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(54) Title: METHOD FOR MODULATING CAR-INDUCED IMMUNE CELLS ACTIVITY

(57) Abrégé/Abstract:
The present invention relates to a method to modulate the level of activation of an engineered immune cell (such as a Chimeric Antigen Receptor T-cell) for immunotherapy. The present invention also relates to cells obtained by the present method, preferably comprising said modulable/tunable chimeric antigen receptors for use in therapeutic or prophylactic treatment.

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(54) **Title**: METHOD FOR MODULATING CAR-INDUCED IMMUNE CELLS ACTIVITY

(57) **Abstract**: The present invention relates to a method to modulate the level of activation of an engineered immune cell (such as a Chimeric Antigen Receptor T-cell) for immunotherapy. The present invention also relates to cells obtained by the present method, preferably comprising said modulable/tunable chimeric antigen receptors for use in therapeutic or prophylactic treatment.

METHOD FOR MODULATING CAR-INDUCED IMMUNE CELLS ACTIVITY

Field of the description

The present invention relates to a method to modulate the transduction signal of a CAR (chimeric antigen receptor) the level of this transduction signal determining the level of activation of an engineered immune cell,such as a Chimeric Antigen Receptor T-cell used in immunotherapy. In particular, the inventors have developed a molecular switch system and new CAR architectures allowing a tunable activation of said cells upon administration of a soluble compound that induces co-localization of inhibitory and activating signaling domains, especially via chemical induced dimerization (CID). Consequently, the activation of an engineered immune cell triggered by a Chimeric Antigen Receptor (CAR), can be monitored or shunted via inhibitory signaling domain(s) on demand. The invention opens the way to a prospect of a tunable and safer adoptive immunotherapy.

Background of the invention

Adoptive immunotherapy, which involves the transfer of autologous antigen-specific T-cells generated *ex vivo*, is a promising strategy to treat cancer. The T-cells used for adoptive immunotherapy can be generated either by expansion of antigen-specific T cells or redirection of T-cells through genetic engineering (Park, Rosenberg et al. 2011). Transfer of viral antigen specific T-cells is a well-established procedure used for the treatment of transplant associated viral infections and rare viral-related malignancies. Similarly, isolation and transfer of tumor specific T-cells has been shown to be successful in treating melanoma. Novel specificities in T-cells have been successfully generated through the genetic transfer of transgenic T cell receptors or chimeric antigen receptors (CARs). CARs are synthetic receptors consisting of a targeting moiety that is associated with one or more signaling domains in a single fusion molecule. CARs have successfully allowed T-cells to be redirected against antigens expressed at the surface of tumor cells from various malignancies including lymphomas and solid tumors (Jena, Dotti et al. 2010).

The first generation of CAR-modified T cell showed success in pre-clinical trials and has entered phase I clinical trials. Clinical trials have commenced in ovarian cancer, neuroblastoma and various types of leukemia and lymphoma (https://clinicaltrials.gov/). The clinical trials showed little evidence of anti-tumor activity with insufficient activation, persistence and homing to cancer tissue.

Diverse studies have reported partial first-generation CARs in the absence of costimulation leads to anergy and failure of *in vivo* expansion.

To overcome these limitations, second and the third generation CAR-modification T cells were designed in order to enhance the activation signal, proliferation, production of cytokines and effector function of CAR-modified T cell in preclinical trials. Second-generation CARs were developed to incorporate the intracellular domains of one or more costimulatory molecules such as CD28, OX40, and 4-1BB within the endodomain, and these improved antigen-specific T-cell activation and expansion. Third-generation CARs include a combination of costimulatory endodomains. Both the second and the third generation CAR-modified T cell have entered clinical trials now. The first clinical trial, which has involved T-cells expressing a CAR combining an anti-CD19binding domain with a 4-1BB costimulatory domain and CD3zeta as an activating signaling domain has led some patients to a complete remission, which has been ongoing 10 months after treatment. The CAR-modified T cells were found to expand 3-logs in these patients, infiltrating and lysing cancer tissue. Interestingly, a fraction of these cells displayed a memory phenotype of T cell for preventive tumor relapses. Although these CAR-modified T cell produced significant therapeutic effect, their activity led to life-threatening tumor lysis 3 weeks after the first infusion of CAR-modified T cell.

Recently adverse events were reported which stress the requirement of special precautions while using second and third generation of CAR-modified T cells. One patient died 5 days after cyclophosphamide chemotherapy followed by infusion of CAR-modified T cells recognizing the antigen ERBB2 (HER-2/neu) (Morgan et al. 2010). The toxicity leads to a clinically significant release of pro-inflammatory cytokines, pulmonary toxicity, multi-organ failure and eventual death of the patient. This and other adverse events highlight the need for caution when employing CAR-modified T cells, as unlike antibodies against tumor-associated antigens, these cells are not cleared from the body within a short amount of time.

There are many on-going researches to develop a safer CAR-based immunotherapy. Several studies reports diverse systems which aim to improve the efficacy and safety of T immunotherapy. T-cell mediated immunity in healthy persons includes multiple sequential steps regulated by a balance between co-stimulatory and inhibitory signals that fine-tune the immunity response. The inhibitory signals referred to as immune checkpoints (such as CTLA-4– or PD-1) are crucial for the maintenance of self-tolerance and also to limit immune-mediated collateral tissue damage (Dolan et al, 2014).

Recently, inhibitory chimeric antigen receptors (iCARs) were designed having as objective to put the brakes on T cell function upon encountering off-target cells. The iCAR is made up of an antigen-specific single-chain variable fragment (scFv) fused to a T cell inhibitory signaling domain. Cells expressing a tumor-associated antigen but not a normal-tissue antigen would induce T cell activation, cytotoxicity and cytokine signaling to kill the on-target cells. In a study (Federov et al. 2013), CTLA-4– or PD-1–based iCARs were shown to selectively limit cytokine secretion, cytotoxicity, and proliferation induced through the endogenous T cell receptor or an activating chimeric receptor. Therefore, to function, the iCAR technology relies on a preliminary selection of 2 antigens: one tumor associated antigen and one normal-tissue antigen. Moreover, the inhibitory effect of PD-1 or CTLA-4 is operating only on off-target cells.

Another system is described in Budde et al. (2013) in which a CD20 Chimeric Antigen Receptor is combined with an inducible caspase 9 (iC9) suicide switch. In the application US 2014/0286987, the latter gene is made functional in the presence of the prodrug AP1903 (tacrolimus) by binding to the mutated FK506-binding protein (FKBP1). A clinical trial is ongoing sponsored by the company Bellicum in which the above capsase technology (CaspaCID™) is engineered into GD2 targeted third generation CAR T cells. Viral transduction transfers DNA from a vector into the target cell and the vector-derived DNA directs expression of chemical induction dimerization (CID) and accessory proteins. In presence of the AP1903 drug, there will be a dimerization of the CID proteins, thus turning on the signal cascade. In the event of a serious of life-threatening toxicity caused by the administered T cells, AP1903 will be infused to trigger rapid destruction and elimination of the CaspaCID™-enabled cells. One important characteristic is that this expression is restricted to the cytoplasm of the cell. Moreover, according to the Bellicum's system, there is no possibility to modulate the activity of the T-cells, since the expression of CaspaCID™ in contact with the drug leads to the death of the T-cells. A similar apoptosis-inducing system based on a multimerizing agent is described in the application WO 2014/152177.

There is a need of a CAR-based immunotherapy technology which is able to inhibit or modulate, by addition of a soluble compound, the activation of chimeric antigen receptor (CAR) immune cells without killing them; which is flexible as the effect of the soluble compound can be either intracellular or extracellular, and which is independent of on/off target cells selection.

The present invention here provides such immunotherapy by which activation can be specifically inhibited/modulated in case of their cytotoxicity (i.e. when needed) by administration of a particular soluble compound.

Summary of the invention

In one aspect, the present invention provides methods for modulating the level of activation of a Chimeric Antigen Receptor-engineered immune cell, such as a CAR-T cell by administration of a soluble compound.

The latter, which may be, in a preferred aspect, a small molecule (such as rapalogs), or a bispecific antibody, triggers a co-localization of at least one binding domain, and consequently enhances a modulation of CAR-engineered immune cell via the action of inhibitory signaling domain(s).

According to one aspect of the invention, an inhibitory membrane protein (IMP) comprising a binding (or dimerization) domain is co-expressed with a CAR into an immune cell. The CAR and the IMP are made both reactive to a soluble compound, especially through a second binding domain comprised within the CAR, thereby allowing the co-localization, by dimerization or ligand recognition, of the inhibitory signaling domain borne by the IMP and of the signal transducing domain borne by the CAR, having the effect of turning down the CAR activation. The inhibitory signaling domain is preferably the programmed death-1 (PD-1), which attenuates T-cell receptor (TCR)-mediated activation of IL-2 production and T-cell proliferation. In Sheppard et al. (2004), it is shown that PD-1 modulation of T-cell function involves inhibition of TCR-mediated phosphorylation through ZAP70 and association with CD3zeta.

The invention provides with several conformations that fall within the scope of the invention - for instance, depending of the type of soluble compound used, either the binding (or dimerization) domains can be extracellular or intracellular (Figures 1 and 2). On another hand, the Chimeric Antigen Receptor (CAR) expressed in the immune cell can be either a multi-chain CAR or a single-chain CAR.

The invention also provides a switch system independent from the selected CAR architecture, which does not necessarily requires inclusion of a second binding or dimerization domain into the CAR. According to this later aspect, the first and second binding (or dimerizing) domains are part of at least two heterologous inhibition membrane proteins (IMPs) expressed at the surface of the immune cells, allowing the dimerization and the activation of an inhibitory signaling domain acting as a (indirect) molecular switch inhibiting CAR induced activation. An example of such switch involves the IL-10

pathway as shown in Figure 3. The dimerization inducer such as rapamycin allows the co-localization of the two dimerization domains. Once dimerized, IL-10R inhibits synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNFα and GM-CSF. It appears that this inhibition is mediated by a cascade of reactions involving several intermediates, such as receptor-associated Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) kinases, leading to tyrosine phosphorylation of STAT proteins as described in Finbloom et al. (1995).

Also, the present invention encompasses the isolated cells or cell lines obtainable by the method of the invention, more particularly isolated immune cells comprising or expressing any of the proteins, polypeptides, allelic variants, altered or deleted genes or vectors described herein. According to previous developments carried out by the applicants and described in WO 2013176915, the immune cells of the present invention or cell lines can be further engineered for allogeneous implantation into patients, for instance by disrupting T-cell receptors.

Following from the above, a further aspect of the invention concerns methods for treating or preventing conditions, such as cancer, where CAR induced immune cells are useful for targeting pathological cells in a patient, and where there is a need to keep the immune cell proliferation under control using a soluble compound.

Brief description of the figures and tables

Figure 1: Schematic representation of an extracellular based chemical induced dimerization (CID) strategy using PD-1 as inhibitory signaling domain, when both binding domains of the inhibitory membrane protein (IMP) complex and the multi-chain Chimeric Antigen Receptor (CAR) are localized extracellularly.

The system is composed of 2 parts: a first one comprises a polypeptide called inhibitory membrane protein (IMP) and the second one corresponds to a Chimeric Antigen Receptor (CAR). The IMP is a transmembrane protein with an extracellular moiety which is a dimerization domain capable to bind to a chemical inducer dimerization (CID) agent, and with an intracellular moiety which contains an inhibitory signaling domain (PD1 here).

The CAR represented in the drawing is a multichain CAR (mcCAR here) having 3 chains (α, β and γ) derived from Fc Receptor as described in WO 2014039523. The α chain bears a scFv binding domain recognizing a surface marker ligand, the β chain bears a co-stimulatory domain (41-BB here)

and the γ chain bears a intracellular transduction signaling domain (CD3ζ here). Figure 1A shows dimerization domains borne on the extracellular part of the γ chain; Figure 1B shows a dimerization domain borne on the extracellular part of the α chain.

When the immune cell expressing the IMP molecule and the mcCAR is not in contact with a CID agent, the mcCAR can function in a normal way, i.e. *in fine* destroy the target cells which are recognized by the scFv specific moiety. In case of an inappropriate or excessive action of the mcCAR, CID agent (rapamycin here) may be administered to the patient in order to trigger the modulable inhibition switch such as explained thereafter.

In both configurations (Figure 1A and Figure 1B), the presence of rapamycin allows a co-localization of the 2 dimerization domains (one on IMP molecule and the other on α- or γ- chain of mcCAR), i.e. these 2 dimerization domains come closer each other. By this action, the inhibitory signaling domain PD1 colocalizes with the transduction signaling domain CD3ζ of the mcCAR. A dephosphorylation of the latter enhances a reactions cascade starting from CD3ζ and ultimately leads to an inhibition of the mcCAR.

Figure 2: Schematic representation of an intracellular based chemical induced dimerization (CID) strategy using PD-1 as inhibitory signaling domain, when both binding domains of the inhibitory membrane protein (IMP) complex and the multi-chain Chimeric Antigen Receptor (CAR) are localized intracellularly.

The system is composed of 2 parts such as presented in Figure 1, Figure 2A and Figure 2B corresponding to the cases when the dimerization domains are borne on the α chain and on the γ chain of mcCAR respectively. A difference with Figure 1 is that the dimerization domains of the IMP protein and mcCAR are located intracellularly. The functioning process is the same than that of Figure 1, excepted that the rapamycin needs to traverse the membrane of the immune cell to be able to bind to the binding domains.

