAL4 transcription factor separated by a TeV cleavage site. This fusion protein allows release of the VP16/GAL4 transcription factor (which contains a nuclear localizing signal) upon TeV cleavage.

These proteins were both expressed in a T-cell which also expressed a CD19-specific chimeric antigen receptor. To demonstrate that the ZAP70-TeV approach is needed, the transcription factor was co-expressed with a CD19 CAR whose endodomain was replaced by TeV (Figure 11).

T-cells were exposed to CD19 negative and positive targets. Transcriptional activation was measured by a Luciferase cassette responsive to GALv/VP16. Only the condition where a standard CD19 CAR was co-expressed with ZAP-TeV and the membrane tethered transcription factor resulted in selective transcriptional activation upon CD19 recognition. The CD19 CAR fused directly to TeV resulted in constitutive transcriptional activation (Figure 12).

Example 3 - PD-1 signal blockade using truncated SHP-1 (PTPN6) or truncated SHP-2

PBMC cells were transduced as shown in the following table:

Name on Figure 15 key	Description	Construct(s)
NT	Untransduced	-
FMC63	Transduced with CD19 CAR only	SFG.aCD19_fmc63-HCH2CH3w-CD28tmZw
PD1	Transduced with PD1 only	pDual-PD1-GFP
FMC63+PD1	Co-transduced with CD19CAR and PD1	SFG.aCD19_fmc63-HCH2CH3w-CD28tmZw and pDual-PD1-GFP
FMC63-SHP1+PD1	Co-transduced with a) bicistronic construct encoding CD19CAR and truncated SHP1, and b) PD1	SFG.aCD19_fmc63-HCH2CH3w-CD28tm-Zeta_w-2A-dualSH2_SHP-1 and pDual-PD1-GFP
FMC63-SHP2+PD1	Co-transduced with a) bicistronic construct encoding CD19CAR and truncated SHP1, and b) PD1	SFG.aCD19_fmc63-HCH2CH3w-CD28tm-Zeta_w-2A-dualSH2_SHP-2 and pDual-PD1-GFP

The cells were co-cultured for 48 hours with SupT1 cells transduced with CD19, PDL1 or both and IFNγ release measured by ELISA. The results are shown in Figure 15.

The presence of PDL1 on SupT1 target cells caused a reduction in IFNγ release. There was increased IFNγ release with PBMC which expressed CAR together with the truncated SHP-1 or truncated SHP-2 construct compared with those which expressed CAR alone. This indicates that the truncated SHp-1 and SHP-2 constructs successfully inhibited the PDL1 inhibitory signal from the target cells.

Example 4 – PD-1 signal hijack using a fusion of SHP-2 SH2 domains and Zap70 kinase

PBMC cells were transduced as shown in the following table:

Name on Figure 16 key	Description	Construct(s)
NT	Untransduced	-
FMC63	Transduced with CD19 CAR only	SFG.aCD19_fmc63-HCH2CH3w-CD28tmZw
PD1	Transduced with PD1 only	pDual-PD1-GFP
FMC63+PD1	Co-transduced with CD19CAR	SFG.aCD19_fmc63-HCH2CH3w-

	and PD1	CD28tmZw and pDual-PD1-GFP
FMC63-SHP2Zap70+PD1	Co-transduced with a) bicistronic construct encoding CD19CAR and fusion of SHP2 SH2domains and Zap70 kinase, and b) PD1	SFG.aCD19_fmc63-HCH2CH3w-CD28tm-Zeta_w-2A-dualSH2_SHP-2-Zap70_Kinase and pDual-PD1-GFP

The cells were co-cultured in a 1:1 ratio for 24 hours with SupT1 cells transduced with CD19 or PDL1. IFNγ release was measured by ELISA (Figure 16A). An increase in IFN-γ production in was seen co-cultures of CAR-SHP2.Zap70 + PD1 transduced T cells with PDL1 SupT1 target cells compared with CAR + PD1 transduced T cells.

A cytotoxicity assay was also conducted in which killing of SupT1 cells was quantified by FACS (Figure 16B). Near complete killing of PDL1 SupT1 targets was observed in co-cultures of PDL1 positive target cells with CAR-SHP2.Zap70 + PD1 transduced T cells. By contrast, killing was not seen with CAR + PD1 alone construct. This indicated that replacing the phosphatase domain of SHP2 with the kinase domain of Zap70 successfully converted the inhibitory PD1 signal to an activatory signal. The SHP-2-Zap70kinase fusion protein therefore successfully hijacked the inhibitory PDL1-PD1 signal and turned it into a T-cell activation signal.

Various modifications and variations of the described methods 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 molecular biology, cellular immunology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. An immune cell which comprises a chimeric antigen receptor (CAR) and a truncated protein which comprises an SH2 domain from a protein which binds a phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM) but lacks a phosphatase domain.

2. An immune cell according to claim 1, wherein the truncated protein comprises the PTPN6 SH2 domain but lacks the PTPN6 phosphatase domain.

3. An immune cell according to claim 1, wherein the truncated protein comprises the SHP-2 SH2 domain but lacks the SHP-2 phosphatase domain.

4. An immune cell according to claim 1, wherein the truncated protein comprises the sequence shown as SEQ ID No. 6 or one or both of the sequences shown as SEQ ID No. 7 and 8.

5. An immune cell according to claim 1, wherein the truncated protein comprises or consists of the sequence shown as SEQ ID No. 10, 11 or 12.

6. A nucleic acid construct which comprises a nucleic acid sequence which encodes a truncated protein as defined in any one of claims 1 to 5; and a nucleic acid sequence encoding a chimeric antigen receptor.

7. A vector comprising a nucleic acid construct according to claim 6.

8. A set of vectors wherein each vector comprises a nucleic acid sequence which encodes a truncated protein as defined in any one of claims 1 to 5; and a nucleic acid sequence encoding a chimeric antigen receptor.

9. A pharmaceutical composition comprising a plurality of immune cells according to any one of claims 1 to 5.

10. A pharmaceutical composition according to claim 9 for use in treating and/or preventing cancer, viral infections or pathogenic immune responses.

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11. A pharmaceutical composition for use according to claim 10, wherein the immune cells in the composition are prepared *ex vivo* by a method comprising the following steps:

(i) provision of an immune cell containing sample from a subject; and

(ii) transduction or transfection of the immune cells with a nucleic acid construct according to claim 6, a vector according to claim 7 or set of vectors according to claim 8.

12. A pharmaceutical composition for use according to claim 10 or 11, wherein the disease is cancer.

13. A method for making an immune cell according to any one of claims 1 to 5, which comprises the step of introducing: a nucleic acid construct according to claim 6, a vector according to claim 7 or a set of vectors according to claim 8, into the immune cell *ex vivo*.

14. A method according to claim 13, wherein the immune cell is from a sample isolated from a subject.

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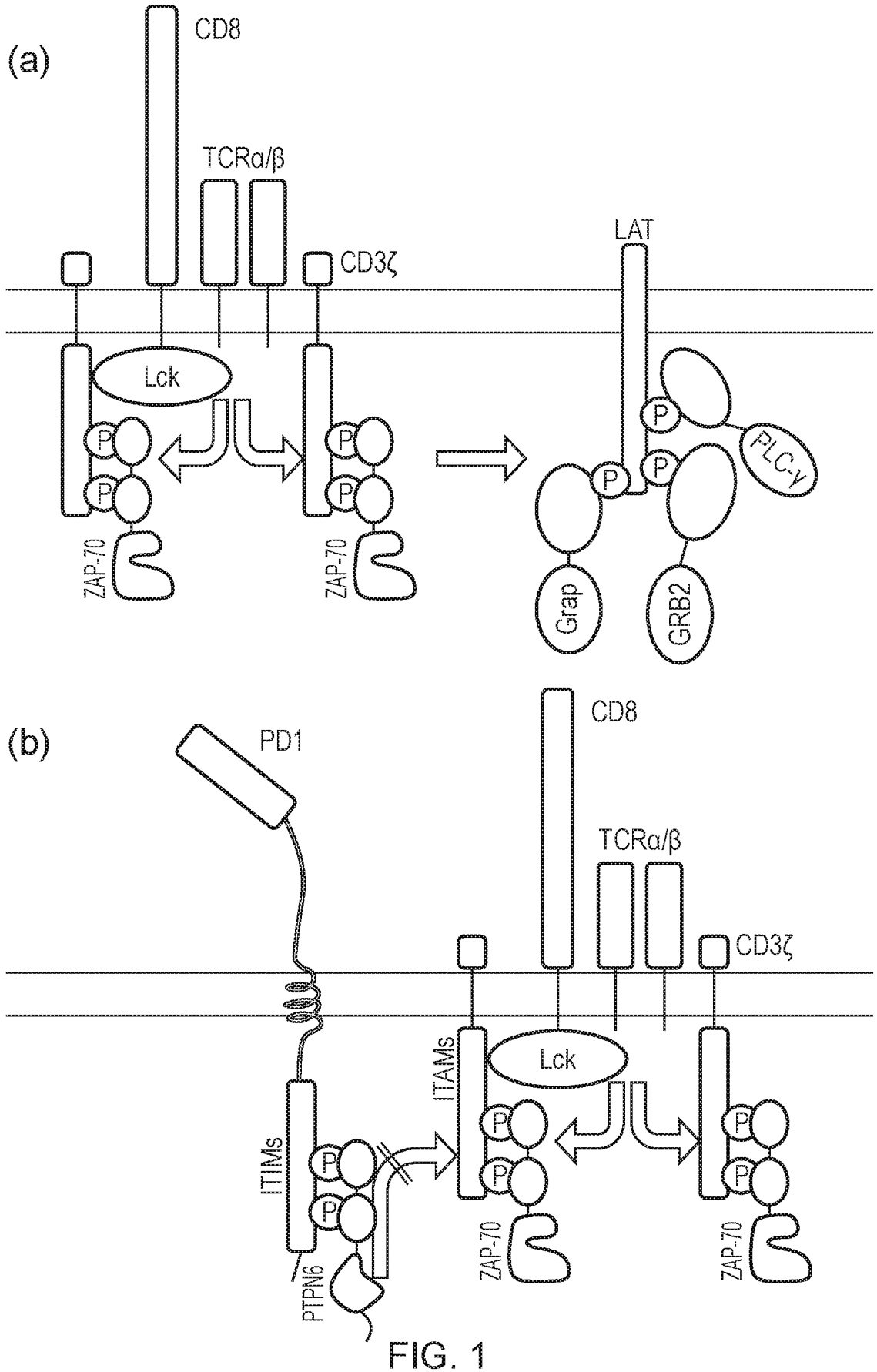
(a)
CD8
TCRα/β
CD3ζ
Lck
P
ZAP-70
LAT
PLC-γ
Grap
GRB2
(b)
PD1
CD8
TCRα/β
CD3ζ
ITAMs
Lck
ITIMs
PTPN6