Figure 3: Schematic representation of an extracellular based chemical induced dimerization (CID) strategy using IL-10R as inhibitory signaling domain, when both binding domains of the inhibitory membrane protein (IMP) P complex are localized extracellularly. The system presented here is a different of those presented in Figure 1 and 2 in the sense that the dimerization domain are

borne on the IMP complex and not on the mcCAR. It is based on an IMP complex and a Chimeric Antigen Receptor (here a mcCAR). The IMP complex is composed of 2 independent proteins, each one transmembrane, having extracellularly a dimerization domain, and intracellularly one monomer of the IL-10R inhibitory signaling domain (monomer IL-10Rα and monomer IL-10Rβ). The mcCAR is the same that the one presented in Figure 1 and 2.

Figure 4: Schematic representation of an extracellular based chemical induced dimerization (CID) strategy using PD-1 as inhibitory signaling domain in the IMP molecule, and a single-chain CAR; when both binding domains in the IMP molecule and scCAR are localized extracellularly. In the absence of small molecule, the scCAR can function when it meets the tumoral cell surface antigen. In the presence of the small molecule, there is a dimerization of the CID binding domains, allowing a "co-localization" of the 2 polypeptides chains, and then the PD-1 can play its inhibitory role on the scCAR. The latter stops to function.

Figure 5: Schematic representation Schematic representation of an intracellular based chemical induced dimerization (CID) strategy using PD-1 as inhibitory signaling domain in the IMP molecule, and a single-chain CAR; when both binding domains in the IMP molecule and scCAR are localized intracellularly. This is the same principle than that of the extracellular one depicted in Figure 4, excepted that the small molecule must cross the membrane to be able to dimerize the 2 intracellular CID binding domains.

When needed, i.e. in case of an inappropriate or excessive effect of the CAR, the rapamycin is administered to the patient. The rapamycin allows the dimerization of the 2 dimerization domains borne on the IMP complex. This enhances the colocalization of the 2 monomers of IL-10R, and consequently its inhibitory activity on the CAR via a reaction cascade involving interferon IFN-γ and JAK-STAT signaling pathway. Therefore, the functional IL-10R dimer can indirectly monitor or shunt the activation of an immune cell by the Chimeric Antigen Receptor (CAR).

The following tables show the sequences for all components which are encompassed in the construction of the IMP complex and of the Chimeric Antigen Receptor (CAR).

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Table 1: Exemplary sequences of modulable/tunable multi-chain CAR based on PD-1 inhibitory signaling domain presented in Figure 1 Extracellular CID strategy-, when the CID protein is located on the gamma chain.

Component	Functional domains	Description	SEQ ID #	Raw amino acid sequence
Multi-chain CAR				
Alpha-chain	Signal sequence	FcεR1α-SP	SEQ ID NO.1	MAPAMESPTLLCVALLFFAPDGVLA
	scFv	CD123-scFv	SEQ ID NO.2	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGK SFKWMGWINTYTGESTYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQ GTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQK PGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYCQQSNEDPPTFGAGTKLELKRS DP
		Klo43-3-scFv	SEQ ID NO.3	MAPAMESPTLLCVALLFFAPDGVLAEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPG KALEWLALIRSKADGYTTEYSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEG AMDYWGQGTSVTVSSGGGGSGGGGSGGGGSMADYKDIVMTQSHKFMSTSVGDRVNITCKASQN VDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSVQAEDLAVYYCQQYYSTPW TFGGGTKLEIKRSDP
		5T4WT19-scFv	SEQ ID NO.4	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPDLVKPGASVKISCKASGYSFTGYYMHWVKQSHG KSLEWIGRINPNNGVTLYNQKFKDKAILTVDKSSTTAYMELRSLTSEDSAVYYCARSTMITNYVMDYW GQVTSVTVSSGGGGSGGGGSGGGGSSIVMTQTPTFLLVSAGDRVTITCKASQSVSNDVAWYQQKPG QSPTLLISYTSSRYAGVPDRFIGSGYGTDFTFTISTLQAEDLAVYFCQQDYNSPPTFGGGTKLEIKRSDP
		VH-4G7-scFv	SEQ ID NO.5	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKP GQGLEWIGYINPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDY WGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWF LQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGAGTKL ELKRSDP
	Hinge	CD8a	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
		IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGKKD
		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRT RYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD

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	Transmembrane + intracellular domain - with hinge-	Alpha-TI-A	SEQ ID NO.9	IFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNR
	Transmembrane + intracellular domain - without hinge-	Alpha-TI-B	SEQ ID NO.10	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNR
	CD19 alpha chain		SEQ ID NO.11	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGAGTKLELKRADTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNN
Beta-chain	beta-41BB chain		SEQ ID NO.12	MDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVLDISHIEGDIFSSFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNKVPEKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
Gamma-chain	Signal sequence	Gamma-ss	SEQ ID NO.13	MAIPAVVLLLLLLVEQAAAG
	CID_Protein	FKBP	SEQ ID NO.14	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAP
		FRB	SEQ ID NO.15	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAP
	Hinge	CD8a	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
		IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD
		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRT

				RYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD
	Transmembrane + intracellular domain –with hinge-	Gamma-TI-A	SEQ ID NO.16	IGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	Transmembrane+ intracellular domain –without hinge-	Gamma-TI-B	SEQ ID NO.17	LGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
IMP complex				
Sequence signal		VH-4G7-ss	SEQ ID NO.18	MALPVTALLLPLALLLHAARPG
Dimerization part	CID_Protein	FKBP	SEQ ID NO.14	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAP
		FRB	SEQ ID NO.15	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAP
Hinge		CD8a	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
		IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD
		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD
Trans membrane domain		CD8a	SEQ ID NO.19	IYIWAPLAGTCGVLLLSLVITLYCR
		4-1BB	SEQ ID NO.20	IISFFLALTSTALLFLLFFLTLRFSVVKRGR
		DAP10	SEQ ID NO.21	ILLAGLVAADAVASLLIVGAVFLCARR
		CD28	SEQ ID NO.22	IFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRR
Inhibitory signaling domain		PD1	SEQ ID NO.23	SRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE

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Table 2: Exemplary sequences of modulable/tunable multi-chain CAR based on PD-1 inhibitory signaling domain presented in Figure 1.

Extracellular CID strategy-, when the CID protein is located on the α chain.

Component	Functional domains	Description	SEQ ID #	Raw amino acid sequence
Multi-chain CAR				
Alpha-chain	Signal sequence	FcεR1α-SP	SEQ ID NO.1	MAPAMESPTLLCVALLFFAPDGVLA
	scFv	CD123-scFv	SEQ ID NO.2	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGK SFKWMGWINTYTGESTYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQ GTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQK PGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYCQQSNEDPPTFGAGTKLELKRS DPG
		Klo43-3-scFv	SEQ ID NO.3	MAPAMESPTLLCVALLFFAPDGVLAEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPG KALEWLALIRSKADGYTTEYSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEG AMDYWGQGTSVTVSSGGGGSGGGGSGGGGSMADYKDIVMTQSHKFMSTSVGDRVNITCKASQN VDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSVQAEDLAVYYCQQYYSTPW TFGGGTKLEIKRSDPG
		5T4WT19-scFv	SEQ ID NO.4	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPDLVKPGASVKISCKASGYSFTGYYMHWVKQSHG KSLEWIGRINPNNGVTLYNQKFKDKAILTVDKSSTTAYMELRSLTSEDSAVYYCARSTMITNYVMDYW GQVTSVTVSSGGGGSGGGGSGGGGSSIVMTQTPTFLLVSAGDRVTITCKASQSVSNDVAWYQQKPG QSPTLLISYTSSRYAGVPDRFIGSGYGTDFTFTISTLQAEDLAVYFCQQDYNSPPTFGGGTKLEIKRSDPG
		VH-4G7-scFv	SEQ ID NO.5	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKP GQGLEWIGYINPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDY WGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWF LQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGAGTKL ELKRSDPG
	CID protein	FKBP	SEQ ID NO.14	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVA QMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAP
		FRB	SEQ ID NO.15	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAP
		CD8α	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
		IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP

	Hinge			SRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGKKD
		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRT RYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD
	Transmembrane + intracellular domain –with hinge-	Gamma-TI-A	SEQ ID NO.9	IGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRRE EYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPRE
	Transmembrane + intracellular –without hinge-	Gamma-TI-B	SEQ ID NO.10	LGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPRE
Beta-chain	beta-41BB chain		SEQ ID NO.12	MDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAM ICLCFGTVVCSVLDISHIEGDIFSSFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGT GITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNKVPEKR GRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
Gamma-chain	Signal sequence	Gamma-ss	SEQ ID NO.13	MAIPAVVLLLLLLVEQAAAG
	CID_Protein	FKBP	SEQ ID NO.14	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVA QMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAP
		FRB	SEQ ID NO.15	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAP
	Hinge	CD8a	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
		IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGKKD
		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRT RYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD
	Transmembrane + intracellular domain -with hinge-	Gamma-TI-A	SEQ ID NO.16	IGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRRE EYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPRE
	Transmembrane + intracellular domain -without hinge-	Gamma-TI-B	SEQ ID NO.17	LGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPRE

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	CD19 alpha chain		SEQ ID NO.11	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKP GQGLEWIGYINPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDY WGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWF LQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGAGTKL ELKRADTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFIST QQQVTFLLKIKRTRKGFRLLNPHPKPNPKNN
IMP complex				
Sequence signal		VH-4G7-ss	SEQ ID NO.18	MALPVTALLLPLALLLHAARPG
Dimerization part	CID_Protein	FKBP	SEQ ID NO.14	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVA QMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAP
		FRB	SEQ ID NO.15	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAP
Hinge		CD8a	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
		IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGKKD
		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRT RYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD
Transmembrane domain		CD8a	SEQ ID NO.19	IYIWAPLAGTCGVLLLSLVITLYCR
		4-1BB	SEQ ID NO.20	IISFFLALTSTALLFLLFFLTLRFSVVKRGR
		DAP10	SEQ ID NO.21	ILLAGLVAADAVASLLIVGAVFLCARR
		CD28	SEQ ID NO.22	IFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRR
Inhibitory signaling domain		PD1	SEQ ID NO.23	SRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTS SPARRGSADGPRSAQPLRPEDGHCSWPLE

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Table 3: Exemplary sequences of modulable/tunable multi-chain CAR based on PD-1 inhibitory signaling domain presented in Figure 1.

Intracellular CID strategy-, when the CID protein is located on the alpha chain.

Several IMP molecules are possible depending of the positioning of the CID protein (dimerization part) and of the PD1 protein.

Component	Functional domains	Description	SEQ ID #	Raw amino acid sequence
Multi-chain CAR				
Alpha-chain	Signal sequence	FcεR1α-SP	SEQ ID NO.1	MAPAMESPTLLCVALLFFAPDGVLA
	svFc	CD123-scFv	SEQ ID NO.2	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGK SFKWMGWINTYTGESTYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQ GTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQK PGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYCQQSNEDPPTFGAGTKLELKRS DP
		Klo43-3-scFv	SEQ ID NO.3	MAPAMESPTLLCVALLFFAPDGVLAEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPG KALEWLALIRSKADGYTTEYSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEG AMDYWGQGTSVTVSSGGGGSGGGGSGGGGSMADYKDIVMTQSHKFMSTSVGDRVNITCKASQN VDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSVQAEDLAVYYCQQYYSTPW TFGGGTKLEIKRSDP
		5T4WT19-scFv	SEQ ID NO.4	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPDLVKPGASVKISCKASGYSFTGYYMHWVKQSHG KSLEWIGRINPNNGVTLYNQKFKDKAILTVDKSSTTAYMELRSLTSEDSAVYYCARSTMITNYVMDYW GQVTSVTVSSGGGGSGGGGSGGGGSSIVMTQTPTFLLVSAGDRVTITCKASQSVSNDVAWYQQKPG QSPTLLISYTSSRYAGVPDRFIGSGYGTDFTFTISTLQAEDLAVYFCQQDYNSPPTFGGGTKLEIKRSDP
		VH-4G7-scFv	SEQ ID NO.5	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKP GQGLEWIGYINPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDY WGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWF LQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGAGTKL ELKRSDP
		CD8a	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD

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	Hinge	IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP SRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN VFSCSVMHEALHNHYTQKSLSLSPGKKD
		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRT RYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD
	Transmembrane + intracellular domain - with hinge-	Alpha-TI-A	SEQ ID NO.9	IFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNR
	Transmembrane + intracellular domain - without hinge-	Alpha-TI-B	SEQ ID NO.10	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVT FLLKIKRTRKGFRLLNPHPKPNPKNNR
	Dimerization part	Without linker		
		FKBP_pos4-ter-alpha	SEQ ID NO.24	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVA QMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
		FRB_pos4-ter-alpha	SEQ ID NO.25	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE
		With linker		
		CD28	SEQ ID NO.26	SRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSGSGSGS
		4-1BB	SEQ ID NO.27	GRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGSGSGSGS
		OX40	SEQ ID NO.28	ALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGSGSGSGS
		DAP10	SEQ ID NO.29	PRRSPAQEDGKVYINMPGRGGSGSGSGS
		CD18	SEQ ID NO.30	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAESGSGSGSGS
		CD28	SEQ ID NO.31	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSGSGSGS

		CD275	SEQ ID NO.32	RDRCLQHSYAGAWAVSPETELTGHVGSGSGSGS
		HVEM	SEQ ID NO.33	CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIPSFTGRSPNHGSGSGSGS
		LIGHT	SEQ ID NO.34	MEESVVRPSVFVVDGQTDIPFTRLGRSHRRQSCSVARGSGSGSGS
		CD40L	SEQ ID NO.35	MIETYNQTSPRSAATGLPISMKGSGSGSGS
		GITR	SEQ ID NO.36	QLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWVGSGSGSGS
		TIM1	SEQ ID NO.37	KKYFFKKEVQQLSVSFSSLQIKALQNAVEKEVQAEDNIYIENSLYATDGSGSGSGS
		SLAM	SEQ ID NO.38	QLRRRGKTNHYQTTVEKKSLTIYAQVQKPGPLQKKLDSFPAQDPCTTIYVAATEPVPESVQETNSITV YASVTLPESGSGSGSGS
		CD2	SEQ ID NO.39	KRKKQRSRRNDEELETRAHRVATEERGRKPHQIPASTPQNPATSQHPPPPPGHRSQAPSHRPPPPGH RVQHQPQKRPPAPSGTQVHQQKGPPLPRPRVQPKPPHGAAENSLSPSSNGSGSGSGS
		TLT-2	SEQ ID NO.40	KKRHMASYSMCSDPSTRDPPGRPEPYVEVYLIGSGSGSGS
		LAG3	SEQ ID NO.41	HLWRRQWRPRRFSALEQGIHPPQAQSKIEELEQEPEPEPEPEPEPEPEPEPEQLGSGSGSGS
		DAP12	SEQ ID NO.42	YFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDVYSDLNTQRPYYKGSGSGSGS
		CD84	SEQ ID NO.43	RLFKRRQGRIFPEGSCLNTFTKNPYAASKKTIYTYIMASRNTQPAESRIYDEILQSKVLPSKEEPVNTVYS EVQFADKMGKASTQDSKPPGTSSYEIVIGSGSGSGS
		CD244	SEQ ID NO.44	EFLTIYEDVKDLKTRRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSP SFNSTIYEVIGKSQPKAQNPARLSRKELENFDVYSGSGSGSGS
		CD229	SEQ ID NO.45	LYSVLSQGYEKLDTPLRPARQQPTPTSDSSSDSNLTTEEDEDRPEVHKPISGRYEVFDQVTQEGAGHD PAPEGQADYDPVTPYVTEVESVVGENTMYAQVFNLQGKTPVSQKEESSATIYCSIRKPQVVPPPQQN DLEIPESPTYENFTGSGSGSGS
		LTBR	SEQ ID NO.46	KAHPYFPDLVQPLLPISGDVSPVSTGLPAAPVLEAGVPQQQSPLDLTREPQLEPGEQSQVAHGTNGIH VTGGSMTITGNIYIYNGPVLGGPPGPGDLPATPEPPYPIPEEGDPGPPGLSTPHQEDGKAWHLAETE HCGATPSNGSGSGSGS
			FKBP_pos5-ter-alpha	SEQ ID NO.47	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVA QMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
			FRB_pos5-ter-alpha	SEQ ID NO.48	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE

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Beta-chain	beta-41BB chain		SEQ ID NO.12	MDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTAM ICLCFGTVVCSVLDISHIEGDIFSSFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIAGGT GITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNKVPEKR GRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
Gamma-chain	gamma-CD3z chain		SEQ ID NO.49	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAY QQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKG ERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
IMP complex (IMP molecule #1)				
Sequence signal		VH-4G7-ss	SEQ ID NO.18	MALPVTALLLPLALLLHAARPGSD
Transmembrane domain		CD8a	SEQ ID NO.19	IYIWAPLAGTCGVLLLSLVITLYCR
		4-1BB	SEQ ID NO.20	IISFFLALTSTALLFLLFFLTLRFSVVKRGR
		DAP10	SEQ ID NO.21	ILLAGLVAADAVASLLIVGAVFLCARR
		CD28	SEQ ID NO.22	IFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRR
Dimerization part	**CID_Protein**	FKBP_pos3-IMP	SEQ ID NO.24	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVA QMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGSS
		FRB_pos3-IMP	SEQ ID NO.25	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGSS
Inhibitory signaling domain		PD1	SEQ ID NO.23	SRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTS SPARRGSADGPRSAQPLRPEDGHCSWPLE
IMP complex (IMP molecule #2)				
Sequence signal		VH-4G7-ss	SEQ ID NO.18	MALPVTALLLPLALLLHAARPGSD
Transmembrane domain		CD8a	SEQ ID NO.26	IYIWAPLAGTCGVLLLSLVITLYCR
		4-1BB	SEQ ID NO.27	IISFFLALTSTALLFLLFFLTLRFSVVKRGR
		DAP10	SEQ ID NO.28	ILLAGLVAADAVASLLIVGAVFLCARR
		CD28	SEQ ID NO.29	IFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRR
		CD28 N	SEQ ID NO.26	SRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSGSGSGS
		4-1BB	SEQ ID NO.27	GRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGSGSGSGS
		OX40	SEQ ID NO.28	ALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGSGSGSGS

Linker		DAP10	SEQ ID NO.29	PRRSPAQEDGKVYINMPGRGGSGSGSGS
		CD18	SEQ ID NO.30	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAESGSGSGSGS
		CD28	SEQ ID NO.31	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSGSGSGS
		CD275	SEQ ID NO.32	RDRCLQHSYAGAWAVSPETELTGHVGSGSGSGS
		HVEM	SEQ ID NO.33	CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIPSFTGRSPNHGSGSGSGS
		LIGHT	SEQ ID NO.34	MEESVVRPSVFVVDGQTDIPFTRLGRSHRRQSCSVARGSGSGSGS
		CD40L	SEQ ID NO.35	MIETYNQTSPRSAATGLPISMKGSGSGSGS
		GITR	SEQ ID NO.36	QLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWVGSGSGSGS
		TIM1	SEQ ID NO.37	KKYFFKKEVQQLSVSFSSLQIKALQNAVEKEVQAEDNIYIENSLYATDGSGSGSGS
		SLAM	SEQ ID NO.38	QLRRRGKTNHYQTTVEKKSLTIYAQVQKPGPLQKKLDSFPAQDPCTTIYVAATEPVPESVQETNSITV YASVTLPESGSGSGSGS
		CD2	SEQ ID NO.39	KRKKQRSRRNDEELETRAHRVATEERGRKPHQIPASTPQNPATSQHPPPPPGHRSQAPSHRPPPPGH RVQHQPQKRPPAPSGTQVHQQKGPPLPRPRVQPKPPHGAAENSLSPSSNGSGSGSGS
		TLT-2	SEQ ID NO.40	KKRHMASYSMCSDPSTRDPPGRPEPYVEVYLIGSGSGSGS
		LAG3	SEQ ID NO.41	HLWRRQWRPRRFSALEQGIHPPQAQSKIEELEQEPEPEPEPEPEPEPEPEPEQLGSGSGSGS
		DAP12	SEQ ID NO.42	YFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDVYSDLNTQRPYYKGSGSGSGS
		CD84	SEQ ID NO.43	RLFKRRQGRIFPEGSCLNTFTKNPYAASKKTIYTYIMASRNTQPAESRIYDEILQSKVLPSKEEPVNTVYS EVQFADKMGKASTQDSKPPGTSSYEIVIGSGSGSGS
		CD244	SEQ ID NO.44	EFLTIYEDVKDLKTRRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSP SFNSTIYEVIGKSQPKAQNPARLSRKELENFDVYSGSGSGSGS
		CD229	SEQ ID NO.45	LYSVLSQGYEKLDTPLRPARQQPTPTSDSSSDSNLTTEEDEDRPEVHKPISGRYEVFDQVTQEGAGHD PAPEGQADYDPVTPYVTEVESVVGENTMYAQVFNLQGKTPVSQKEESSATIYCSIRKPQVVPPPQQN DLEIPESPTYENFTGSGSGSGS
		LTBR	SEQ ID NO.46	KAHPYFPDLVQPLLPISGDVSPVSTGLPAAPVLEAGVPQQQSPLDLTREPQLEPGEQSQVAHGTNGIH VTGGSMTITGNIYIYNGPVLGGPPGPGDLPATPEPPYPIPEEGDPGPPGLSTPHQEDGKAWHLAETE HCGATPSNGSGSGSGS
Dimerization part	CID_Protein	FKBP_pos4-IMP	SEQ ID NO.51	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVA QMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGSG
		FRB_pos4-IMP	SEQ ID NO.52	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGSG
Inhibitory signaling domain		PD1_pos5-ter	SEQ ID NO.53	SRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTS SPARRGSADGPRSAQPLRPEDGHCSWPLE

IMP complex (IMP molecule #3)			
Sequence signal	VH-4G7-ss	SEQ ID NO.18	MALPVTALLLPLALLLHAARPGSD
Transmembrane domain	CD8a	SEQ ID NO.26	IYIWAPLAGTCGVLLLSLVITLYCR
	4-1BB	SEQ ID NO.27	IISFFLALTSTALLFLLFFLTLRFSVVKRGR
	DAP10	SEQ ID NO.28	ILLAGLVAADAVASLLIVGAVFLCARR
	CD28	SEQ ID NO.29	IFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRR
Inhibitory signaling domain	PD1_pos3	SEQ ID NO.50	SRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTS SPARRGSADGPRSAQPLRPEDGHCSWPLGSGSS
Linker	CD28	SEQ ID NO.26	SRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSGSGSGS
	4-1BB	SEQ ID NO.27	GRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGSGSGSGS
	OX40	SEQ ID NO.28	ALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGSGSGSGS
	DAP10	SEQ ID NO.29	PRRSPAQEDGKVYINMPGRGGSGSGSGS
	CD18	SEQ ID NO.30	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAESGSGSGSGS
	CD28	SEQ ID NO.31	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSGSGSGS
	CD275	SEQ ID NO.32	RDRCLQHSYAGAWAVSPETELTGHVGSGSGSGS
	HVEM	SEQ ID NO.33	CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIPSFTGRSPNHGSGSGSGS
	LIGHT	SEQ ID NO.34	MEESVVRPSVFVVDGQTDIPFTRLGRSHRRQSCSVARGSGSGSGS
	CD40L	SEQ ID NO.35	MIETYNQTSPRSAATGLPISMKGSGSGSGS
	GITR	SEQ ID NO.36	QLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWVGSGSGSGS
	TIM1	SEQ ID NO.37	KKYFFKKEVQQLSVSFSSLQIKALQNAVEKEVQAEDNIYIENSLYATDGSGSGSGS
	SLAM	SEQ ID NO.38	QLRRRGKTNHYQTTVEKKSLTIYAQVQKPGPLQKKLDSFPAQDPCTTIYVAATEPVPESVQETNSITV YASVTLPESGSGSGSGS
	CD2	SEQ ID NO.39	KRKKQRSRRNDEELETRAHRVATEERGRKPHQIPASTPQNPATSQHPPPPPGHRSQAPSHRPPPPGH RVQHQPQKRPPAPSGTQVHQQKGPPLPRPRVQPKPPHGAAENSLSPSSNGSGSGSGS
	TLT-2	SEQ ID NO.40	KKRHMASYSMCSDPSTRDPPGRPEPYVEVYLIGSGSGSGS
	LAG3	SEQ ID NO.41	HLWRRQWRPRRFSALEQGIHPPQAQSKIEELEQEPEPEPEPEPEPEPEPEPEQLGSGSGSGS
	DAP12	SEQ ID NO.42	YFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDVYSDLNTQRPYYKGSGSGSGS
	CD84	SEQ ID NO.43	RLFKRRQGRIFPEGSCLNTFTKNPYAASKKTIYTYIMASRNTQPAESRIYDEILQSKVLPSKEEPVNTVYS EVQFADKMGKASTQDSKPPGTSSYEIVIGSGSGSGS
	CD244	SEQ ID NO.44	EFLTIYEDVKDLKTRRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSP SFNSTIYEVIGKSQPKAQNPARLSRKELENFDVYSGSGSGSGS

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		CD229	SEQ ID NO.45	LYSVLSQGYEKLDTPLRPARQQPTPTSDSSSDSNLTTEEDEDRPEVHKPISGRYEVFDQVTQEGAGHD PAPEGQADYDPVTPYVTEVESVVGENTMYAQVFNLQGKTPVSQKEESSATIYCSIRKPQVVPPPQQN DLEIPESPTYENFTGSGSGSGS
		LTBR	SEQ ID NO.46	KAHPYFPDLVQPLLPISGDVSPVSTGLPAAPVLEAGVPQQQSPLDLTREPQLEPGEQSQVAHGTNGIH VTGGSMTITGNIYIYNGPVLGGPPGPGDLPATPEPPYPIPEEGDPGPPGLSTPHQEDGKAWHLAETE HCGATPSNGSGSGSGS
Dimerization part	CID_Protein	FKBP_pos4-ter-IMP	SEQ ID NO.54	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVA QMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
		FRB_pos4-ter-IMP MTOR_HUMAN	SEQ ID NO.55	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE
IMP complex (IMP molecule #4)				
Sequence signal		VH-4G7-ss	SEQ ID NO.18	MALPVTALLLPLALLLHAARPGSD
Transmembrane domain		CD8a	SEQ ID NO.19	IYIWAPLAGTCGVLLLSLVITLYCR
		4-1BB	SEQ ID NO.20	IISFFLALTSTALLFLLFFLTLRFSVVKRGR
		DAP10	SEQ ID NO.21	ILLAGLVAADAVASLLIVGAVFLCARR
		CD28 CD28_HUMAN	SEQ ID NO.22	IFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRR
		CD28	SEQ ID NO.26	SRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSGSGSGS
		4-1BB	SEQ ID NO.27	GRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGSGSGSGS
		OX40	SEQ ID NO.28	ALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGSGSGSGS
		DAP10	SEQ ID NO.29	PRRSPAQEDGKVYINMPGRGGSGSGSGS
		CD18	SEQ ID NO.30	KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAESGSGSGSGS
		CD28	SEQ ID NO.31	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGSGSGSGS
		CD275	SEQ ID NO.32	RDRCLQHSYAGAWAVSPETELTGHVGSGSGSGS
		HVEM	SEQ ID NO.33	CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIPSFTGRSPNHGSGSGSGS
		LIGHT	SEQ ID NO.34	MEESVVRPSVFVVDGQTDIPFTRLGRSHRRQSCSVARGSGSGSGS
		CD40L	SEQ ID NO.35	MIETYNQTSPRSAATGLPISMKGSGSGSGS
		GITR	SEQ ID NO.36	QLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWVGSGSGSGS
		TIM1	SEQ ID NO.37	KKYFFKKEVQQLSVSFSSLQIKALQNAVEKEVQAEDNIYIENSLYATDGSGSGSGS
		SLAM	SEQ ID NO.38	QLRRRGKTNHYQTTVEKKSLTIYAQVQKPGPLQKKLDSFPAQDPCTTIYVAATEPVPESVQETNSITV