FIG. 1

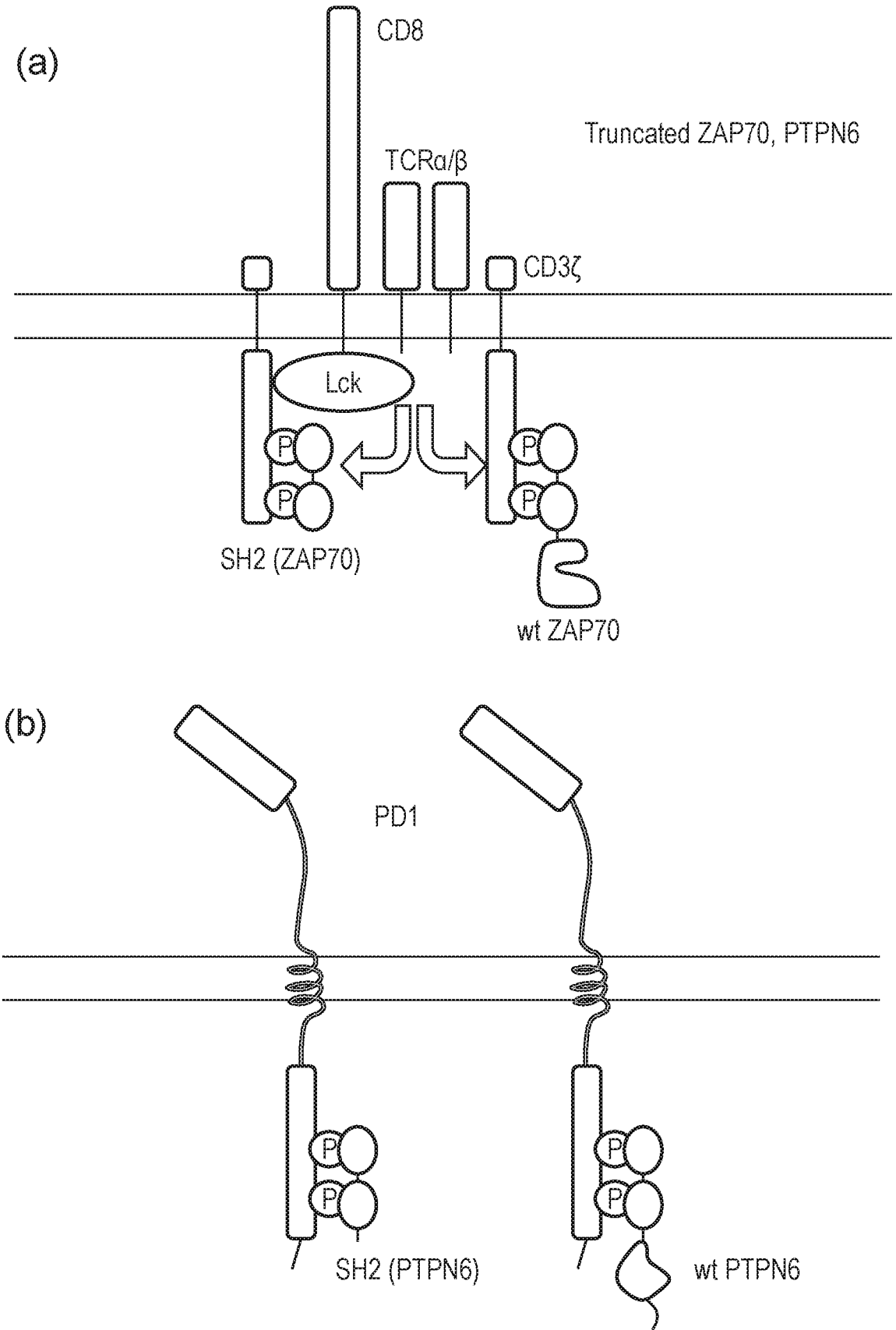
(a)
CD8
Truncated ZAP70, PTPN6
TCRα/β
CD3ζ
Lck
P
P
P
P
SH2 (ZAP70)
wt ZAP70
(b)
PD1
P
P
P
P
SH2 (PTPN6)
wt PTPN6

FIG. 2

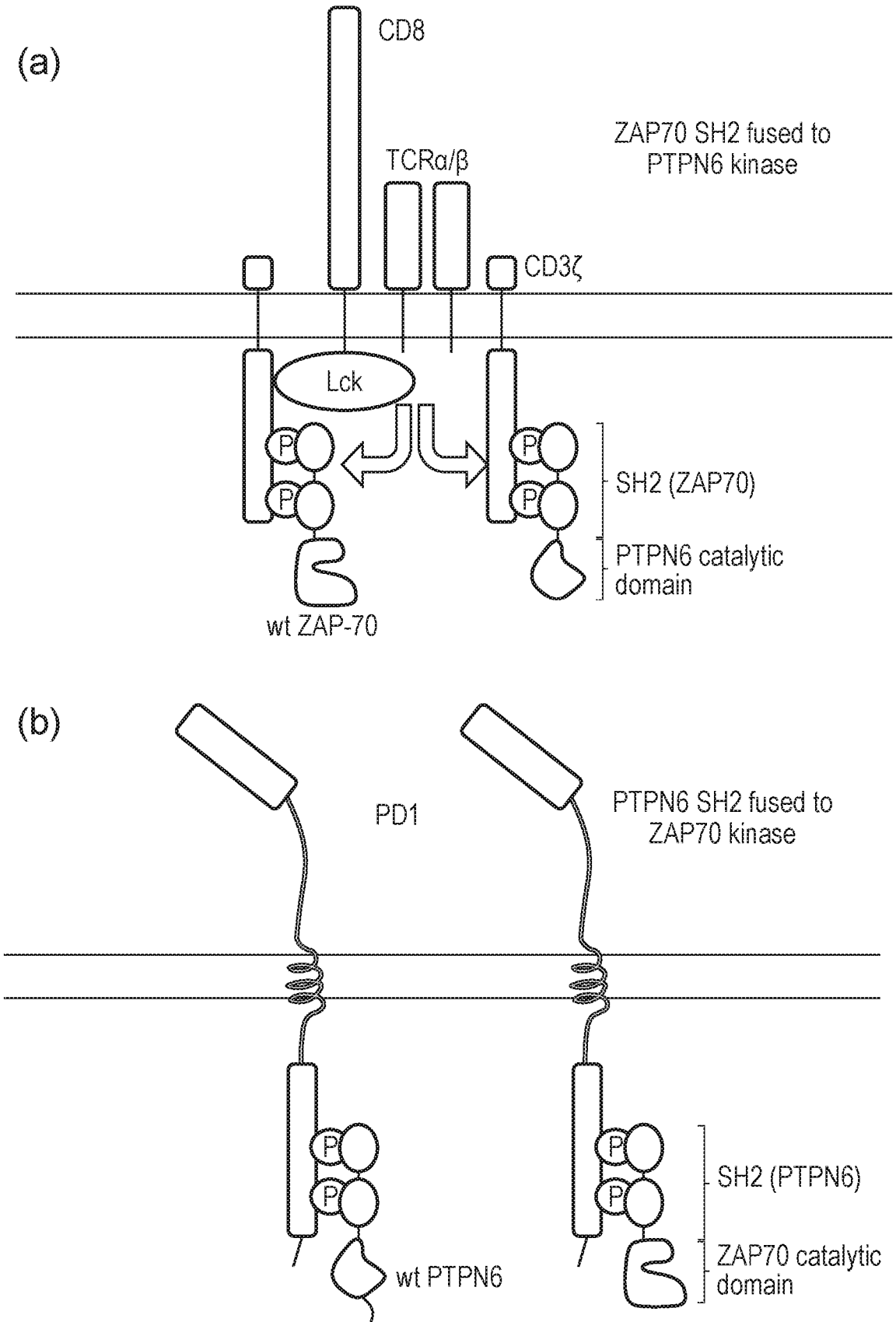
(a)
CD8
TCRα/β
CD3ζ
ZAP70 SH2 fused to
PTPN6 kinase
Lck
P
P
P
P
SH2 (ZAP70)
PTPN6 catalytic
domain
wt ZAP-70
(b)
PD1
PTPN6 SH2 fused to
ZAP70 kinase
P
P
P
P
SH2 (PTPN6)
ZAP70 catalytic
domain
wt PTPN6

FIG. 3

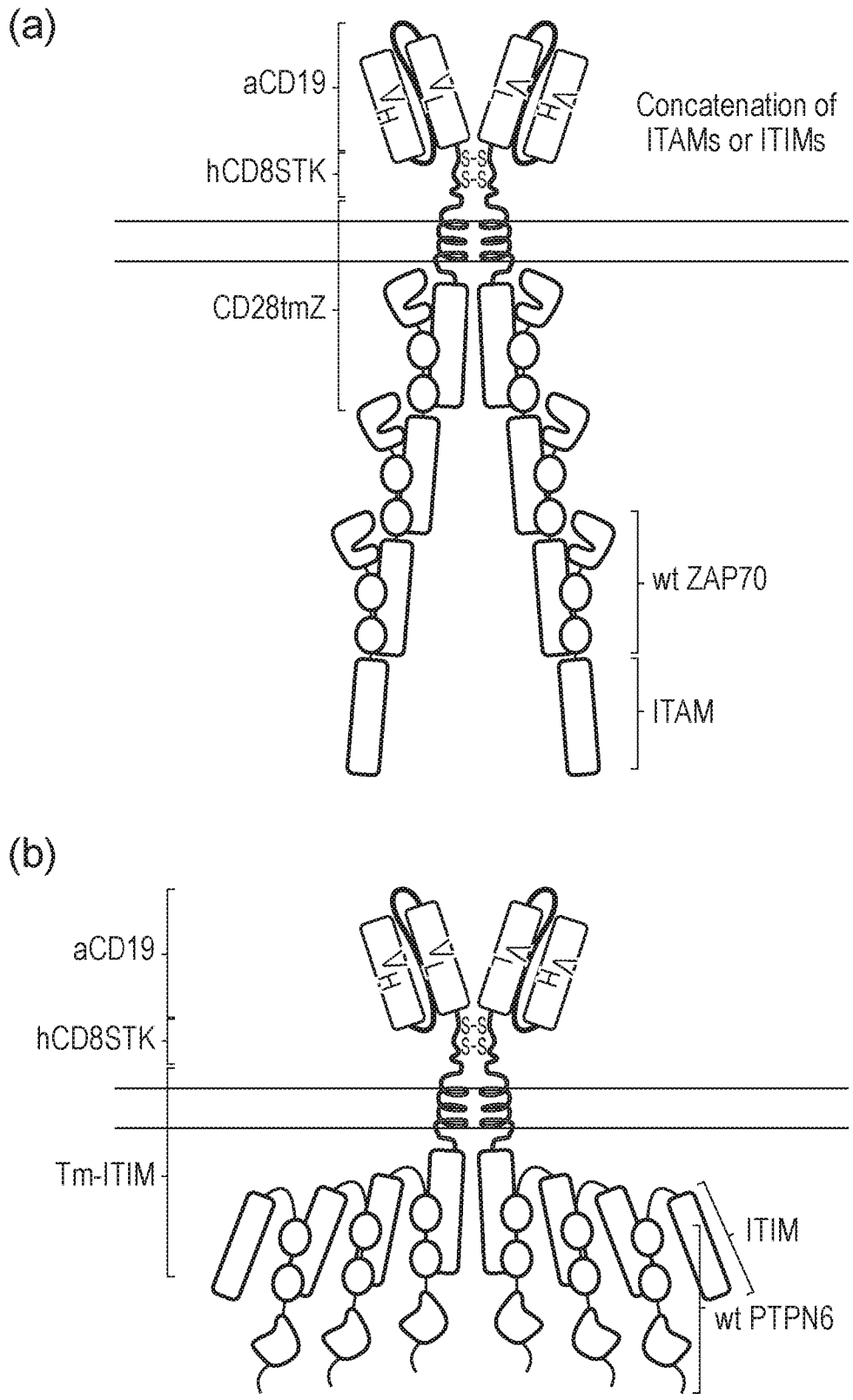
(a)
aCD19
hCD8STK
CD28tmZ
Concatenation of ITAMs or ITIMs
wt ZAP70
ITAM
(b)
aCD19
hCD8STK
Tm-ITIM
ITIM
wt PTPN6

FIG. 4

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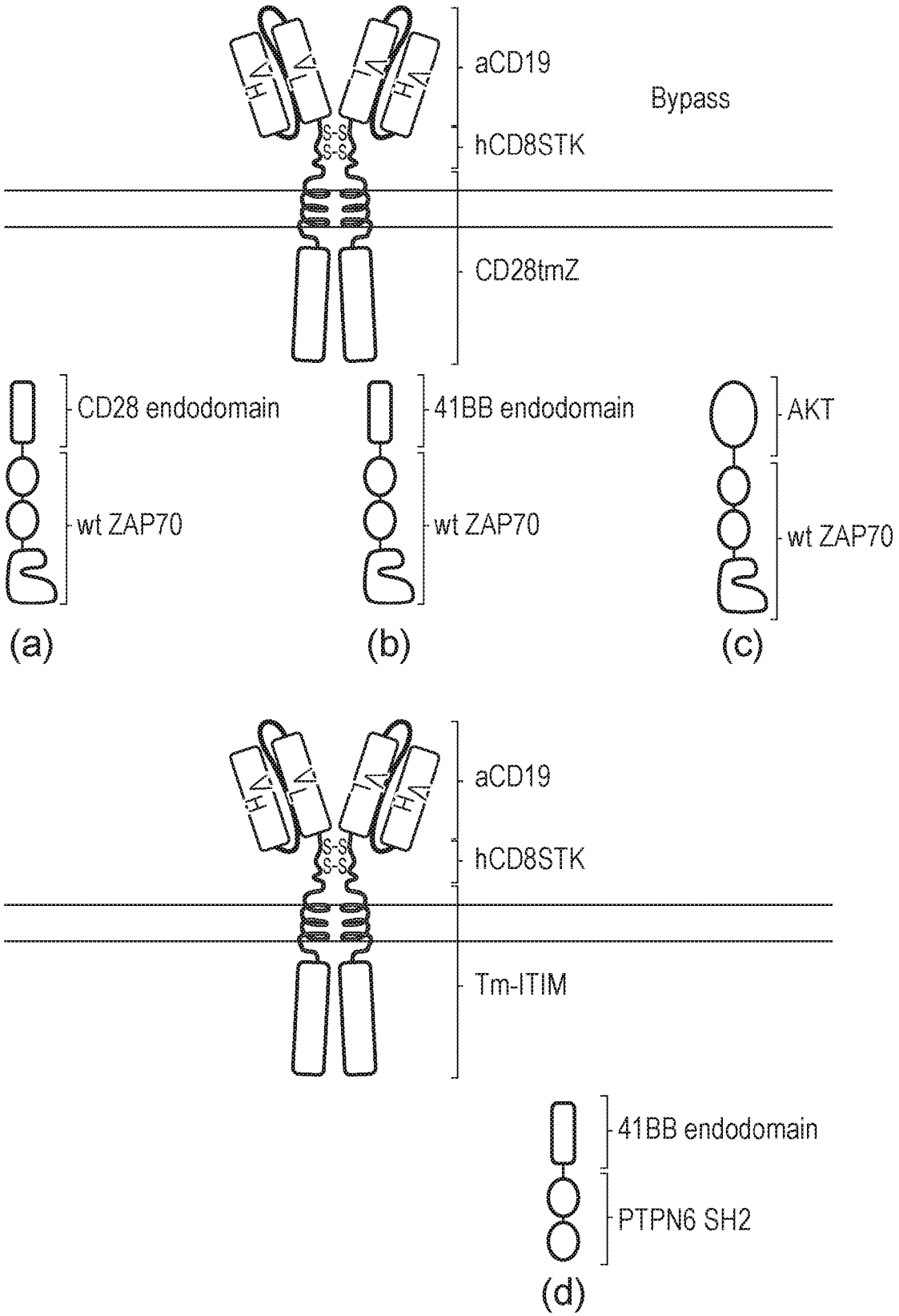
aCD19
hCD8STK
Bypass
CD28tmZ
CD28 endodomain
wt ZAP70
(a)
41BB endodomain
wt ZAP70
(b)
AKT
wt ZAP70
(c)
aCD19
hCD8STK
Tm-ITIM
41BB endodomain
PTPN6 SH2
(d)

FIG. 5

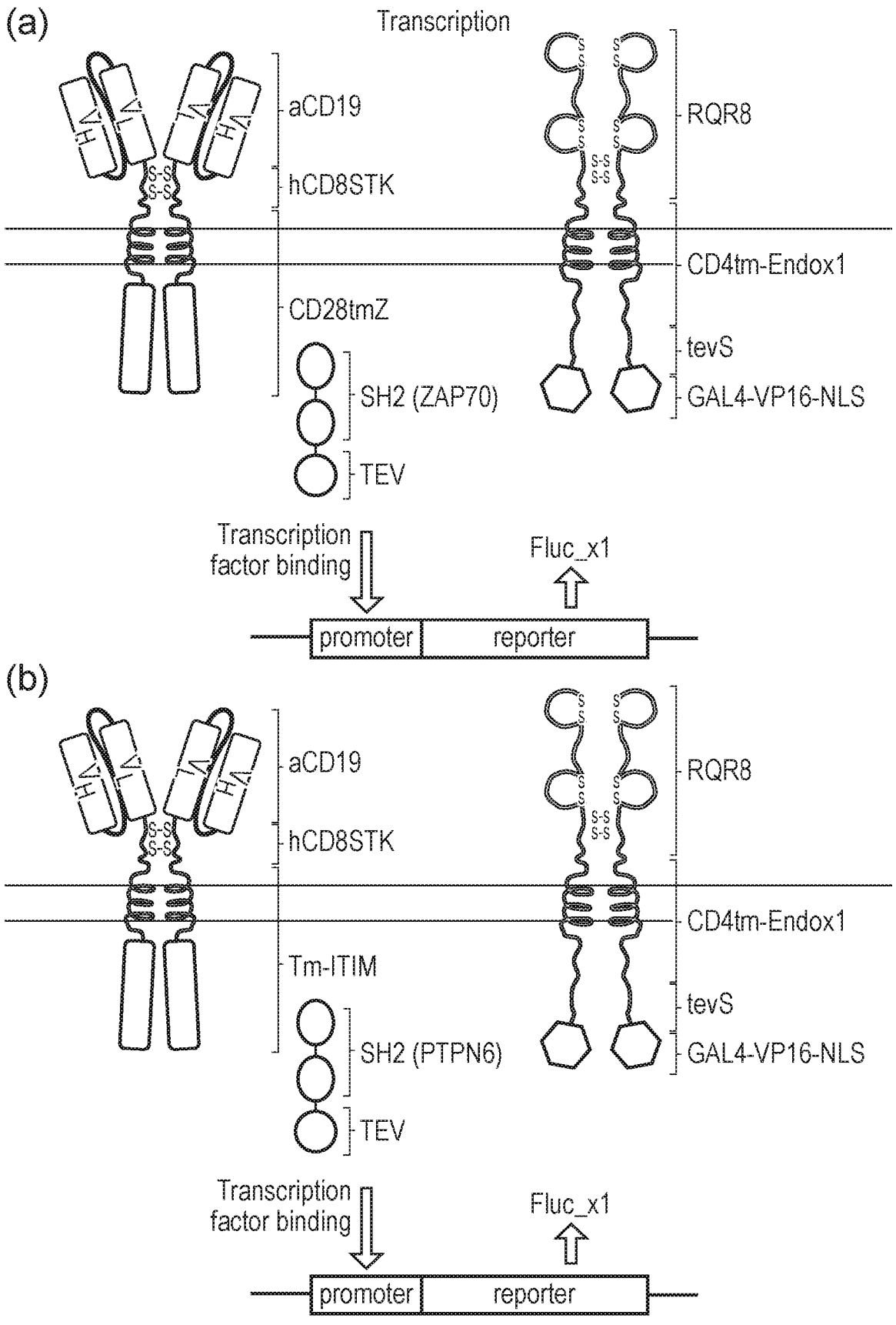
(a)
Transcription
aCD19
hCD8STK
CD28tmZ
SH2 (ZAP70)
TEV
RQR8
CD4tm-Endox1
tevS
GAL4-VP16-NLS
Transcription factor binding
Fluc_x1
promoter
reporter
(b)
aCD19
hCD8STK
Tm-ITIM
SH2 (PTPN6)
TEV
RQR8
CD4tm-Endox1
tevS
GAL4-VP16-NLS
Transcription factor binding
Fluc_x1
promoter
reporter