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Linker				YASVTLPESGSGSGSGS
		CD2	SEQ ID NO.39	KRKKQRSRRNDEELETRAHRVATEERGRKPHQIPASTPQNPATSQHPPPPPGHRSQAPSHRPPPPGHRVQHQPQKRPPAPSGTQVHQQKGPPLPRPRVQPKPPHGAAENSLSPSSNGSGSGSGS
		TLT-2	SEQ ID NO.40	KKRHMASYSMCSDPSTRDPPGRPEPYVEVYLIGSGSGSGS
		LAG3	SEQ ID NO.41	HLWRRQWRPRRFSALEQGIHPPQAQSKIEELEQEPEPEPEPEPEPEPEPEPEQLGSGSGSGS
		DAP12	SEQ ID NO.42	YFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDVYSDLNTQRPYYKGSGSGSGS
		CD84	SEQ ID NO.43	RLFKRRQGRIFPEGSCLNTFTKNPYAASKKTIYTYIMASRNTQPAESRIYDEILQSKVLPSKEEPVNTVYSEVQFADKMGKASTQDSKPPGTSSYEIVIGSGSGSGS
		CD244	SEQ ID NO.44	EFLTIYEDVKDLKTRRNHEQEQTFPGGGSTIYSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSPSFNSTIYEVIGKSQPKAQNPARLSRKELENFDVYSGSGSGSGS
		CD229	SEQ ID NO.45	LYSVLSQGYEKLDTPLRPARQQPTPTSDSSSDSNLTTEEDEDRPEVHKPISGRYEVFDQVTQEGAGHDPAPEGQADYDPVTPYVTEVESVVGENTMYAQVFNLQGKTPVSQKEESSATIYCSIRKPQVVPPPQQNDLEIPESPTYENFTGSGSGSGS
		LTBR	SEQ ID NO.46	KAHPYFPDLVQPLLPISGDVSPVSTGLPAAPVLEAGVPQQQSPLDLTREPQLEPGEQSQVAHGTNGIHVTGGSMTITGNIYIYNGPVLGGPPGPGDLPATPEPPYPIPEEGDPGPPGLSTPHQEDGKAWHLAETEHCGATPSNGSGSGSGS
Inhibitory signaling domain		PD1_pos4	SEQ ID NO.56	SRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLGSGSG
Dimerization part	CID_Protein	FKBP_pos5-ter-IMP	SEQ ID NO.57	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
		FRB_pos5-ter-IMP	SEQ ID NO.58	GRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE

Table 4: Exemplary sequences of modulable/tunable multi-chain CAR based on IL-10R inhibitory signaling domain presented in Figure 3

Component	Functional domains	Description	SEQ ID #	Raw amino acid sequence
Multi-chain CAR				
Alpha-chain	Signal sequence	FcεR1α-SP	SEQ ID NO.1	MAPAMESPTLLCVALLFFAPDGVLA
		CD123-scFv	SEQ ID NO.2	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAP GKSFKWMGWINTYTGESTYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDY WGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAVSLGQRATISCRASESVDNYGNTFMH WYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYCQQSNEDPPTFGAGT KLELKRSDP
		Klo43-3-scFv	SEQ ID NO.3	MAPAMESPTLLCVALLFFAPDGVLAEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPP GKALEWLALIRSKADGYTTEYSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSP EGAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSMADYKDIVMTQSHKFMSTSVGDRVNITCKA SQNVDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSVQAEDLAVYYCQQYY STPWTFGGGTKLEIKRSDP
		5T4WT19-scFv	SEQ ID NO.4	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPDLVKPGASVKISCKASGYSFTGYYMHWVKQSH GKSLEWIGRINPNNGVTLYNQKFKDKAILTVDKSSTTAYMELRSLTSEDSAVYYCARSTMITNYVMDY WGQVTSVTVSSGGGGSGGGGSGGGGSSIVMTQTPTFLLVSAGDRVTITCKASQSVSNDVAWYQQ KPGQSPTLLISYTSSRYAGVPDRFIGSGYGTDFTFTISTLQAEDLAVYFCQQDYNSPPTFGGGTKLEIKR SDP
		VH-4G7-scFv	SEQ ID NO.5	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQK PGQGLEWIGYINPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVF DYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYL YWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGA GTKLELKRSDP
	Hinge	CD8a	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
		IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD

		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRG RTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD
	Transmembrane + intracellular domain - with hinge-	Alpha-TI-A	SEQ ID NO.9	IFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNR
	Transmembrane + intracellular domain - without hinge-	Alpha-TI-B	SEQ ID NO.10	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTF LLKIKRTRKGFRLLNPHPKPNPKNNR
	CD19 alpha chain		SEQ ID NO.11	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQK PGQGLEWIGYINPYNDGTKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVF DYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVMTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYL YWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPFTFGA GTKLELKRADTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDT GLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNN
Gamma-chain		gamma-CD3z chain	SEQ ID NO.49	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPA YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
Beta-chain		beta-41BB chain	SEQ ID NO.12	MDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSASSPPLHTWLTVLKKEQEFLGVTQILTA MICLCFGTVVCSVLDISHIEGDIFSSFKAGYPFWGAIFFSISGMLSIISERRNATYLVRGSLGANTASSIA GGTGITILIINLKKSLAYIHIHSCQKFFETKCFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNK VPEKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
IL-10A- and IL-10B based IMP molecules				
Signal sequence		VH-4G7-ss	SEQ ID NO.18	MALPVTALLLPLALLLHAARPG
Dimerization part		FKBP	SEQ ID NO.14	SGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGV AQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAP
		FRB	SEQ ID NO.15	SGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDL MEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAP

Hinge		CD8a	SEQ ID NO.6	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
		IgG1	SEQ ID NO.7	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD
		EpoR_D2	SEQ ID NO.8	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRG RTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD
Transmembrane domain + inhibitory signaling domain	IR-10RA inhibitory domain	IL10R1_HUMAN	SEQ ID NO.59	IVIIFFAFVLLLSGALAYCLALQLYVRRRKKLPSVLLFKKPSPFIFISQRPSPETQDTIHPLDEEAFLKVSPEL KNLDLHGSTDSGFGSTKPSLQTEEPQFLLPDPHPQADRTLGNREPPVLGDSCSSGSSNSTDSGICLQE PSLSPSTGPTWEQQVGSNSRGQDDSGIDLVQNSEGRAGDTQGGSALGHHSPPEPEVPGEEDPAAV AFQGYLRQTRCAEEKATKTGCLEEESPLTDGLGPKFGRCLVDEAGLHPPALAKGYLKQDPLEMTLASS GAPTGQWNQPTEEWSLLALSSCSDLGISDWSFAHDLAPLGCVAAPGGLLGSFNSDLVTLPLISSLQS SE
	IR-10B inhibitory domain	II10R2_HUMAN	SEQ ID NO.60	IWMVAVILMASVFMVCLALLGCFALLWCVYKKTKYAFSPRNSLPQHLKEFLGHPHHNTLLFFSFPLS DENDVFDKLSVIAEDSESGKQNPGDSCSLGTPPGQGPQSE

Detailed description of the invention

The ability to control functional responses in adoptive T cell therapy is a key issue. In such therapeutic strategies, T cells are engineered by expressing surface-exposed chimeric antigen receptor (CAR) that achieves high tumor specific target cell recognition. However, to control and minimize potential toxic effects, design of modulable switch systems is highly desirable.

The inventors developed methods of engineering such immune cells based on the design of a chimeric multi-protein complex which is able to inactivate the Chimeric Antigen Receptor (CAR) through the addition of a soluble compound, such as rapalogs.

In particular, the present invention provides a method for modulating the level of activation of an engineered immune cell, said method comprising the following steps of:

(a) transfecting an immune cell with at least a first polynucleotide encoding a Chimeric Antigen Receptor (CAR) , which can be activated in-vivo and/or in vitro by an external ligand; said CAR comprising a first binding domain and ;

(b) further transfecting said immune cell with at least a second polynucleotide encoding an engineered inhibitory membrane protein (IMP) complex, comprising at least one intracellular inhibitory signaling domain and one second binding domain,

such that said immune cell co-expresses, said CAR, and said inhibitory membrane protein (IMP) complex, and;

(c) contacting said engineered immune cell with said external ligand, so that a signal is transduced by the CAR, and then;

(d) reducing the level of said signal transduction of said CAR by adding a soluble compound that binds the binding domain of said IMP and the binding domain of the CAR molecule to co-localize said IMP and said CAR,

thereby modulating the level of activation of the engineered immune cell.

By “modulating” the level of activation, it is intended that an inhibition response is obtained on the CAR-engineered immune cells by the administration of a soluble compound, and preferably not a destructive response in contrast to other systems from the prior art. This is particularly useful to

adjust the CAR induced activation to patient's needs and more critically, when a severe case of adverse event occurs in a patient.

According to a preferred embodiment, said signal of transduction of the CAR is an activation of the immune cell and said immune cell is activated.

According to another preferred embodiment, the co-localization of the IMP and CAR molecules has the effect of switching off the CAR signal transduction.

One interesting feature of the system of the invention is its flexibility based on the co-localization of proteins i.e no-covalent binding. Thus, after a short while following the discontinuation of administration of the soluble compound (such as a rapalog), one can expect the soluble compound will not bind anymore the binding domains, the "switch off" will cease to be operational, and the CAR engineered immune cells will be able to retrieve their potential.

According to a preferred embodiment, first and second binding domains are CID binding domains.

In the present invention, it is also contemplated the possibility to perform a « fine tuning » of a CAR transduction signal: a dose of soluble can be adjusted; depending of the severity of the adverse event following an inadequate/excessive activity of the CAR engineered immune cells administrated to the patient.

The signal transduced by a CAR in an engineered immune cell may be an activation signal or an inactivation signal on the engineered immune cell, depending of the nature of the signal. This depends on the transduction signaling domain included in the CAR (activating or inhibiting signaling domain, which is expressed by the immune cell When the immune cell expresses a "positive CAR" which usually comprise an activation domain comprising ITAM component(s), with the effect of activatingsaid cell (i.e. its immune function) upon recognition of a tumoral surface specific-antigen, then the IMP will act as a brake on the cell activation, and further as a "switch-off" system,. Conversly, in the event the transduction signal is generated by a "negative CAR" that comprises, for instance, an inhibitory signaling domain, such as immunoreceptor tyrosine-based inhibition motif (ITIM) , then the inhibition will be partially or totally lifted, acting as a "switch-on" system.

Inhibitory membrane protein (IMP) complex

According to another aspect, the present invention discloses an inhibitory dual CAR/IMP complex, comprising at least two transmembrane chimeric polypeptides:

- a first one encoding a Chimeric Antigen Receptor which comprises one dimerization binding domain;

-the second one encoding an engineered inhibitory membrane protein (IMP) complex, said IMP complex comprising at least one intracellular inhibitory signaling domain and one dimerization binding domain.

According to a preferred embodiment, said inhibitory dual CAR/IMP complex comprises dimerization binding domains which are located extracellularly.

According to an embodiment, the CAR which is part of said inhibitory dual CAR/IMP complex is a multi-chain CAR.

According to an embodiment, the CAR which is part of said inhibitory dual CAR/IMP complex is a single-chain CAR.

In one embodiment, the intra inhibitory domain which is part of the engineered inhibitory membrane protein (IMP) complex is the PD-1 protein (also called “Programmed cell death protein 1” or CD279).

According to a preferred embodiment, the dimerization binding domains are FKBP or FRB.According to a more preferred embodiment, said first and second binding domains have at least 80 % identity with SEQ ID NO 14 and SEQ ID NO 15.

According to the invention, the inhibitory membrane (IMP) complex corresponds to at least one transmembrane chimeric protein; the latter containing at least one intracellular inhibitory signaling domain and one binding domain.

In one embodiment, the IMP complex involves one transmembrane chimeric protein, comprising at least one inhibitory signaling domain and a first binding domain that can bind to a second binding domain borne on the Chimeric Antigen Receptor (CAR) through the presence of a soluble compound (Figures 1 and 2).

These inhibitory signaling domains may belong to the “immune checkpoints” molecules effectively serve as "brakes" to down-modulate or inhibit an immune response.

Amongst these inhibitory signaling domains, some can be “functional” inhibitors because they can interact by (reversible) post-translational modification, such as by dephosphorylation

mechanism (i.e. for PD-1), some can be “structural” inhibitors, such as antibodies (preferably an humanized intrabodies) which can physically block the protein.

According to the invention, these inhibitory signaling domains can be from, but are not limited to, Programmed Death 1 (PD-1, also known as PDCD1 or CD279, accession number: NM_005018), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4, also known as CD152, GenBank accession number AF414120.1), LAG3 (also known as CD223, accession number: NM_002286.5), Tim3 (also known as HAVCR2, GenBank accession number: JX049979.1), BTLA (also known as CD272, accession number: NM_181780.3), BY55 (also known as CD160, GenBank accession number: CR541888.1), TIGIT (also known as VSTM3, accession number: NM_173799), LAIR1 (also known as CD305, GenBank accession number: CR542051.1, (Meyaard, Adema et al. 1997)), SIGLEC10 (GeneBank accession number: AY358337.1), 2B4 (also known as CD244, accession number: NM_001166664.1), PPP2CA, PPP2CB, PTPN6, PTPN22, CD96, CRTAM, SIGLEC7 (Nicoll, Ni et al. 1999), SIGLEC9 (Zhang, Nicoll et al. 2000; Ikehara, Ikehara et al. 2004), TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFRBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF (Quigley, Pereyra et al. 2010), GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, which proteins have been described as having ability to inhibit immune cells activation.