FIG. 6

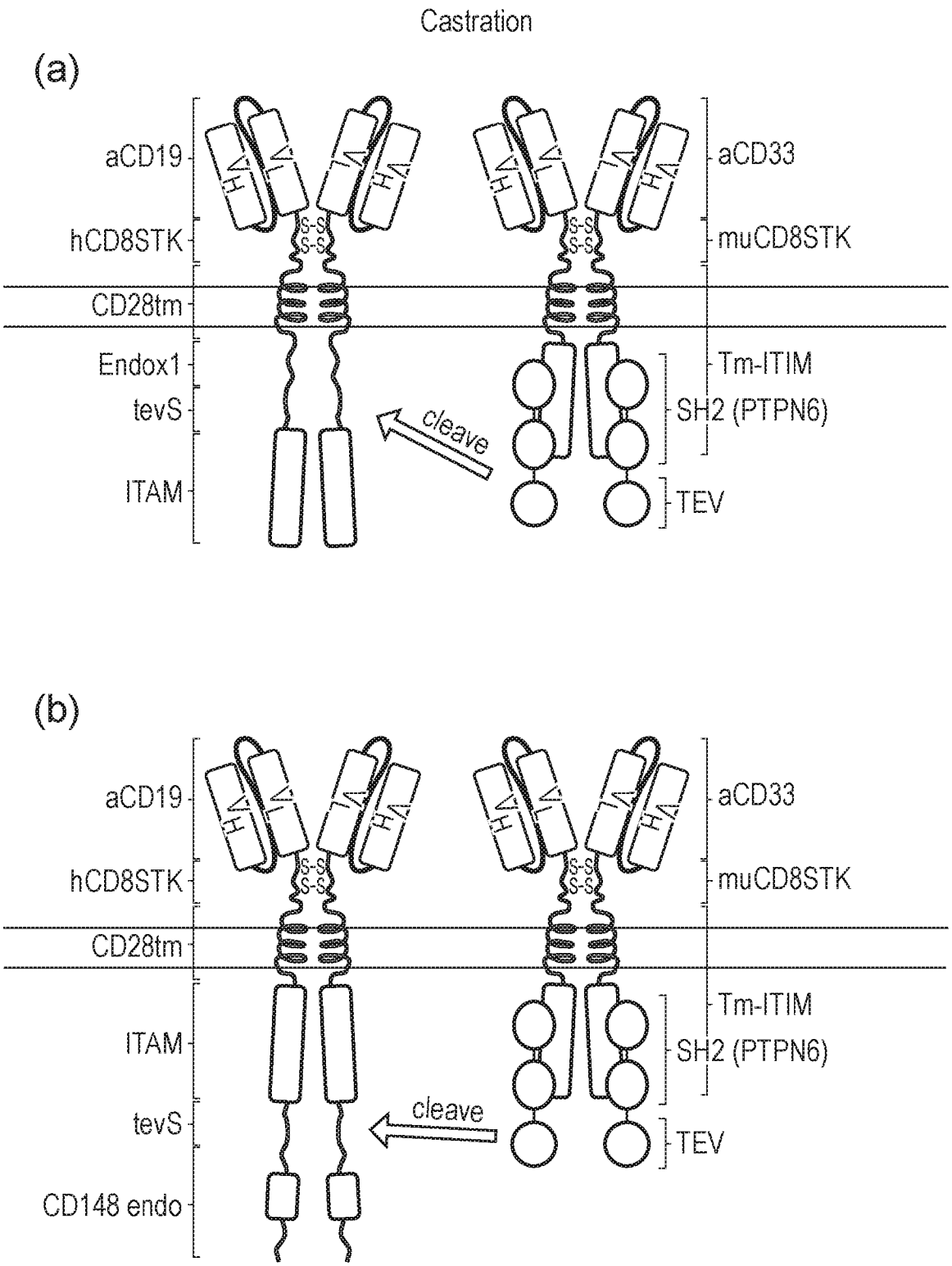
Castration
(a)
aCD19
hCD8STK
CD28tm
Endox1
tevS
ITAM
cleave
aCD33
muCD8STK
Tm-ITIM
SH2 (PTPN6)
TEV
(b)
aCD19
hCD8STK
CD28tm
ITAM
tevS
CD148 endo
cleave
aCD33
muCD8STK
Tm-ITIM
SH2 (PTPN6)
TEV

FIG. 7

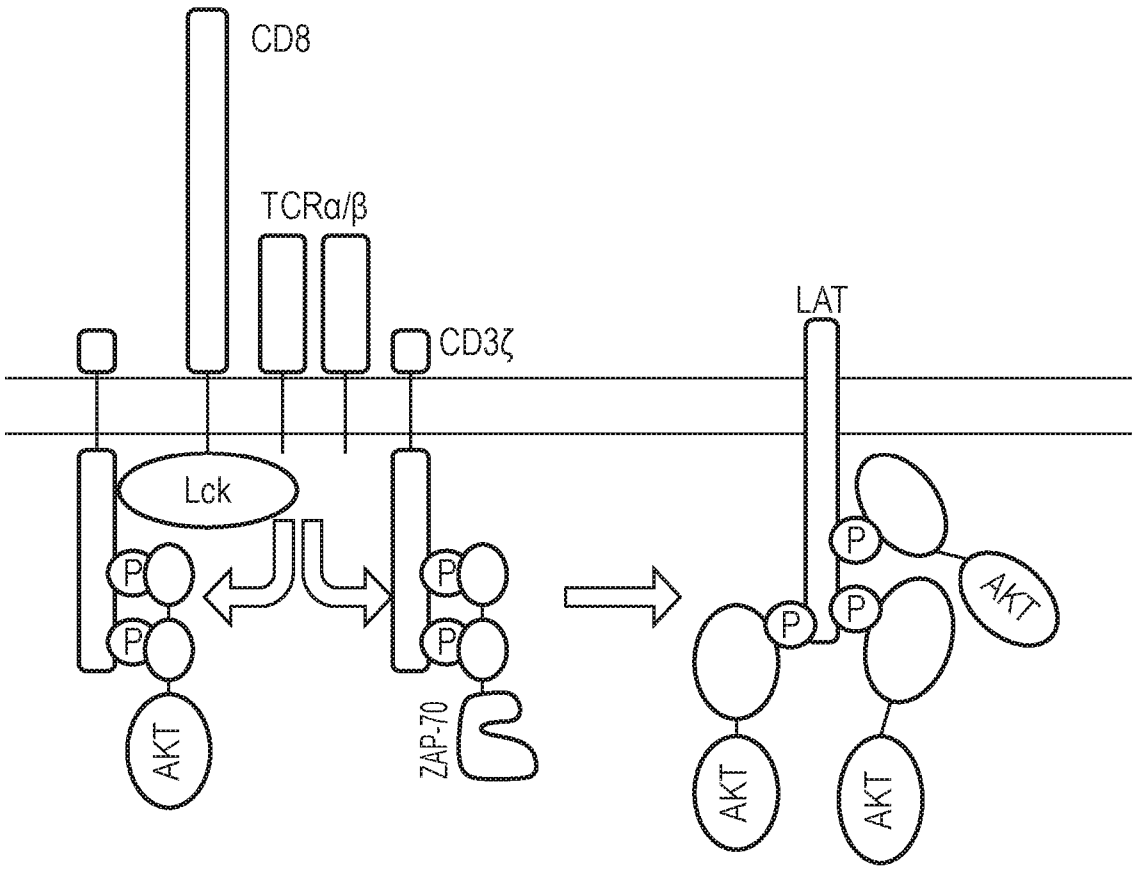

FIG. 8

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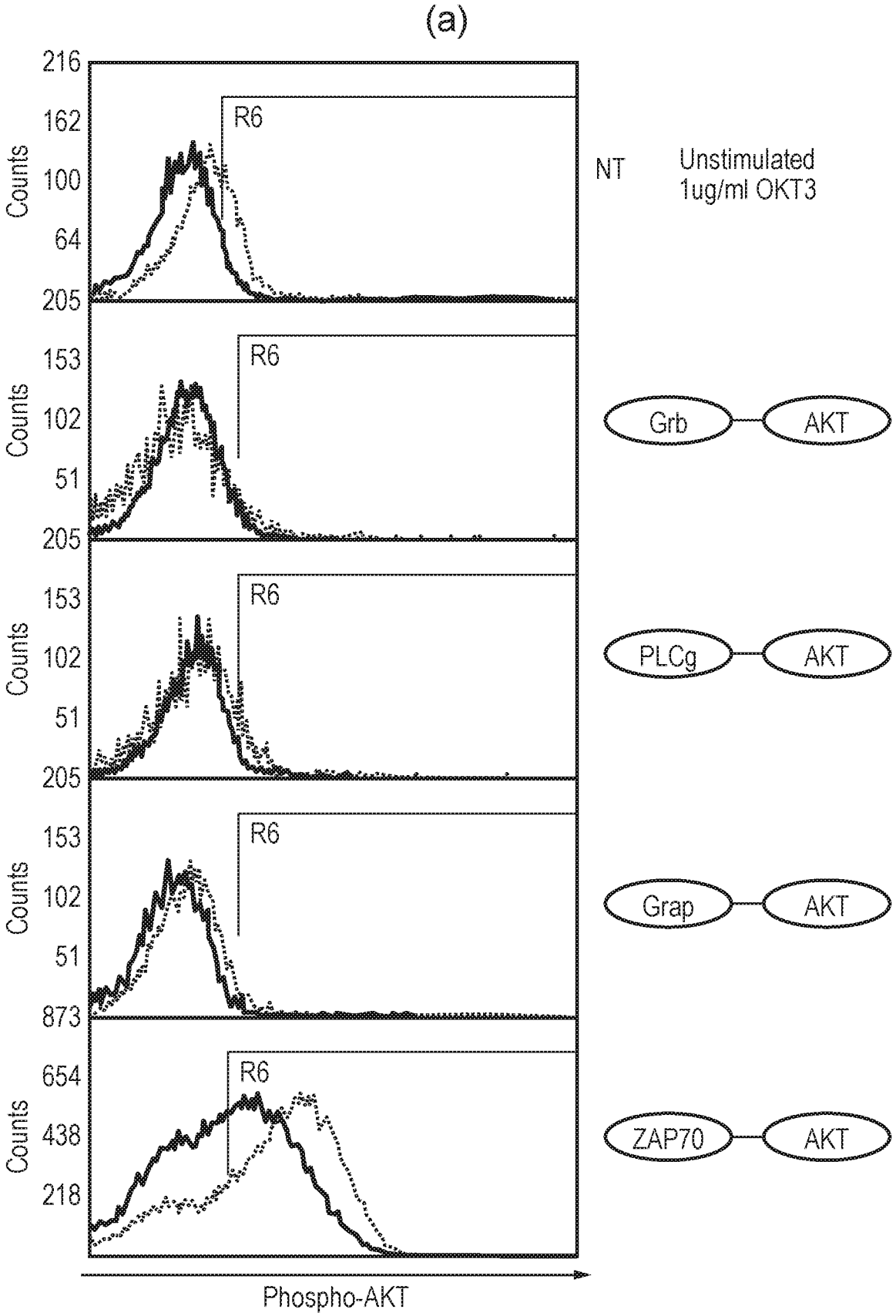
(a)
Counts
216
162
100
64
205
R6
NT
Unstimulated
1ug/ml OKT3
153
102
51
205
Grb
AKT
PLCg
873
Grap
654
438
218
ZAP70
Phospho-AKT

FIG. 9

(b)

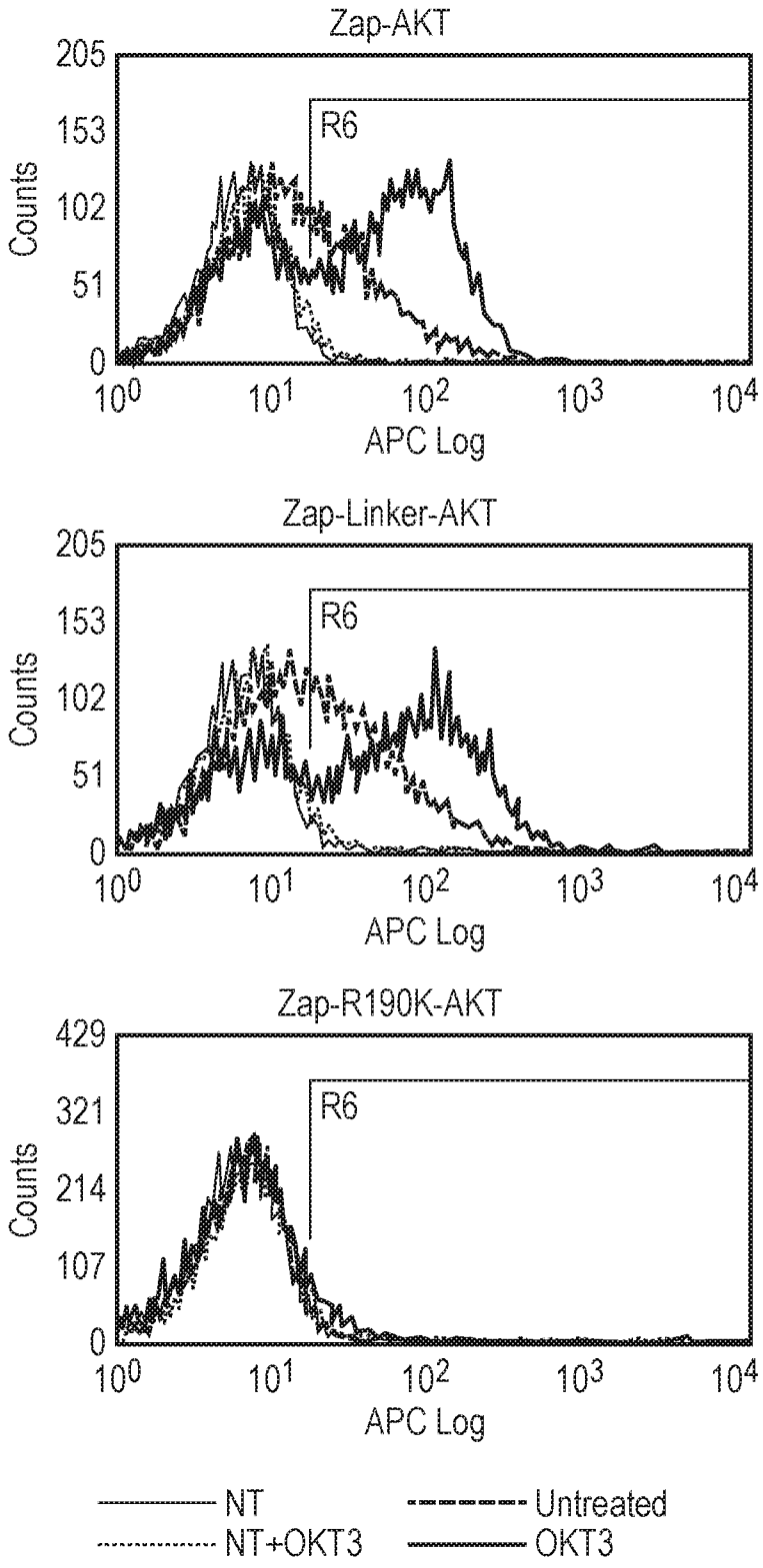

FIG. 9 (Continued)

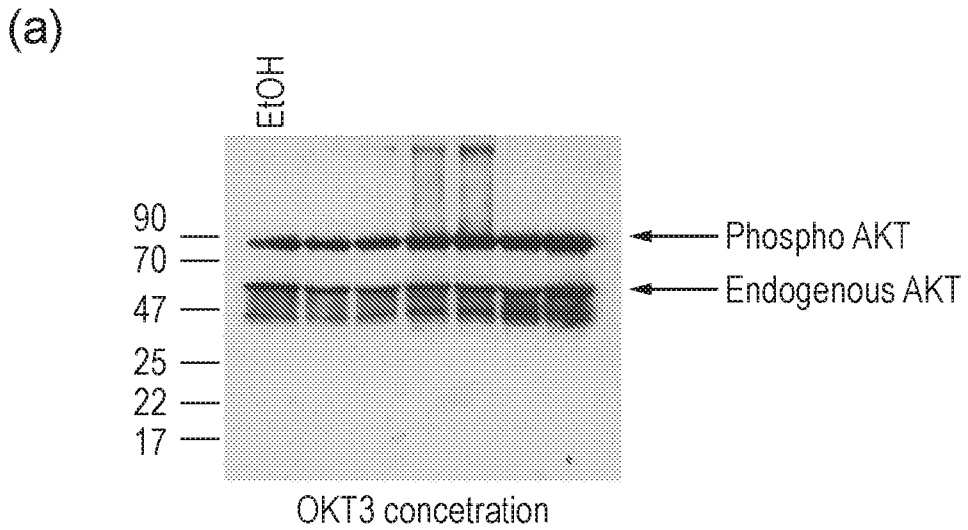
(a)
EtOH
90
70
47
25
22
17
Phospho AKT
Endogenous AKT
OKT3 concetration
(b)
+IL2
+OKT3
+OKT3
NT
ZAP-AKT

FIG. 10

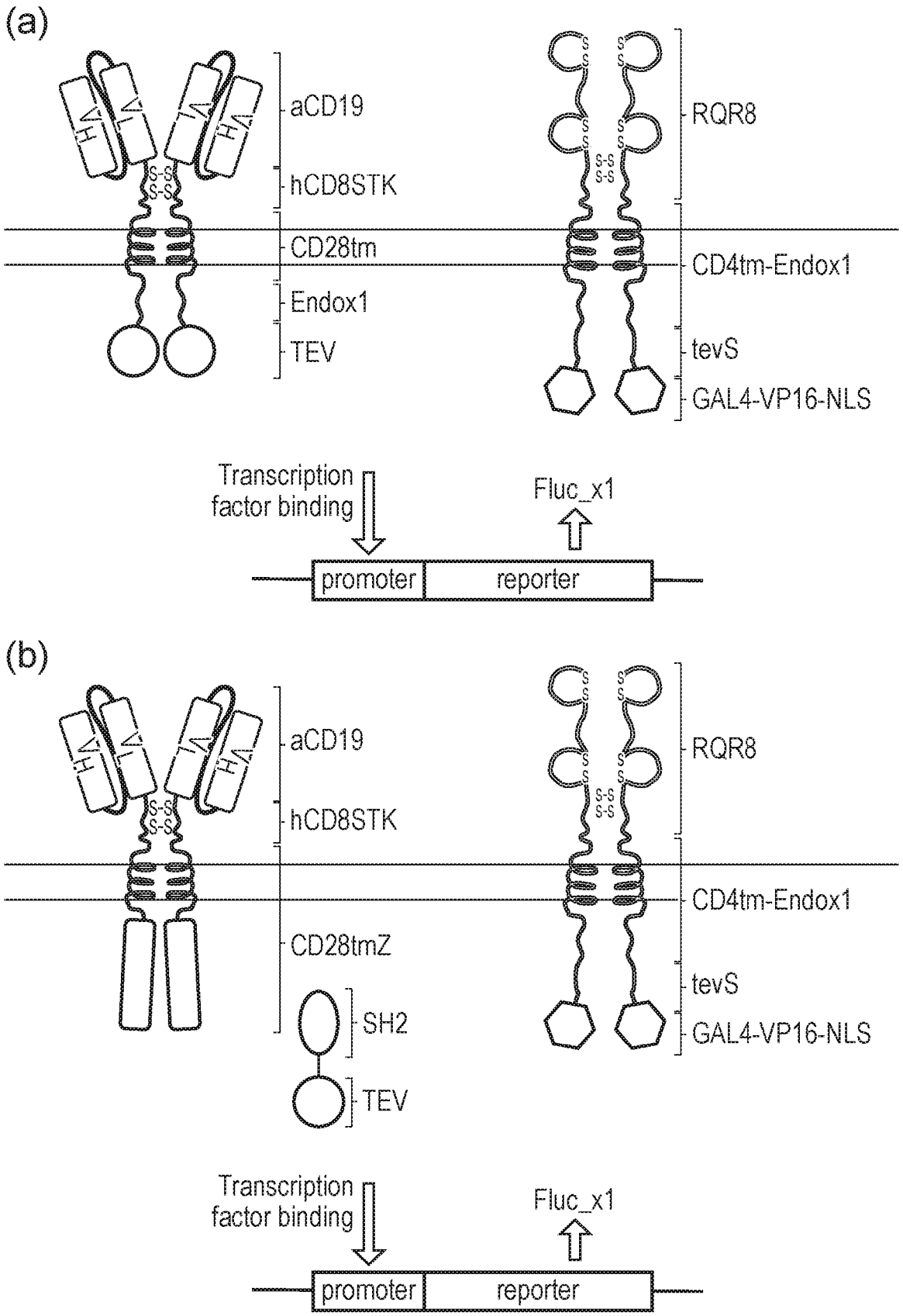
(a)
aCD19
hCD8STK
CD28tm
Endox1
TEV
RQR8
CD4tm-Endox1
tevS
GAL4-VP16-NLS
Transcription factor binding
Fluc_x1
promoter
reporter
(b)
aCD19
hCD8STK
CD28tmZ
SH2
TEV
RQR8
CD4tm-Endox1
tevS
GAL4-VP16-NLS
Transcription factor binding
Fluc_x1
promoter
reporter