A particular preferred inhibitory signaling domain is from PD-1 and preferably comprises the amino acid sequence that has at least 80% sequence identity with SEQ ID NO.23.

Beside these immune checkpoints (such as PD-1), other inhibitors towards the immune response are encompassed within the invention: for instance, some cytokines.

According to an alternative embodiment, the IMP complex of the invention involves two transmembrane chimeric proteins, each comprising a binding domain that may be bound by a soluble compound (Figure 3).

In accordance with one preferred embodiment, inhibitory signaling domains are the interleukin IL-10Rα and IL-10Rβ monomers, and preferably comprise the amino acid sequences that have at least 80% sequence identity with SEQ ID NO.59 and SEQ ID NO.60 respectively.

In general, IL-10R is composed of two subunits to be active: IL-10Rα (Ref. Uniprot: Q13651; RefSeq: NP_001549); and IL-10Rβ (Ref.Uniprot: Q08334, RefSeq: NM_000628 for the human species). According to Satoshi et al. 1999), the inhibitory action of IL-10 on immune cells -such as the CAR-

lymphocytes T of the invention- is obtained via the inhibition of expression of both interferon α– and interferon γ– induced genes.

Typically, IL10RA/IL10B polypeptides of the invention respectively comprise the amino acid sequence has at least 80% sequence identity with SEQ ID NO: 59 and SEQ ID NO: 60 respectively.

The inhibitory action of the IL-10R occurs after the dimerization of the IL10RA/IL10B polypeptides and leads to the inhibition of cytokines such as interferon IFN-γ, IL-2, IL-3 or TNF-α involving the JAK-STAT signaling pathway (Finbloom et al. 1995). Therefore, the functional IL-10R dimer can indirectly monitor or shunt the activation of an immune cell by the Chimeric Antigen Receptor (CAR).

In addition to IL-10R interleukin, other examples of switches can be used according to the system presented in Figure 3, as long as they are negative regulators on immune cells and they are multimeric. Amongst them, one can find TGF-β (transforming growth factor beta), VEGF (Vascular endothelial growth factor), or the apoptosis inducing-multimeric receptors such as TNFR (tumor necrosis factor receptor) or DR3 (Death-receptor-3).

In view of the preceding, one of the particularities of an IMP according to the invention comprising an inhibitory signaling domain such as IL-10R is that this inhibitory system does not require any component from the CAR to initiate its action. Consequently, it can be used in combination with any CAR regardless its specificity and architecture, to down regulate activated CAR T-cell, acting as a “universal” switch. Subsequently, the invention contemplates the combined use of a CAR and a molecular switch as previously described whatever be the antigen targeted by the CAR.

The transmembrane part of the IMP complex is chosen amongst not only those used for the Chimeric Antigen Receptor (CAR) as presented in the following section “Chimeric Antigen Receptor” but also by many others which appear suitable for the skilled person in the construction of such multipart polypeptide.

The binding domain of the IMP complex according to the invention is a protein which can bind to a soluble compound which, via the signaling domain, mediates a modulation on the activation of the CAR. Preferably, the system of the invention is composed of two binding domains which can co-localized or dimerized by the intervention of soluble compound.

The binding domain of the present invention is preferably a dimerization domain.

According to one or the other above embodiments, either these two binding domains are borne on two different IMPs, or one binding domain is part of one IMP and the other on the CAR, depending of the type and action of the inhibitory signaling domain used.

According to a preferred embodiment, both binding domains interact with the same soluble compound.

By “co-localization” or “dimerization” (also called “chemically induced dimerization” or CID) of the two binding domains is meant that the two proteins come close together to make is a macromolecular complex, non-covalently bound, in the presence of a certain small molecule, enzyme or other dimerizing agent. This CID system allows the manipulation of signaling pathways in the case of the present invention.

According to one embodiment, the binding or dimerization domains are extracellular. This configuration is favored/more favorable when the small molecule acting as dimerizing agent is not immunogenic and/ or cannot come easily across the membrane of the immune cell. The first and second binding or dimerization domains can be borne respectively on the IMP protein and on the CAR structure (Figure 1), or both on two separate 2 polypeptides from the IMP complex (Figure 3) depending of the inhibiting signaling domain used (by instance, IL10R versus PD-1). In the first situation, where one binding domain is present on the CAR structure, the action of the inhibitory signaling domain will be directly on the CAR, in contrast to the second situation, where the IMP complex has an inhibitory function on the immune cell activation that is deemed independent from the activation by the CAR.

According to the alternative embodiment, the binding or dimerization domains are intracellular. This conformation applies when one binding or dimerization domain is part of one IMP (having an inhibitory signaling domain) and a second one is part of the CAR (Figure 2). This chemical induction dimerization (CID) strategy will be preferred, for instance, when potential immunogenic small molecules are used.

The localization of the binding/dimerization domain on the alpha chain may be advantageous for a structural or conformational reason.

Still within the scope of the invention, dimerization domains are the preferred binding domains, therefore one binding domain binds to another domain, when they are put in presence of a dimerizing agent.

Amongst binding domains which may be contemplated, one can find number of couples such as FKBP/FKBP with the dimerizing agent FK1012 (Spencer et al, 1993); FKBP/CAN with the dimerizing agent FK506 (Ho et al, 1996); FKBP/CyP-Fas with the dimerizing agent FKCsA (Belshaw et al, 1996); FKBP/FRB with the dimerizing agent rapamycin (Rivera et al, 1996); GyrB/GyrB with the dimerizing agent coumermycin (Farrar et al, 1996); GAI/GID1 with the dimerizing agent gibberellin (Miyamoto et al, 2012). In the last publication, the system has been shown functioning not only in higher plant but in mammalian cells.

According to a preferred embodiment, the binding domains are FKBP/FRB, and the dimerizing agent is a rapalog, and according to a more preferred embodiment, the rapalog is rapamycin.

In accordance with particular embodiments, the binding domains FKBP/FRB are polypeptides comprising the amino acid sequence that has at least 80% sequence identity with SEQ ID NO:14 and SEQ ID NO: 15 respectively.

According to one embodiment, the present invention relates to a retroviral vector comprising the polynucleotide as described previously.

Soluble compounds

By « soluble compound » is meant compounds that will be soluble in the serum of a patient, so that it will not precipitate and will act on the various engineered immune cells present in patient's blood circulation. A soluble compound according to the invention is preferably a small molecules", but may also be bi-specific antibodies. A "small molecule", as used herein, is a low molecular weight (<2000 daltons) organic compound. Non-limiting examples of small molecules which find application in the present invention include the macrolide rapamycin and its analogs, also known as "rapalogs", such as AP21967, Deforolimus (AP23573), everolimus (RAD001), and temsirolimus (CCI-779). Other non-limiting examples of small molecules which find application in the present invention include tacrolimus (FK506), FK506 derivatives, such as FK1012, FK506 analogs, such as AP1903. Yet other non-limiting examples of small molecules which find application in the present invention include coumermycin, gibberellin, HaXs, AP1510, AP20187 and AP21967. These "small molecules" can rapidly diffuse across cell membranes so that they can reach intracellular sites of action and to bind to a specific protein; they act as an effector, modifying the activity of function of the protein. Libraries of

such compounds can be found, for instance, in http://www.nlm.nih.gov/cgi/mesh/2011/MB_cgi?mode=&term=Small+Molecule+Libraries.

Amongst preferred small molecules, the ones which are particularly suited to the invention are the agents able to dimerize binding domains, called also dimerizing agents, such as rapalogs.

Rapalogs correspond to rapamycin analogs, including rapamycin (sirolimus), CCI779 (temsirolimus) and RAD001 (everolimus). Rapamycin is a macrocyclic antibiotic produced by the bacterium *Streptomyces hygroscopicus* found in the soil of Easter Island. Rapamycin was discovered as a potent antifungal agent, but it also exhibited what was at first considered to be an undesirable immunosuppressive effect, which subsequently led to its development as a clinically useful drug. Amongst the "soluble compounds" envisioned by the invention, one may also contemplate the use of antibodies, and more particularly bispecific and monoclonal antibodies. Like the small molecules and dimerizing agents, bispecific monoclonal antibodies are designed in such a way that their epitopes can bind simultaneously both binding domains borne on IMP complex and/or the CAR structure. Their bindings generally allow co-localization of the inhibitory signaling domains of the IMP complex and/or the CAR, and consequently a modulated action on the CAR.

As few examples, preferred bispecific monoclonal antibodies can be directed to an epitope of the FRB protein and one of the FKBP protein; or to two different epitopes of the FKBP protein; or to an epitope on the KBP protein and one on the CAN protein; or to an epitope on the FKBP protein and one on the CyP-Fas protein; or to two different epitopes of the GyrB protein; or to one epitope on the GAI and one of the GID1 protein. Methods for obtaining bi-specific monoclonal antibodies are well known in the art (Springer T.A, 1985).

Chimeric Antigen Receptors

The present invention aims to produce engineered immune cells in which the level of activation can be modulated through the IMP/CAR system. In order to allow this modulation, the present invention provides with specific design for the production of Chimeric Antigen Receptors (CAR) which are more prompt to interact with the IMP complex, either directly (Figures 1-2) or indirectly (Figure 3).

In general, CAR consists of an extracellular single chain antibody (scFv) fused to the intracellular signaling domain of the T-cell antigen receptor complex zeta chain (scFv:ζ) and have the ability, when expressed in T-cells, to redirect antigen recognition based on the monoclonal antibody's

specificity as described elsewhere (WO2010025177A, WO 2012079000A WO2013126712A, EP2126054A) Several examples of CAR which may be used in the present invention is a CAR directing against CD123 (clones #43 and # 32716 from Cellectis), CS1 and TPBG (5T4) and can comprise as non-limiting example the amino acid sequences : SEQ ID NO:2, SEQ ID NO.3, SEQ ID NO.4 and SEQ ID NO.5.

Recent advances in the immunophenotyping of AML cells have revealed several AML associated cell surface antigens that may act as targets for future therapies. The interleukin 3 receptor alpha chain (IL-3Rα; CD123 – NCBI reference: NP_001254642) has been identified as a potential immunotherapeutic target since it is over-expressed on AML tumor cells compared to normal hematopoietic stem cells. Additionally, two phase I trials for CD123-specific therapeutics have been completed with both drugs displaying good safety profiles (ClinicalTrials.gov ID: NCT00401739 and NCT00397579).

One candidate antigen of immunotherapies for solid tumors, including the colorectal, ovarian and gastric and also for non-solid tumors such as childhood acute lymphoblastic leukemia (ALL) is the trophoblast glycoprotein, also known as TPBG or 5T4 (UniProt: Q13641). 5T4 is often referred to as an oncofetal antigen due to its expression in foetal trophoblast (where it was first discovered) or trophoblast glycoprotein (TPBG). 5T4 protein is an N-glycosylated transmembrane 72 kDa glycoprotein containing seven leucine-rich repeat regions (Hole et al, 1988). The 5T4 antigen was found to be expressed in number of carcinoma including gastric (Starzynska et al. 1995), ovarian and carcinoma (Wrigley et al. 1995). Also, 5T4 oncofetal antigen is expressed in high risk of relapse childhood pre-B acute lymphoblastic leukemia (Castro et al. 2012). It has very limited expression in normal tissue but is widespread in malignant tumors throughout their development (Carsberg et al. 1995).

CS1 (Gene ref: CS1 UNQ576/PRO1138, Uniprot ref: Q9NQ25) is a cell surface glycoprotein belonging to the CD2 subset of the immunoglobulin superfamily (IgSF) and is highly expressed by multiple myeloma cells, but minimally expressed by normal cells (Chu et al. 2013).The new Chimeric Antigen Receptor architectures according to the invention include an additional binding domain, distinct from that antigen recognition domain directed against a component present on the target cell, and that will be reactive with the same soluble component that acts on the IMP protein.

Single chain CAR according to the invention is basically a conventional single chain generation CAR which contains in supplement a binding (or dimerization) domain to the soluble molecule.

Depending of the CID strategy used, the binding (or dimerization) domain may be located extracellularly or intracellularly.

Both 2nd generation CAR (no combination of co-stimulatory molecules) and 3rd generation CARs (combination of co-stimulatory molecules) are contemplated in the scope of the present invention.

In one configuration of the present invention where the binding (or dimerization) domain is extracellular, a CAR according to the invention can contain one or several of the following components:

(a) a signal sequence and one of the 2 antigen-specific targeting regions (either the heavy chain or the light chain of scFV);

(b) optionally a linker;

(c) a binding (or dimerizing) domain

(d) the remaining antigen-specific targeting region (either the heavy chain or the light chain of scFV);

(e) optionally a hinge (or interspacer);

(f) a transmembrane domain;

(g) an intracellular domain containing at least one co-stimulatory molecule.

From the above structure, the position of the binding (or dimerization) domain relative to that the other extracellular components may change.

The invention may also involve a multi chain CAR as previously described by the applicant in WO 2014039523A or in PCT/EP2014/059662.

Both multichain Chimeric Antigen Receptor (mcCAR) and single chain Chimeric Antigen Receptor (scCAR) configurations are encompassed in the scope of the invention. Multi chain CAR structure according to the invention can be adapted from previous multi chain CAR, by introducing a binding (or dimerization) domain, which is sensitive to a soluble compound.