FIG. 11

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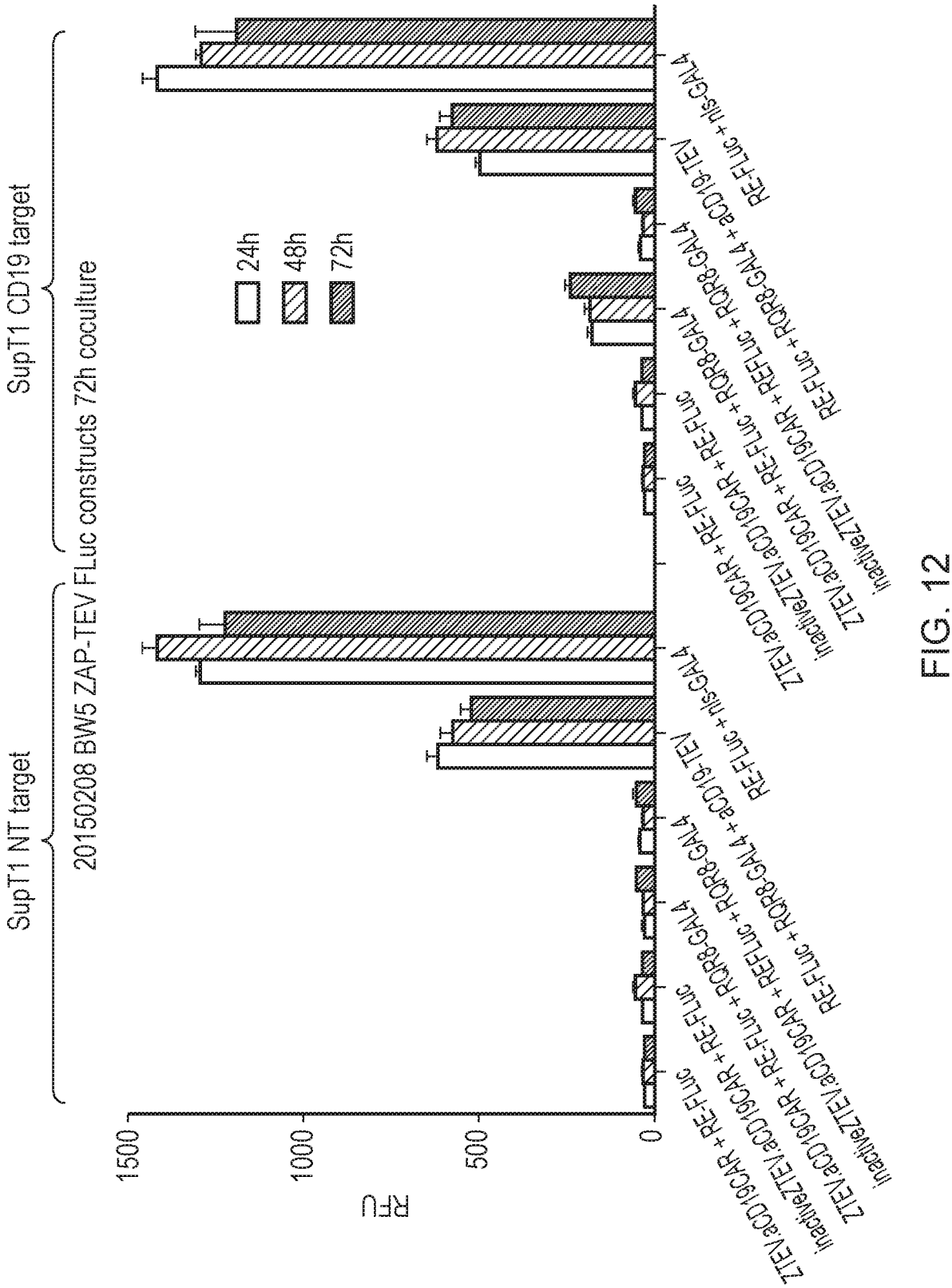
SupT1 NT target
SupT1 CD19 target
20150208 BW5 ZAP-TEV FLuc constructs 72h coculture
24h
48h
72h
RFU
1500
1000
500
0
RE-FLuc + his-GAL4
RE-FLuc + RORβ-GAL4 + aCD19-TEV
inactiveZTEV.aCD19CAR + RE-FLuc + RORβ-GAL4
ZTEV.aCD19CAR + RE-FLuc + RORβ-GAL4
inactiveZTEV.aCD19CAR + RE-FLuc
ZTEV.aCD19CAR + RE-FLuc
RE-FLuc + his-GAL4
RE-FLuc + RORβ-GAL4 + aCD19-TEV
inactiveZTEV.aCD19CAR + RE-FLuc + RORβ-GAL4
ZTEV.aCD19CAR + RE-FLuc + RORβ-GAL4
inactiveZTEV.aCD19CAR + RE-FLuc
ZTEV.aCD19CAR + RE-FLuc

FIG. 12

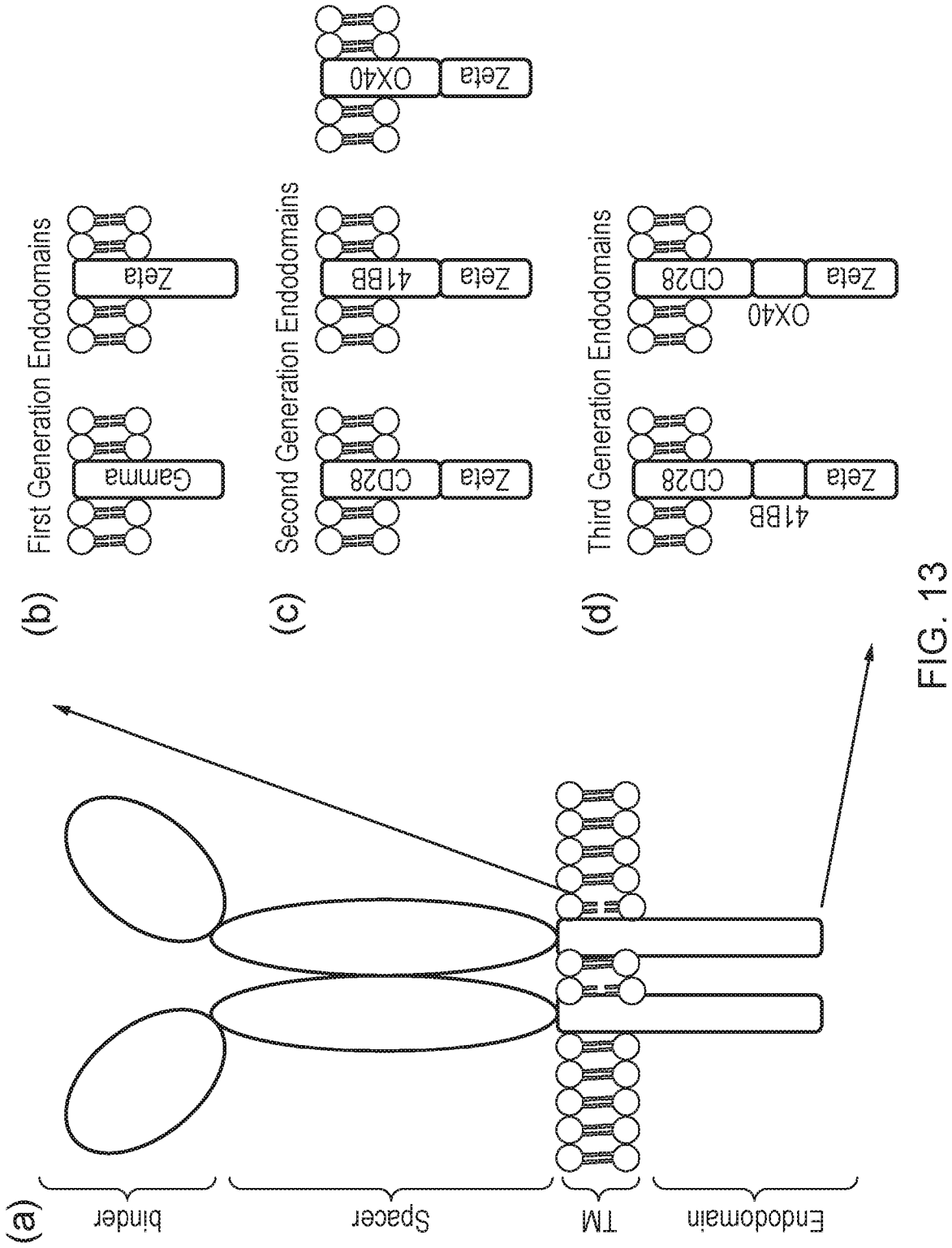
(a)
binder
Spacer
TM
Endodomain
(b)
First Generation Endodomains
Zeta
Gamma
(c)
Second Generation Endodomains
Zeta
4 1BB
Zeta
CD28
Zeta
OX40
(d)
Third Generation Endodomains
Zeta
CD28
OX40
Zeta
CD28
4 1BB

FIG. 13

>dZAP_SH2 (SEQ ID No. 2)
MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAI
AGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALE
QAIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHY
LISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHP

MPD – ZAP70 SH2 domain

>dPTPN6_SH2 (SEQ ID No. 6)
MVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKF
ATLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRES
LSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYL
RQPYY

MVR – PTPN6 SH2 domain

>ZAP_SH2-PTPN6 (SEQ ID No. 30)
MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAI
AGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALE
QAIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHY
LISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPSGG
GGSGGGGSGGGGSGGGGSFWEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGRD
SNIPGSDYINANYIKNQLLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRNKC
VPYWPEVGMQRAYGPYSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGVPSEPGGV
LSFLDQINQRQESLPHAGPIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMVQ
TEAQYKFIYVAIAQFIETTKKKL

MPD – ZAP70 SH2 domain
SGG - Linker
FEW - PTPN6 kinase

>PTPN6_SH2-ZAP (SEQ ID No. 29)
MVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKF
ATLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRES
LSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYL
RQPYYSGGGGSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMRKKQIDVAIKVLKQGTEKAD
TEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFLVGKREEIPVSNVAELLHQVS
MGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARSAGKWPLKWYAPECINFRK
FSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPELYALMSDCWIYKWED
RPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

MVR - PTPN6 SH2 domain
SGG - Linker

FIG. 14

DPE – ZAP70 kinase

>ZETA-ZAP (SEQ ID No. 14)

MRRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKD
KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRSGGGGSGGGGSGGGGSGGG
GSMPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTY
AIAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEAL
EQAIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYH
YLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQR
RIDTLNSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQ
GVYRMRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGP
LHKFLVGKREEIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDS
YYTARSAGKWPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRM
ECPPECPPELYALMSDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

MRR - CD3Zeta endodomain
SGG - Linker
MPD - full-length ZAP70

> PD1_endo-PTPN6 (SEQ ID No. 25)

MTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPR
SAQPLRPEDGHCSWPLSGGGGSGGGGSGGGGSGGGGSMVRWFHRDLSGLDAETLLKGRGVHGSFLA
RPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLTELVEYYTQQQGVLQDRDGTIIHLKYPL
NCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFVLSVLSDQPKAGPGSPLRVTHIKV
MCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPYYATRVNAADIENRVLELNKKQESEDTA
KAGFWEEFESLQKQEVKNLHQRLEGQRPENKGKNRYKNILPFDHSRVILQGRDSNIPGSDYINANYIKNQ
LLGPDENAKTYIASQGCLEATVNDFWQMAWQENSRVIVMTTREVEKGRNKCVPYWPEVGMQRAYGP
YSVTNCGEHDTTEYKLRTLQVSPLDNGDLIREIWHYQYLSWPDHGVPSEPGGVLSFLDQINQRQESLPHA
GPIIVHCSAGIGRTGTIIVIDMLMENISTKGLDCDIDIQKTIQMVRAQRSGMVQTEAQYKFIYVAIAQFIETT
KKKLEVLQSQKGQESEYGNITYPPAMKNAHAKASRTSSKHKEDVYENLHTKNKREEKVKKQRSADKEKSK
GSLKRK

MTG - PD1 endodomain
SGG - Linker
MVR - full length PTPN6

>CD28-ZAP (SEQ ID No. 40)

MRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSSGGGGSGGGGSGGGGSGGGGSMPD
PAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGG
KAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQAIIS
QAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHYLISQ
DKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTL
NSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYR
MRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKF
LVGKREEIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTA

FIG. 14 (continued)

RSAGKWPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPP
ECPPELYALMSDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

MRS - CD28 endodomain
SGG - Linker
MPD - full-length ZAP70

>41BB-ZAP (SEQ ID No. 41)

MKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELSGGGGSGGGGSGGGGSGGGGSMPD
PAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGG
KAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQAIIS
QAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHYLISQ
DKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTL
NSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYR
MRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKF
LVGKREEIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTA
RSAGKWPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPP
ECPPELYALMSDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

MKR - 41BB endodomain
SGG - Linker
MPD - full-length ZAP70

>OX40-ZAP (SEQ ID No. 42)

MRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKISGGGGSGGGGSGGGGSGGGGSMPDPAAHLP
FFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGGKAHCGP
AELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQAIISQAPQV
EKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHYLISQDKAGKY
CIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTLNSDGYT
PEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMRKKQI
DVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFLVGKRE
EIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARSAGK
WPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPE
LYALMSDCWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA

MRD - OX40 endodomain
SGG - Linker
MPD - full-length ZAP70

>CD28-PTPN6_SH2 (SEQ ID No. 43)

MRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSSGGGGSGGGGSGGGGSGGGGSMVR
WFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLT
ELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLSQP
GDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPY

FIG. 14 (continued)

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MRS - CD28 endodomain
SGG - Linker
MVR - amino-terminus of PTPN6 SH2 domain

>41BB-PTPN6_SH2 (SEQ ID No. 44)
MKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELSGGGGSGGGGSGGGGSGGGGSGGGGSMVR
WFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLT
ELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLSQP
GDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPY

MKR - 41BB endodomain
SGG - Linker
MVR - amino-terminus of PTPN6 SH2 domain

>OX40-PTPN6_SH2 (SEQ ID No. 45)
MRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKISGGGGSGGGGSGGGGSGGGGSGGGGSMVRWFHRD
LSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKFATLTELVEYY
TQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRESLSQPGDFVL
SVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYLRQPY

MRD - OX40 endodomain
SGG - Linker
MVR - amino-terminus of PTPN6 SH2 domain

>ZAP_SH2-AKT (SEQ ID No. 47)
MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAI
AGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALE
QAIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHY
LISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPAEE
MEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENRVLQN
SRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLK
LENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMM
CGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFAGIVWQ
HVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDSERRPHFPQFSYSASGTA

MPD - ZAP70 SH2 domain
AEE - AKT kinase

>ZAP_SH2-L-AKT (SEQ ID No. 48)
fused with via a linker
MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAI
AGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALE
QAIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHY
LISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPSGG
GGSGGGGSGGGGSGGGGSAEEMEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKIL

FIG. 14 (continued)

KKEVIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARF
YGAEIVSALDYLHSEKNVVYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLED
NDYGRAVDWWGLGVVMYEMMCGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQR
LGGGSEDAKEIMQHRFFAGIVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMEC
VDSERRPHFPQFSYSASGTA

MPD - ZAP70 SH2 domain
SGG - Linker
AEE - AKT kinase domain

>ZAPnf-AKT (SEQ ID No. 49)

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAI
AGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALE
QAIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLKPRKEQGTYALSLIYGKTVYHY
LISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPAEE
MEVSLAKPKHRVTMNEFEYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENRVLQN
SRHPFLTALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLK
LENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMM
CGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRFFAGIVWQ
HVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDSERRPHFPQFSYSASGTA

MPD - ZAP70 mutated to be non-functional
AEE - AKT kinase

>ZAP_SH2-TEV (SEQ ID No. 53)

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQLNGTYAI
AGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALE
QAIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYALSLIYGKTVYHY
LISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLTHPSGG
GGSGGGGSGGGGSGGGGSSLFKGPRDYNPISSTICHLTNESDGHTTSLYGIGFGPFIITNKHLFRRNNGTLI
LVQSLHGVFKVKNTTTLQQHLIDGRDMIIIRMPKDFPPFPQKLKFREPQREERICLVTTNFQTKSMSSMVS
DTSCTFPSSDGIFWKHWIQTKDGQCGSPLVSTRDGFIVGIHSASNFTNTNNYFTSVPKNFMELLTNQEAQ
QWVSGWRLNADSVLWGGHKVFMSKPEEPFQPVKEATQLMNELVYSQ

MPD - ZAP70 SH2 domain
SLF - TEV protease

>PTPN6_SH2-TEV (SEQ ID No. 54)

MVRWFHRDLSGLDAETLLKGRGVHGSFLARPSRKNQGDFSLSVRVGDQVTHIRIQNSGDFYDLYGGEKF
ATLTELVEYYTQQQGVLQDRDGTIIHLKYPLNCSDPTSERWYHGHMSGGQAETLLQAKGEPWTFLVRES
LSQPGDFVLSVLSDQPKAGPGSPLRVTHIKVMCEGGRYTVGGLETFDSLTDLVEHFKKTGIEEASGAFVYL
RQPYYSGGGGSSLFKGPRDYNPISSTICHLTNESDGHTTSLYGIGFGPFIITNKHLFRRNNGTLLVQSLHGVF
KVKNTTTLQQHLIDGRDMIIIRMPKDFPPFPQKLKFREPQREERICLVTTNFQTKSMSSMVSDTSCTFPSS
DGIFWKHWIQTKDGQCGSPLVSTRDGFIVGIHSASNFTNTNNYFTSVPKNFMELLTNQEAQQWVSGW
RLNADSVLWGGHKVFMSKPEEPFQPVKEATQLMNELVYSQ

FIG. 14 (continued)

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MVR - PTPN6 SH2 domain
SGG - Linker
SLH - TEV protease

>RQR8-CD4tm-Endox1-tevS-VP16-GAL4 (SEQ ID No. 55)
MGTSLLCWMALCLLGADHADACPYSNPSLCSGGGGSELPTQGTFSNVSTNVSPAKPTTTACPYSNPSLC
SGGGGSPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDMALIVLGGVAGLLLFIGLGIFFC
VRCRHRRRQAERMAQIKRVVSEKKTAQAPHRFQKTCSPSGGGGSENLYFQMPKKKRKVAPPTDVSLGD
ELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYG
GSGGGSMQILVASDATMKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLT
EVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNVNKDAVTDRLASVETDMPLTLRQHR
ISATSSSEESSNKGQRQLTV

MGT – RQR8 domain
RCR - CD4tm
SGG - Linker
ENL – TEV recognition site
PKK - VP16
MQI - GAL4

>aCD33-muCD8STK-PD1_tm_endo (SEQ ID No. 56)
MAVPTQVLGLLLLWLTDARCDIQMTQSPSSLSASVGDRVTITCRASEDIYFNLVWYQQKPGKAPKLLIYD
TNRLADGVPSRFSGSGSGTQYTLTISSLQPEDFATYYCQHYKNYPLTFGQGTKLEIKRSGGGGSGGGGSG
GGGSGGGGSRSEVQLVESGGGLVQPGGSLRLSCAASGFTLSNYGMHWIRQAPGKGLEWVSSISLNGGS
TYYRDSVKGRFTISRDNAKSTLYLQMNSLRAEDTAVYYCAAQDAYTGGYFDYWGQGTLVTVSSMDPAIT
TKPVLRTPSPVHPTGTSQPQRPEDCRPRGSVKGTGLDFACDIYVGVVGGLLGSLVLLVWVLAVICSRAAR
GTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGS
ADGPRSAQPLRPEDGHCSWPL

MAV – anti-CD33
ITT - Mouse CD8alpha stalk
VGV – PD-1 endodomain

>aCD19-hCD8STK-CD28tm-Endox1-tevS-Z (SEQ ID No. 57)
CAR against CD19 with cleavable Zeta endodomain
MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHT
SRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITKAGGGGSGGGGSGGG
GSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSAL
KSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTP
APTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRCRHRRRQ
AERMAQIKRVVSEKKTAQAPHRFQKTCSPSGGGGSENLYFQMRRVKFSRSADAPAYQQGQNQLYNEL
NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ
GLSTATKDTYDALHMQALPPR

FIG. 14 (continued)

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MSL – anti-CD19
PTT - CD8alpha stalk
FWV - CD28 transmembrane domain
RCR - Endox1
ENL – TEV recognition site
RRV – CD3 zeta endodomain

>aCD19-hCD8STK-CD28tmZ-tevS-CD148endo (SEQ ID No. 58)
CAR against CD19 with Zeta endodomain and cleavable CD148 catalytic domain

MSLPVTALLLPLALLLHAARPDIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHT
SRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEITKAGGGGSGGGGSGGG
GSGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNSAL
KSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSSDPTTTPAPRPPTP
APTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSA
DAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG
MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRENLYFQMAVFGCIFGALVIVTVGGFIFWRKK
RKDAKNNEVSFSQIKPKKSKLIRVENFEAYFKKQQADSNCGFAEEYEDLKLVGISQPKYAAELAENRGKNR
YNNVLPYDISRVKLSVQTHSTDDYINANYMPGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAIIMLT
KCVEQGRTKCEEYWPSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHPLRQFHFTSWPDHGV
PDTTDLLINFRYLVRDYMKQSPPESPILVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYDLRMHRPL
MVQTEDQYVFLNQCVLDIVRSQKDSKVDLIYQNTTAMTIYENLAPVTTFGKTNGYIA

MSL - anti-CD19
PTT - CD8alpha stalk
FWV - CD28 transmembrane domain
RRV - CD3 zeta endodomain
ENL - TEV recognition site
AVF - CD148 endodomain

>dualSH2 SHP-1-ZAP70 kinase (SEQ ID No. 61)

WFHPNITGVEAENLLLTRGVDGSFLARPSKSNPGDFTLSVRRNGAVTHIKIQNTGDYYDLYG
GEKFATLAELVQYYMEHHGQLKEKNGDVIELKYPLNCADPTSERWFHGHLSGKEAEKLLTE
KGKHGSFLVRESQSHPGDFVLSVRTGDDKGESNDGKSKVTHVMIRCQELKYDVGGGERFD
SLTDLVEHYKKNPMVETLGTVLQLKQPLNTTRINPNSSASNASGAAAPTLPAHPSTLTHPQR
RIDTLNSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGC
GNFGSVRQGVYRMRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLIGVCQAE
ALMLVMEMAGGGPLHKFLVGKREEIPVSNVAELLHQVSMGMKYLEEKNFVHRDLAARNVLL
VNRHYAKISDFGLSKALGADDSYYTARSAGKWPLKWYAPECINFRKFSSRSDVWSYGVTM
WEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPELYALMSDCWIYKWEDRPDFLTV
EQRMRACYYSL