Depending of the CID strategy used, the binding domain may be located extracellularly or intracellularly as shown in Figures 1-2.

In the situation where the multi chain CAR is derived from a Fc receptor as described in WO 2014039523A, the binding (or dimerization) domain may be positioned either on the alpha chain (FCεRα), beta chain FCεRβ) or gamma chain (FCεRγ).

According to a particular embodiment, the engineered immune cells are transfected by a multi-chain based chimeric antigen receptor (CAR) wherein one of the dimerization domains is part of the α-chain.

Such mcCAR can comprise one or a subset of the following components:

- an alpha chain (FCεRα) containing :

(a) a signal sequence and an antigen-specific targeting region;
(b) a binding (or dimerization) domain;
(c) an extracellular spacer domain (hinge);
(d) a transmembrane domain;

- a beta chain (FCεRβ) containing:

(a) a signal sequence;
(b) an extracellular spacer domain (hinge);
(c) a transmembrane domain;
(d) a co-stimulatory ligand;

- a gamma chain (FCεRγ) containing :

(a) a signal sequence;
(b) an extracellular spacer domain (hinge);
(c) a transmembrane domain;
(d) a signal transducing domain.

According to a another particular embodiment, the engineered immune cells are transfected by a multi-chain based chimeric antigen receptor (mcCAR) wherein one of the dimerization domains is part of the γ-chain; such mcCAR can comprise one or a subset of the following components:

- an alpha chain (FCεRα) containing:

(a) a signal sequence and an antigen-specific targeting region;

(b) an extracellular spacer domain (hinge);

(c) a transmembrane domain;

(d) optionally, one or more intracellular domain;

- a beta chain (FCεRβ) containing:

(a) a signal sequence;

(b) an extracellular spacer domain (hinge);

(c) a transmembrane domain;

(d) a co-stimulatory ligand;

- a gamma chain (FCεRγ) containing at least:

(a) a signal sequence;

(b) a binding (or dimerization) domain;

(c) an extracellular spacer domain (hinge);

(d) a transmembrane domain;

(e) a signal transducing domain.

The term “extracellular ligand-binding domain” as used herein is defined as an oligo- or polypeptide that is capable of binding a ligand. Preferably, the domain will be capable of interacting with a cell surface molecule.

Said extracellular ligand-binding domain is a single chain antibody fragment (scFv) comprising the light (V_L) and the heavy (V_H) variable fragment of a target antigen specific monoclonal antibody specific to a particular target antigen joined by a flexible linker.. Binding domain specific to a specific antigen other than scFv can also be used for predefined targeting of lymphocytes, such as camelid or shark (VNAR) single-domain antibody fragments or receptor ligands like a vascular endothelial growth factor polypeptide, an integrin-binding peptide, heregulin or an IL-13 mutein, antibody binding domains, antibody hypervariable loops or CDRs as non-limiting examples.

Said first transmembrane polypeptide may further comprise a stalk region between said extracellular ligand-binding domain and said transmembrane domain. The term “stalk region” used herein generally means any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand-binding domain. In particular, stalk region are used to provide more flexibility and accessibility for the extracellular ligand-binding domain. A stalk region may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids. Stalk region

may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4 or CD28, or from all or part of an antibody constant region. Alternatively the stalk region may be a synthetic sequence that corresponds to a naturally occurring stalk sequence, or may be an entirely synthetic stalk sequence. In a preferred embodiment said stalk region is a part of human CD8 alpha chain (e.g. NP_001139345.1).

Thus, the expression of multi-chain CAR in immune cells results in modified cells that selectively and eliminate defined targets, including but not limited to malignant cells carrying a respective tumor-associated surface antigen or virus infected cells carrying a virus-specific surface antigen, or target cells carrying a lineage-specific or tissue-specific surface antigen.

A multi-chain CAR according to the invention can comprise several extracellular ligand-binding domains, to simultaneously bind different elements in target thereby augmenting immune cell activation and function. The extracellular ligand-binding domains may be placed in tandem on the same transmembrane polypeptide, and optionally can be separated by a linker. In another embodiment, said different extracellular ligand-binding domains can be placed on different transmembrane polypeptides composing the multi-chain CAR. The present invention may relate to a population of multi-chain CARs comprising each one different extracellular ligand binding domains.

Also, the invention may encompass engineered immune cells which express at the surface of said cell a population of multi-chain CAR each one comprising different extracellular ligand binding domains. The present invention may relate to engineered immune cell in which are introduced polynucleotides encoding polypeptides composing a population of multi-chain CAR each one comprising different extracellular ligand binding domains. By population of multi-chain CARs, it is meant at least two, three, four, five, six or more multi-chain CARs each one comprising different extracellular ligand binding domains. The different extracellular ligand binding domains according to the present invention may simultaneously bind different elements in target thereby augmenting immune cell activation and function.

The signal transducing domain or intracellular signaling domain of the multi-chain CAR of the invention is responsible for intracellular signaling following the binding of extracellular ligand binding domain to the target resulting in the activation of the immune cell and immune response. In other words, the signal transducing domain is responsible for the activation of at least one of the normal effector functions of the immune cell in which the multi-chain CAR is expressed. For example, the effector function of a T cell can be a cytolytic activity or helper activity including the secretion of cytokines. Thus, the term “signal transducing domain” refers to the portion of a protein which transduces the effector signal function signal and directs the cell to perform a specialized function.

Examples of signal transducing domain for use in multi-chain CAR can be the cytoplasmic sequences of the Fc receptor or T cell receptor and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivate or variant of these sequences and any synthetic sequence that as the same functional capability. Signal transduction domain comprises two distinct classes of cytoplasmic signaling sequence, those that initiate antigen-dependent primary activation, and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal. Primary cytoplasmic signaling sequence can comprise signaling motifs which are known as immunoreceptor tyrosine-based activation motifs of ITAMs. ITAMs are well defined signaling motifs found in the intracytoplasmic tail of a variety of receptors that serve as binding sites for syk/zap70 class tyrosine kinases. Examples of ITAM used in the invention can include as non limiting examples those derived from TCRzeta, FcRgamma, FcRbeta, FcRepsilon, CD3gamma, CD3delta, CD3epsilon, CD5, CD22, CD79a, CD79b and CD66d. In a preferred embodiment, the signaling transducing domain of the multi-chain CAR can comprise the CD3zeta signaling domain, or the intracytoplasmic domain of the FcεRI beta or gamma chains.

The signal transduction domain of the multi-chain CAR of the present invention may comprise a co-stimulatory signal molecule. A co-stimulatory molecule is a cell surface molecule other than an antigen receptor or their ligands that is required for an efficient immune response.

"Co-stimulatory ligand" refers to a molecule on an antigen presenting cell that specifically binds a cognate co-stimulatory molecule on a T-cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation activation, differentiation and the like. A co-stimulatory ligand can include but is not limited to CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory igand (ICOS-L), intercellular adhesion molecule (ICAM, CD30L, CD40, CD70, CD83, HLA-G, MICA, M1CB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as but not limited to, CD27, CD28, 4-IBB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LTGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83.

A "co-stimulatory molecule" refers to the cognate binding partner on a T-cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the cell, such as, but not limited to proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and Toll ligand receptor. Examples of costimulatory molecules include

CD27, CD28, CD8, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3 and a ligand that specifically binds with CD83 and the like.

Said signal transducing domain may be a TNFR-associated Factor 2 (TRAF2) binding motifs, intracytoplasmic tail of costimulatory TNFR member family. Cytoplasmic tail of costimulatory TNFR family member contains TRAF2 binding motifs consisting of the major conserved motif (P/S/A)X(Q/E)E) or the minor motif (PXQXXD), wherein X is any amino acid. TRAF proteins are recruited to the intracellular tails of many TNFRs in response to receptor trimerization.

The signal transduction domain of the multi-chain CAR of the present invention may comprise a part of co-stimulatory signal molecule which is 4-1BB (GenBank: AAA53133.).

The distinguishing features of appropriate transmembrane polypeptides comprise the ability to be expressed at the surface of an immune cell, in particular lymphocyte cells or Natural killer (NK) cells, and to interact together for directing cellular response of immune cell against a predefined target cell. The different transmembrane polypeptides of the multi-chain CAR of the present invention comprising an extracellular ligand-biding domain and/or a signal transducing domain interact together to take part in signal transduction following the binding with a target ligand and induce an immune response. The transmembrane domain can be derived either from a natural or from a synthetic source. The transmembrane domain can be derived from any membrane-bound or transmembrane protein. As non-limiting examples, the transmembrane polypeptide can be a subunit of the T cell receptor such as α, β, γ orζ, polypeptide constituting CD3 complex, IL2 receptor p55 (α chain), p75 (β chain) or γ chain, subunit chain of Fc receptors, in particular Fcγ receptor III or CD proteins. Alternatively the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine.

The multi-chain CAR may comprise a transmembrane polypeptide derived from a FcεRI chain, such as a FcεRI α chain, in which the extracellular domain is replaced by an extracellular ligand-binding domain.

Said multi-chain CAR may comprise a part of FcεRI alpha chain and a part of FcεRI beta chain or variant thereof such that said FcεRI chains spontaneously dimerize together to form a dimeric Chimeric Antigen Receptor. The multi-chain Chimeric Antigen may comprise a part of FcεRI alpha chain and a part of a FcεRI gamma chain or variant thereof such that said FcεRI chains spontaneously trimerize together to form a trimeric Chimeric Antigen Receptor. Another alternative is a multi-chain Chimeric Antigen Receptor which may comprise a part of FcεRI alpha chain, a part of

FcεRI beta chain and a part of FcεRI gamma chain or variants thereof such that said FcεRI chains spontaneously tetramerize together to form a tetrameric Chimeric Antigen Receptor.

Polynucleotides, vectors:

The present invention also relates to polynucleotides, vectors encoding the above described modulable/tunable IMP complex and CAR polypeptide structures according to the invention. The present invention provides polynucleotides, including DNA and RNA molecules that encode the transmembrane polypeptides disclosed herein that can be included in the multi-chain or mono-chain CAR. In particular, the invention relates to a polynucleotide comprising a nucleic acid sequence encoding at least one transmembrane polypeptide composing the multi-chain/mono-chain CAR as described above. More particularly the invention relates to a polynucleotide comprising two or more nucleic acid sequences encoding transmembrane polypeptides composing the multi-chain/mono-chain CAR as described above.

The polynucleotide may consist in an expression cassette or expression vector (e.g. a plasmid for introduction into a bacterial host cell, or a viral vector such as a baculovirus vector for transfection of an insect host cell, or a plasmid or viral vector such as a lentivirus for transfection of a mammalian host cell).

According to one embodiment, the transfection realized during the method of the invention for producing an engineered immune is performed using of a lentiviral or a retroviral vector for a stable integration of the CAR and/or IMP into the immune cells genome

According to another embodiment, said transfection is performed by the use of CAR/IMP encoding polycistronic mRNA for a transitory expression.

The different nucleic acid sequences can be included in one polynucleotide or vector which comprises a nucleic acid sequence encoding ribosomal skip sequence such as a sequence encoding a 2A peptide. 2A peptides, which were identified in the Aphthovirus subgroup of picornaviruses, causes a ribosomal "skip" from one codon to the next without the formation of a peptide bond between the two amino acids encoded by the codons (see Donnelly et al., J. of General Virology 82: 1013-1025 (2001); Donnelly et al., J. of Gen. Virology 78: 13-21 (1997); Doronina et al., Mol. And. Cell. Biology 28(13): 4227-4239 (2008); Atkins et al., RNA 13: 803-810 (2007)). By "codon" is meant three nucleotides on an mRNA (or on the sense strand of a DNA molecule) that are translated by a ribosome into one amino acid residue. Thus, two polypeptides can be synthesized from a single, contiguous open reading frame within an mRNA when the polypeptides are separated by a 2A

oligopeptide sequence that is in frame. Such ribosomal skip mechanisms are well known in the art and are known to be used by several vectors for the expression of several proteins encoded by a single messenger RNA.

To direct, transmembrane polypeptide such as FcεR into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in polynucleotide sequence or vector sequence. The secretory signal sequence may be that of FcεR, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo.* The secretory signal sequence is operably linked to the transmembrane nucleic acid sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleic acid sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleic acid sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830). In a preferred embodiment the signal peptide comprises the residues 1 to 25 of the FcεRI alpha chain (NP_001992.1).

Those skilled in the art will recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. Preferably, the nucleic acid sequences of the present invention are codon-optimized for expression in mammalian cells, preferably for expression in human cells. Codon-optimization refers to the exchange in a sequence of interest of codons that are generally rare in highly expressed genes of a given species by codons that are generally frequent in highly expressed genes of such species, such codons encoding the amino acids as the codons that are being exchanged.

<u>Engineered immune cells</u>

The present invention also relates to an isolated immune cell which is a Chimeric Antigen Receptor (CAR) having at least an engineered (IMP) inhibitory membrane protein.

The immune cell to be used in accordance of the present invention may, for instance, be a T-cell, a cytotoxic T cell, a regulatory T cell or a NK cell.

According to a particular embodiment, the immune cell is a T-cell.

Said engineered immune cells may be infused into a patient upon the step of IMP complex transfection and before the step of incubation with the external ligand (such as a surface antigen).

According to certain embodiment, the present invention provides a modulable/tunable CAR immune cell which is also engineered to be allogeneic. This non-autologous characteristic is particularly useful for the development of a standardized therapy in which allogeneic therapeutic cells could be pre-manufactured and available for immediate administration to patients.

Such methods comprise the step of inactivating at least one gene encoding a T-Cell Receptor (TCR) component, in particular TCRα, TCRβ genes.

The step of inactivation of, for instance the TCR component may be performed at any time during the manufacturing of the modulable/tunable CAR-engineered immune cell of the invention. However, it is preferable to perform it before the transfection steps in order to not interact too much on the growth/expansion of the cells.

Is also encompassed in the scope of the present invention, the possibility to engineer further the immune cells (i.e T-cells) to make them resistant to chemotherapy drugs, such as purine nucleotide analogs (clofarabine, flubarabine…).

One way particularly suitable according to the invention to realize these gene inactivations is the use of exonucleases such as TALE nucleases. This can be performed such as described elsewhere (WO2012138927 and prior art cited in the next section “DEFINITIONS”).

Methods of engineering immune cells

The present invention also relates to a method for producing an modulable/tunable CAR-engineered immune cell comprising two steps of transfection, the first one being of at least a first polynucleotide encoding a Chimeric Antigen Receptor (CAR); and the second one of a second polynucleotide having the inhibitory membrane protein (IMP) complex, and finally a step of selection of immune cells having co-expressed the said polynucleotides.

In another embodiment, the present invention relates to a method of producing engineered immune cells for immunotherapy comprising introducing into said cells the different polypeptides composing said CAR/IMP and expanding said cells. In a preferred embodiment, said polynucleotides are included in lentiviral vectors in view of being stably expressed in the cells. Such lentivirus vectors may comprise a marker gene such as GFP and/or firefly luciferase.

In still another embodiment, said transfection step a) in the method of producing engineered immune cells is performed by insertion of the CAR and IMP polynucleotides via recombination process, preferably homologous. This embodiment has the advantage to a more

specific integration, and less ectopic insertions. In a preferred embodiment, such site-specific insertion is made by expressing an exogenous specific-endonuclease and the addition of at least one polynucleotide template encoding said CAR/IMP peptides. As an example, the site-specific insertion may be done in the TCR gene, which allowsreducing or disabling TCR (T cell receptor) function, with the effect of rendering the immune cells, in particular T-cells less alloreactive .This later aspect is particular sought to reduce the risk of GVHD in a context of immunotherapy where the immune cells are allogeneic.

Specific nucleases usually create double-stranded break (DSBs) at desired locations in the genome, and harness the cell's endogenous mechanisms to repair the induced break by natural processes of homologous recombination (HR) and non-homologous end-joining (NHEJ). Engineered nucleases such as zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), engineered homing endonucleases and RNA or DNA guided endonucleases, such as CRISPR/Cas , CPF1 or Argonaute systems, are particularly appropriate to carry out gene insertion or inactivation according to the present invention.

Among other references, TALEN and Cas9 systems are respectively described in WO 2013/176915 and WO 2014/191128. The Zinc-finger nucleases (ZFNs) have been initially described in Kim, YG; Cha, J.; Chandrasegaran, S. (1996). "Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain". Proc Natl Acad Sci USA 93 (3): 1156–60.

Delivery methods

The different methods described above involve expressing a protein of interest such as IMP complex, Chimeric Antigen Receptor (CAR) into a cell. Polypeptides may be expressed in the cell as a result of the introduction of polynucleotides encoding said polypeptides into the cell. Alternatively, said polypeptides could be produced outside the cell and then introduced thereto. Methods for introducing a polynucleotide construct into cells are known in the art and include as non limiting examples stable transformation methods wherein the polynucleotide construct is integrated into the genome of the cell, transient transformation methods wherein the polynucleotide construct is not integrated into the genome of the cell and virus mediated methods. Said polynucleotides may be introduced into a cell by for example, recombinant viral vectors (e.g. retroviruses, adenoviruses), liposome and the like. For example, transient transformation methods include for example microinjection, electroporation or particle bombardment. Said polynucleotides may be included in

vectors, more particularly plasmids or virus, in view of being expressed in cells. Said plasmid vector can comprise a selection marker which provides for identification and/or selection of cells which received said vector. Different transgenes can be included in one vector. Said vector can comprise a nucleic acid sequence encoding ribosomal skip sequence such as a sequence encoding a 2A peptide. 2A peptides, which were identified in the Aphthovirus subgroup of picornaviruses, causes a ribosomal "skip" from one codon to the next without the formation of a peptide bond between the two amino acids encoded by the codons (see Donnelly et al., J. of General Virology 82: 1013-1025 (2001); Donnelly et al., J. of Gen. Virology 78: 13-21 (1997); Doronina et al., Mol. And. Cell. Biology 28(13): 4227-4239 (2008); Atkins et al., RNA 13: 803-810 (2007)). By "codon" is meant three nucleotides on an mRNA (or on the sense strand of a DNA molecule) that are translated by a ribosome into one amino acid residue. Thus, two polypeptides can be synthesized from a single, contiguous open reading frame within an mRNA when the polypeptides are separated by a 2A oligopeptide sequence that is in frame. Such ribosomal skip mechanisms are well known in the art and are known to be used by several vectors for the expression of several proteins encoded by a single messenger RNA.

Polynucleotides encoding polypeptides according to the present invention can be mRNA which is introduced directly into the cells, for example by electroporation. The inventors determined the optimal condition for mRNA electroporation in T-cell. The inventor used the cytoPulse technology which allows, by the use of pulsed electric fields, to transiently permeabilize living cells for delivery of material into the cells. The technology, based on the use of PulseAgile (BTX Havard Apparatus, 84 October Hill Road, Holliston, MA 01746, USA) electroporation waveforms grants the precise control of pulse duration, intensity as well as the interval between pulses (U.S. patent 6,010,613 and International PCT application WO2004083379). All these parameters can be modified in order to reach the best conditions for high transfection efficiency with minimal mortality. Basically, the first high electric field pulses allow pore formation, while subsequent lower electric field pulses allow to move the polynucleotide into the cell.

Activation and expansion of T-cells

Whether prior to or after genetic modification of the T-cells, the T-cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005. T-cells can be expanded *in vitro* or *in vivo.* Generally, the T cells of the invention are expanded by contact with an agent that stimulates a CD3 TCR complex and a co-

stimulatory molecule on the surface of the T-cells to create an activation signal for the T-cell. For example, chemicals such as calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), or mitogenic lectins like phytohemagglutinin (PHA) can be used to create an activation signal for the T-cell. As non limiting examples, T-cell populations may be stimulated *in vitro* such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T-cells, a ligand that binds the accessory molecule is used. For example, a population of T-cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T-cells. To stimulate proliferation of either CD4+ T-cells or CD8+ T-cells, an anti-CD3 antibody and an anti-CD28 antibody. For example, the agents providing each signal may be in solution or coupled to a surface. As those of ordinary skill in the art can readily appreciate, the ratio of particles to cells may depend on particle size relative to the target cell.

Conditions appropriate for T-cell culture include an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-vivo 5, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-g , 1L-4, 1L-7, GM-CSF, -10, - 2, 1L-15, TGFp, IL-21 and TNF- or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, A1M-V, DMEM, MEM, a-MEM, F-12, X-Vivo 1, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T-cells. Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth; for example, an appropriate temperature (e.g., 37° C) and atmosphere (e.g., air plus 5% C02). T cells that have been exposed to varied stimulation times may exhibit different characteristics.

Therapeutic applications

In another embodiment, isolated immune cells obtained as previously described can be used in adoptive cell immunotherapy.

According to an alternative embodiment, said immunes cells are recovered from the patient himself. According to one embodiment, said immune cells are recovered from donors.

According to another embodiment, said immune cells are rendered allogeneic in order to prepare batch of cells which can be administered to a panel of patients and not only the donor himself. This feature, which represents a real advantage in economic terms, is compulsory to drop the risk of graft rejection.

To achieve this goal, the immune cells such as T-cells undergo a knock-out (KO) in the T-cell receptor (TCR) gene that is responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. By inactivating the TCR gene, the latter cannot engage with antigenic peptide and MHC (peptide/MHC), and therefore there is not activation of the T lymphocyte

According to an embodiment, the inactivation of the TCR gene by knock-out (KO) is performed by endonucleases, preferably by TALE-nucleases.

In particular, said T-cells according to the present invention can be used for treating cancer , infections or auto-immune disease in a patient in need thereof.

In another aspect, the present invention relies on methods for treating patients in need thereof, said method comprising at least one of the following steps:

(a) providing an isolated T-cell obtainable by any one of the methods previously described;

(b) Administrating said cells to said patient.

Said T-cells of the invention can undergo robust *in vivo* expansion and can persist for an extended amount of time.

Said treatment can be ameliorating, curative or prophylactic. This may be done under standard protocols and reproduced as many times as needed. The resulting modified T-cells may be administrated to the patient or to one or several patients depending of its availability as an “off the shelf” therapeutic product.

Cells that can be used with the disclosed methods are described in the previous section. Said treatment can be used to treat patients diagnosed with cancer, viral infection and autoimmune disorders. Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. The cancers may comprise nonsolid tumors

(such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with the allogeneic T-cell resistant to drugs of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included. In an embodiment of the present invention, childhood acute lymphoblastic leukemia (ALL) and amyotrophic myeloma leukemia (AML) diseases are typically treated by allogeneic drug resistant T-cells according to the invention. This can be achieved by using drug resistant KO TRAC $CD19^+$ CAR T-cells and drug resistant KO TRAC $CD123^+$ T-cells respectively. “TRAC” refers to “T cell receptor α constant“» and corresponds to TCRα subunit constant gene.

It can be a treatment in combination with one or more therapies against cancer selected from the group of antibodies therapy, chemotherapy, cytokines therapy, dendritic cell therapy, gene therapy, hormone therapy, laser light therapy and radiation therapy.

Said treatment may be administrated into patients undergoing an immunosuppressive treatment, such as cells or population of cells, which have been made resistant to at least one drug agent due to either expression of a drug resistance gene or the inactivation of a drug sensitizing gene. In this aspect, the drug treatment should help the selection and expansion of the T-cells according to the invention within the patient.

The administration of the cells or population of cells according to the present invention may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermaliy, intratumorally, intranodally, intramedullary, intramuscularly, intracranially, by intravenous or intralymphatic injection, or intraperitoneally. In one embodiment, the cell compositions of the present invention are preferably administered by intravenous injection.

The administration of the cells or population of cells can consist of the administration of 10^3-10^{10} cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. The cells or population of cells can be administrated in one or more doses. In another embodiment, said effective amount of cells are administrated as a single dose. In another embodiment, said effective amount of cells are administrated as more than one dose over a period time. Timing of administration is within the judgment of managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained

from any source, such as a blood bank or a donor. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions within the skill of the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administrated will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired.

Said effective amount of cells or pharmaceutical composition may be preferably administrated parenterally. Said administration can be an intravenous administration. Said administration can be directly done by injection within a tumor.

Cells may be administered to a patient in conjunction with (e.g., before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or nataliziimab treatment for MS patients or efaliztimab treatment for psoriasis patients or other treatments for PML patients. The T-cells of the invention may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycoplienolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin) (Liu et al., Cell 66:807-815, 1 1; Henderson et al., Immun. 73:316-321, 1991; Bierer et al., Citrr. Opin. mm n. 5:763-773, 93). The cell compositions of the present invention may be administered to a patient in conjunction with (e.g., before, simultaneously or following) bone marrow transplantation, T-cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. The cell compositions of the present invention may be administered following B-cell ablative therapy such as agents that react with CD20, e.g., Rituxan. For example, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. Following the transplant, subjects may receive an infusion of the expanded immune cells of the present invention. Expanded cells may be administered before or following surgery.

Pharmaceutical composition

The isolated T-cells of the present invention may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components. Briefly, pharmaceutical compositions of the present invention may comprise T-cells as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g. aluminum hydroxide); and preservatives. Compositions of the present invention are preferably formulated for intravenous administration. Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials

Kit and vector

The present invention encompasses a kit or a vector for engineering immune cells with modulable level of activation, which comprises:

-a first polynucleotide encoding a Chimeric Antigen Receptor (CAR); and

- a second polynucleotide encoding an engineered inhibitory membrane protein (IMP) complex as described above in the “Inhibitory membrane complex” paragraph.

DEFINITIONS

In the description above, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the present embodiments.

- Amino acid residues in a polypeptide sequence are designated herein according to the one-letter code, in which, for example, Q means Gln or Glutamine residue, R means Arg or Arginine residue and D means Asp or Aspartic acid residue.

- Nucleotides are designated as follows: one-letter code is used for designating the base of a nucleoside: a is adenine, t is thymine, c is cytosine, and g is guanine. For the degenerated

nucleotides, r represents g or a (purine nucleotides), k represents g or t, s represents g or c, w represents a or t, m represents a or c, y represents t or c (pyrimidine nucleotides), d represents g, a or t, v represents g, a or c, b represents g, t or c, h represents a, t or c, and n represents g, a, t or c.

- As used herein, “nucleic acid” or “nucleic acid molecule” refers to nucleotides and/or polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Nucleic acids can be either single stranded or double stranded.

- By “gene” is meant the basic unit of heredity, consisting of a segment of DNA arranged in a linear manner along a chromosome, which codes for a specific protein or segment of protein, small RNA and the like. A gene typically includes a promoter, a 5' untranslated region, one or more coding sequences (exons), optionally introns, a 3' untranslated region. The gene may further comprise a terminator, enhancers and/or silencers.

- By “genome” it is meant the entire genetic material contained in a cell such as nuclear genome, chloroplastic genome, mitochondrial genome.

- By “mutation” is intended the substitution, deletion, insertion of one or more nucleotides/amino acids in a polynucleotide (cDNA, gene) or a polypeptide sequence. Said mutation can affect the coding sequence of a gene or its regulatory sequence. It may also affect the structure of the genomic sequence or the structure/stability of the encoded mRNA.