WFH - First SH2 domain from SHP-2
NCA - Linker
WFH - Second SH2 domain from SHP-2
NTT - Linker
LIA - Zap-70 protein kinase

>-dualSH2 SHP-2-Akt Kinase (SEQ ID No. 62)

WFHPNITGVEAENLLLTRGVDGSFLARPSKSNPGDFTLSVRRNGAVTHIKIQNTGDYYDLYG
GEKFATLAELVQYYMEHHGQLKEKNGDVIELKYPLNCADPTSERWFHGHLSGKEAEKLLTE
KGKHGSFLVRESQSHPGDFVLSVRTGDDKGESNDGKSKVTHVMIRCQELKYDVGGGERFD

FIG. 14 (continued)

SLTDLVEHYKKNPMVETLGTVLQLKQPLNTTRINAEEMEVSLAKPKHRVTMNEFEYLKLLGK
GTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENRVLQNSRHPFLTALKYSFQTHD
RLCFVMEYANGGELFFHLSRERVFSEDRARFYGAEIVSALDYLHSEKNVVYRDLKLENLMLD
KDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAVDWWGLGVVMYEMMC
GRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQRLGGGSEDAKEIMQHRF
FAGIVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITITPPDQDDSMECVDSERRPHF
PQFSYSASGTA

WFH - First SH2 domain from SHP-2
NCA - Linker
WFH - Second SH2 domain from SHP-2
NTT - Linker
FEY - AKT protein kinase
RFF – Linker
AGI - AKT AGC-kinase C-terminal

FIG. 14 (continued)

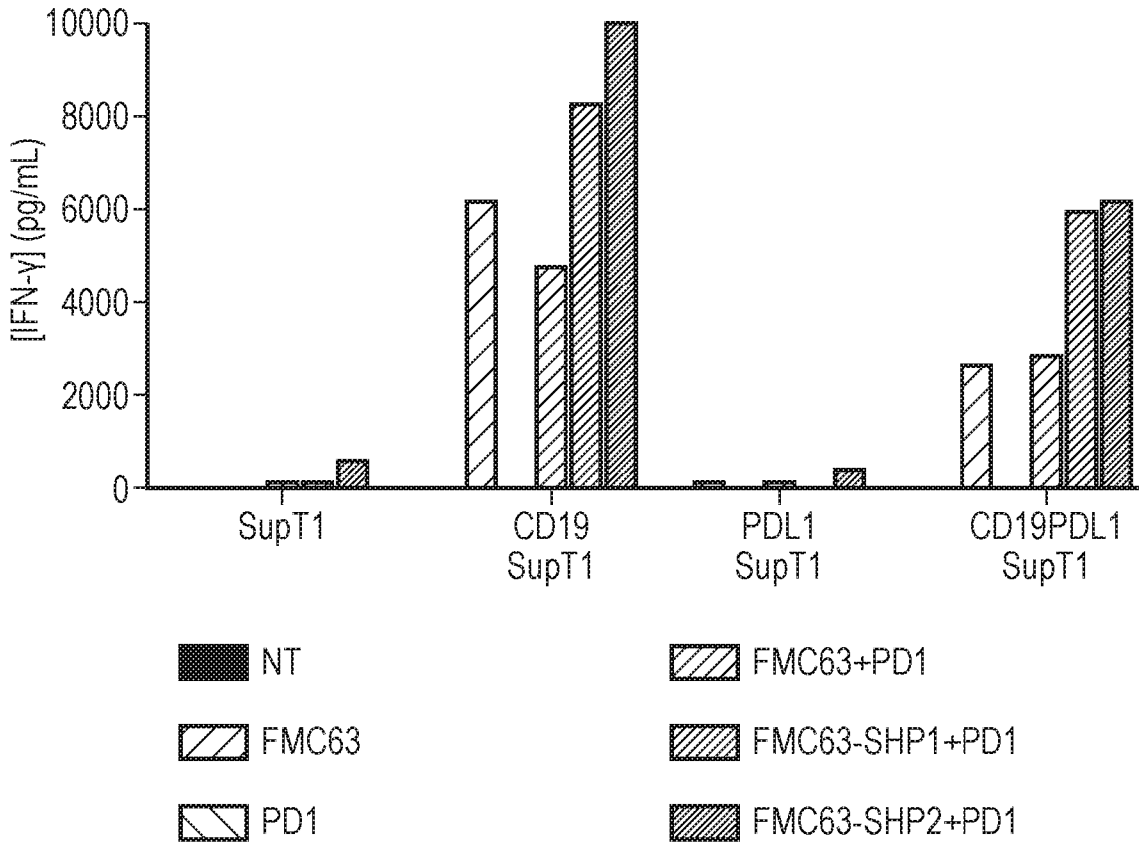

FIG. 15

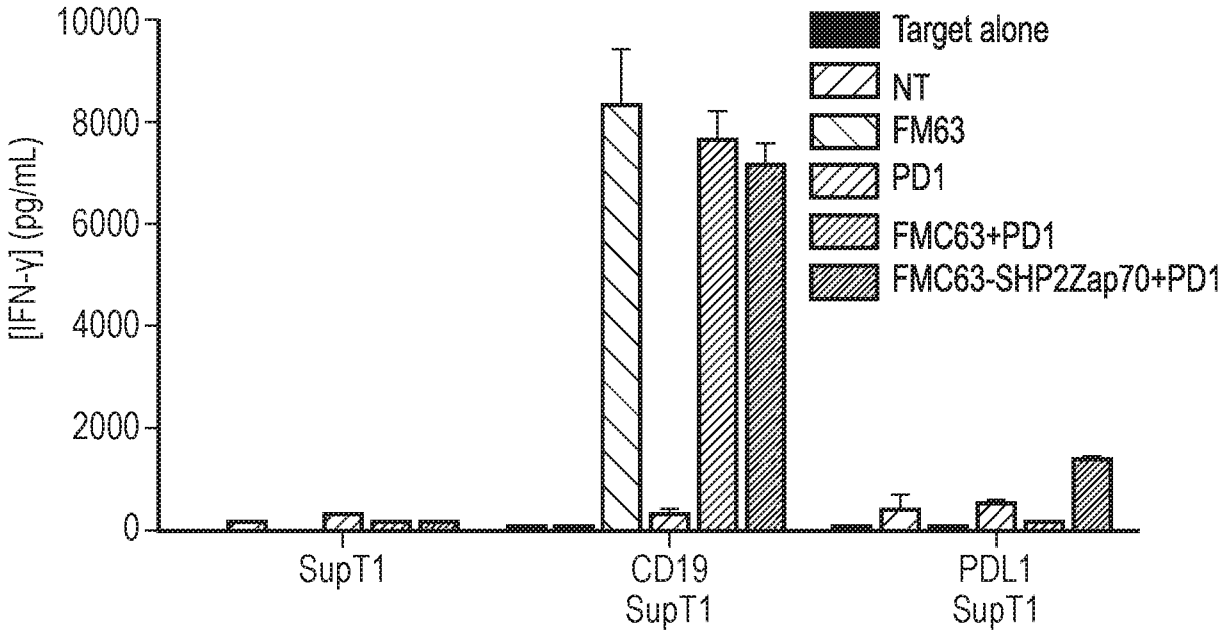

FIG. 16A

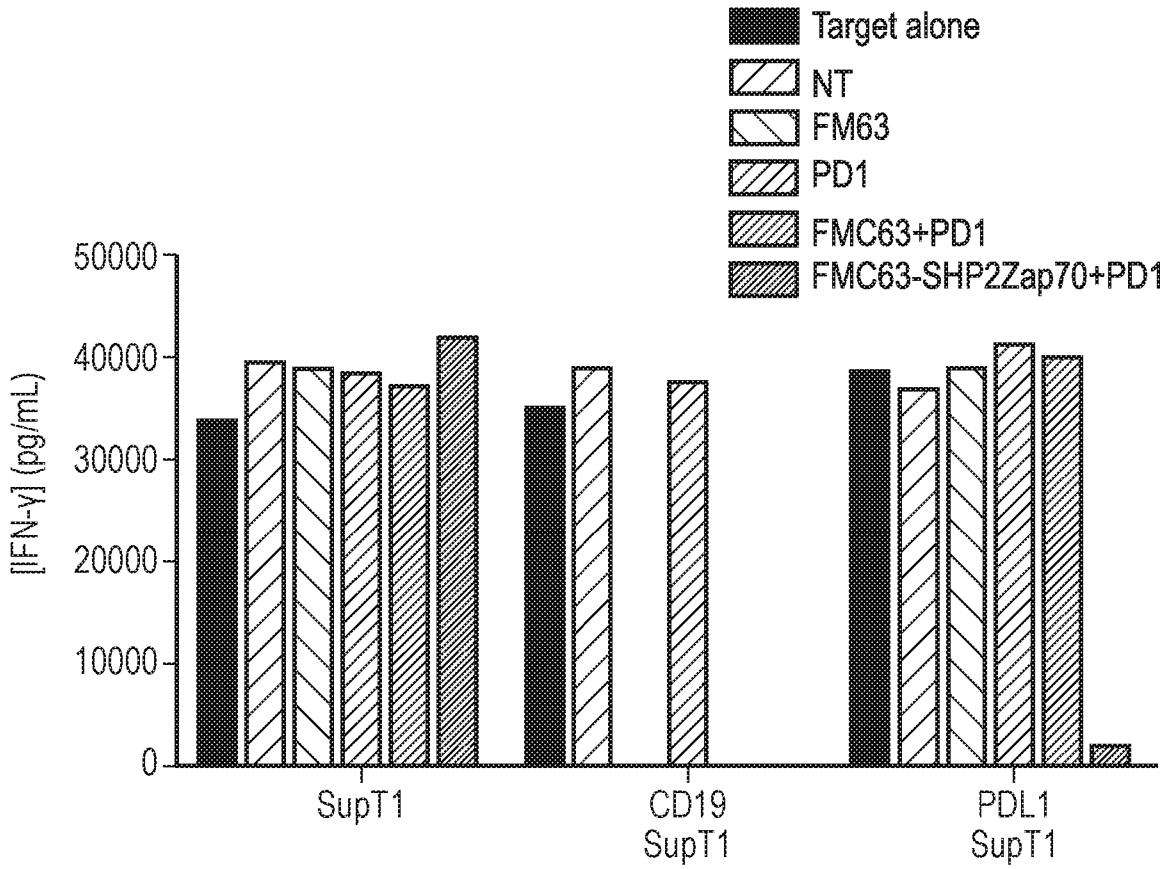

FIG. 16B