“TALE-nuclease” refers to engineered proteins resulting from the fusion of a DNA binding domain typically derived from Transcription Activator like Effector proteins (TALE), with an endonuclease catalytic domain. Such catalytic domain is preferably a nuclease domain and more preferably a domain having endonuclease activity, like for instance I-TevI, ColE7, NucA and Fok-I. In a particular embodiment, said nuclease is a monomeric TALE-Nuclease . A monomeric Nuclease is a nuclease that does not require dimerization for specific recognition and cleavage, such as the fusions of engineered DNA binding domain with the catalytic domain of I-TevI described in WO2012138927. In another particular embodiment, said rare-cutting endonuclease is a dimeric TALE-nuclease, preferably comprising a DNA binding domain fused to FokI. TALE-nuclease have been already described and used to stimulate gene targeting and gene modifications (Boch, Scholze et al. 2009;

Moscou and Bogdanove 2009; Christian, Cermak et al. 2010). Such engineered TALE-nucleases are commercially available under the trade name TALEN™ (Cellectis, 8 rue de la Croix Jarry, 75013 Paris, France).

- The term “cleavage” refers to the breakage of the covalent backbone of a polynucleotide. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. Double stranded DNA, RNA, or DNA/RNA hybrid cleavage can result in the production of either blunt ends or staggered ends.

- By “chimeric antigen receptor “(CAR) it is meant a chimeric receptor which comprises an extracellular ligand-binding domain, a transmembrane domain and a signaling transducing domain.

- The term “extracellular ligand-binding domain” as used herein is defined as an oligo- or polypeptide that is capable of binding a ligand. Preferably, the domain will be capable of interacting with a cell surface molecule. For example, the extracellular ligand-binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state.

In a preferred embodiment, said extracellular ligand-binding domain comprises a single chain antibody fragment (scFv) comprising the light (V_L) and the heavy (V_H) variable fragment of a target antigen specific monoclonal antibody joined by a flexible linker. In a preferred embodiment, said scFV is derived from a CD123, 5T4 or CS1 antibody.

The signal transducing domain or intracellular signaling domain of the CAR according to the present invention is responsible for intracellular signaling following the binding of extracellular ligand binding domain to the target resulting in the activation of the immune cell and immune response. Preferred examples of signal transducing domain for use in a CAR can be the cytoplasmic sequences of the T-cell receptor and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement. Signal transduction domain comprises two distinct classes of cytoplasmic signaling sequence, those that initiate antigen-dependent primary activation, and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal. Primary cytoplasmic signaling sequence can comprise signaling motifs which are known as immunoreceptor tyrosine-based activation motifs of ITAMs. In particular embodiment the signal transduction domain of the CAR of the present invention comprises a co-stimulatory signal molecule. A co-stimulatory

molecule is a cell surface molecule other than an antigen receptor or their ligands that is required for an efficient immune response. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and Toll ligand receptor. Examples of costimulatory molecules include CD27, CD28, CD8, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3 and a ligand that specifically binds with CD83 and the like.

The CAR according to the present invention is expressed on the surface membrane of the cell. Thus, the CAR can comprise a transmembrane domain. The distinguishing features of appropriate transmembrane domains comprise the ability to be expressed at the surface of a cell, preferably in the present invention an immune cell, in particular lymphocyte cells or Natural killer (NK) cells, and to interact together for directing cellular response of immune cell against a predefined target cell. The transmembrane domain can further comprise a stalk region_between said extracellular ligand-binding domain and said transmembrane domain. The term “stalk region” used herein generally means any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand-binding domain. In particular, stalk region are used to provide more flexibility and accessibility for the extracellular ligand-binding domain. A stalk region may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids. Stalk region may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4 or CD28, or from all or part of an antibody constant region. Alternatively the stalk region may be a synthetic sequence that corresponds to a naturally occurring stalk sequence, or may be an entirely synthetic stalk sequence.

Downregulation or mutation of target antigens is commonly observed in cancer cells, creating antigen-loss escape variants. Thus, to offset tumor escape and render immune cells more specific to target, the CD19 specific CAR can comprise another extracellular ligand-binding domains, to simultaneously bind different elements in target thereby augmenting immune cell activation and function. Examples of CD19 specific CAR are ScFv FMC63 (Kochenderfer JN, Wilson WH, Janik JE, et al. *Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. Blood 2010;116(20):4099-410)* or ScFv 4G7 CAR (described in the application filed under the number PCT/EP2014/059662). In one embodiment, the extracellular ligand-binding domains can be placed in tandem on the same transmembrane polypeptide, and optionally can be separated by a linker. In another embodiment, said different extracellular ligand-binding domains can be placed on different transmembrane polypeptides composing the CAR. In another embodiment, the present invention relates to a population of CARs comprising each one different extracellular ligand binding domains. In a particular, the present invention relates to a

method of engineering immune cells comprising providing an immune cell and expressing at the surface of said cell a population of CAR each one comprising different extracellular ligand binding domains. In another particular embodiment, the present invention relates to a method of engineering an immune cell comprising providing an immune cell and introducing into said cell polynucleotides encoding polypeptides composing a population of CAR each one comprising different extracellular ligand binding domains. By population of CARs, it is meant at least two, three, four, five, six or more CARs each one comprising different extracellular ligand binding domains. The different extracellular ligand binding domains according to the present invention can preferably simultaneously bind different elements in target thereby augmenting immune cell activation and function. The present invention also relates to an isolated immune cell which comprises a population of CARs each one comprising different extracellular ligand binding domains.

- The terms "vector" refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. A “vector” in the present invention includes, but is not limited to, a viral vector, a plasmid, a RNA vector or a linear or circular DNA or RNA molecule which may consists of a chromosomal, non chromosomal, semi-synthetic or synthetic nucleic acids. Preferred vectors are those capable of autonomous replication (episomal vector) and/or expression of nucleic acids to which they are linked (expression vectors). Large numbers of suitable vectors are known to those of skill in the art and commercially available.

- By “ delivery vector” is intended any delivery vector which can be used in the present invention to put into cell contact (i.e “contacting”) or deliver inside cells or subcellular compartments (i.e “introducing”) agents/chemicals and molecules (proteins or nucleic acids) needed in the present invention. It includes, but is not limited to liposomal delivery vectors, viral delivery vectors, drug delivery vectors, chemical carriers, polymeric carriers, lipoplexes, polyplexes, dendrimers, microbubbles (ultrasound contrast agents), nanoparticles, emulsions or other appropriate transfer vectors.

- Viral vectors include retrovirus, adenovirus, parvovirus (e. g. adenoassociated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e. g., influenza virus), rhabdovirus (e. g., rabies and vesicular stomatitis virus), paramyxovirus (e. g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus, herpesvirus (e. g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e. g. vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example.

Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J. M., Retroviridae: The viruses and their replication, In Fundamental Virology, Third Edition, B. N. Fields, et al., Eds., Lippincott-Raven Publishers, Philadelphia, 1996).

- By “lentiviral vector” is meant HIV-Based lentiviral vectors that are very promising for gene delivery because of their relatively large packaging capacity, reduced immunogenicity and their ability to stably transduce with high efficiency a large range of different cell types. Lentiviral vectors are usually generated following transient transfection of three (packaging, envelope and transfer) or more plasmids into producer cells. Like HIV, lentiviral vectors enter the target cell through the interaction of viral surface glycoproteins with receptors on the cell surface. On entry, the viral RNA undergoes reverse transcription, which is mediated by the viral reverse transcriptase complex. The product of reverse transcription is a double-stranded linear viral DNA, which is the substrate for viral integration in the DNA of infected cells. By “integrative lentiviral vectors (or LV)”, is meant such vectors as non limiting example, that are able to integrate the genome of a target cell. At the opposite by “non-integrative lentiviral vectors (or NILV)” is meant efficient gene delivery vectors that do not integrate the genome of a target cell through the action of the virus integrase.

- By cell or cells is intended any eukaryotic living cells, primary cells and cell lines derived from these organisms for *in vitro* cultures.

- By “primary cell” or “primary cells” are intended cells taken directly from living tissue (i.e. biopsy material) and established for growth in vitro, that have undergone very few population doublings and are therefore more representative of the main functional components and characteristics of tissues from which they are derived from, in comparison to continuous tumorigenic or artificially immortalized cell lines. As non limiting examples cell lines can be selected from the group consisting of CHO-K1 cells; HEK293 cells; Caco2 cells; U2-OS cells; NIH 3T3 cells; NSO cells; SP2 cells; CHO-S cells; DG44 cells; K-562 cells, U-937 cells; MRC5 cells; IMR90 cells; Jurkat cells; HepG2 cells; HeLa cells; HT-1080 cells; HCT-116 cells; Hu-h7 cells; Huvec cells; Molt 4 cells.

- Because some variability may arise from the genomic data from which these polypeptides derive, and also to take into account the possibility to substitute some of the amino acids present in these polypeptides without significant loss of activity (functional variants), the invention encompasses polypeptides variants of the above polypeptides that share at least 70%, preferably at least 80 %, more preferably at least 90 % and even more preferably at least 95 % identity with the sequences provided in this patent application.

The present invention is thus drawn to polypeptides comprising a polypeptide sequence that has at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with amino acid sequence selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 60.

-"identity" refers to sequence identity between two nucleic acid molecules or polypeptides. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST which are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default setting. For example, polypeptides having at least 70%, 85%, 90%, 95%, 98% or 99% identity to specific polypeptides described herein and preferably exhibiting substantially the same functions, as well as polynucleotide encoding such polypeptides, are contemplated.

In addition to a general method for engineering T-cells resistant to purine nucleotide analogs (PNA) drugs , in particular clofarabine and fludarabine, by endonuclease inactivation of dcK gene(s).

GENERAL METHODS

Primary cells

Peripheral blood mononuclear cells are isolated by density gradient centrifugation from buffy coats from healthy volunteer donors (Etablissement Français du Sang). T lymphocytes are then purified using the EasySep human T cell enrichment kit (Stemcell Technologies), and activated with Dynabeads Human T-Activator CD3/CD28 (Life Technologies) in X-vivo 15 medium (Lonza) supplemented with 20 ng/ml IL-2 (Miltenyi) and 5% human AB serum (Seralab).

Cell lines

The CD123-positive cell lines such as KG1a or MOLM13 and CD123-negative cell line such as RPMI-8226 for multiple myeloma (MM) are obtained from the American Type Culture

Collection. Typically, the cells are cultured in RPMI 1640 supplemented with 10-20% heat–inactivated FCS, 2mmol/L L-glutamine and 100 units/ml penicillin, and 100µg/mL streptomycin.

Synthesis of IMP/CAR encoding mRNAs

All individual chains of the CAR architecture were amplified by PCR to introduce a T7 promoter and a stop codon sequence prior to mRNA synthesis. mRNA encoding the proteins of interest were in vitro transcribed from the PCR product and polyadenylated using the mMessage mMachine T7 Ultra kit (Life technologies) following the manufacturer's instructions. RNAs were purified with RNeasy columns (Qiagen), eluted in cytoporation medium T and quantified by measuring absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer. Quality of the RNA was verified on a denaturing formaldehyde/MOPS agarose gel.

Transiently expression in T cells

The switch system of the present invention constituted of a CAR and a second polynucleotide encoding an engineered inhibitory membrane protein (IMP) complex are expressed in human T cells after electroporation of polycistronic mRNA. T cells were electroporated with capped and polyadenylated polycistronic mRNA that were produced using the mMESSAGE mMACHINE kit and linearized plasmids as template. The plasmids used as template contained the T7 RNA polymerase promoter followed by a polycistronic DNA sequence encoding the different CAR/IMP variants.

The electroporation of the polycistronic mRNAs into the human T cells is proceeded using the CytoLVT-S device (Cellectis), according to the following protocol: $5X10^6$ T cells preactivated several days (3-5) with anti CD3/CD28 coated beads and IL2 are resuspended in cytoporation buffer T, and electroporated with 45µg of mRNA. Twenty-four hours after electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs are labeled with a fixable viability dye eFluor-780 and a PE-conjugated goat anti mouse IgG F(ab')2 fragment specific, and analysed by flow cytometry.

The live T cells engineered using polycistronic mRNAs express the multi-chain CARs on their surface.

Lenviral expression in T cells

In vitro screening of CAR-specific IMP/CAR

The polycistronic genes encoding a CAR and a second polynucleotide encoding an engineered inhibitory membrane protein (IMP) complex are vectorized in human T cells using lentiviral vectors as reported previously. Firstly the cell surface expression profile is assessed over time of the 2 above polynucleotide-encoded peptides in transduced T cells. For that purpose, the anti-CD123 scFv /Fc fusion protein may be used. Typically, it is observed that both peptides are highly expressed on the cell surface 3 days post transduction and remained relatively highly expressed over a 2 weeks period. The capacity of both peptides is then assessed to mediate antigen-dependent T cells activation. To address this issue, activity assays is performed using a CD123 - positive cell line (as KG1 or MOLM13), and a CD123-negative cell line (RPMI-8226). It is generally observed that both peptides are able to activate T cells in the presence of CD123-positive cell line but not in the presence ofCD123-negative cell line as shown with the results of the degranulation assay, the cytotoxicity assay and the IFNγ secretion assay.

EXAMPLES

Example 1. Assembly of IMPs (switch-off receptors) and engineered multi chain CARs (mcCARs)

Assembly of constructs are done using Golden Gate assembly strategies according to published protocols [such as Engler C, Kandzia R, Marillonnet S; (2008) "*A one pot, one step, precision cloning method with high throughput capability*",PLoS One. 3(11)]. Switch-off receptors and engineered CARs are thus cloned in separated shuttle cloning plasmid. Non exhaustive examples for assembled constructs are given in the following Table 5; all of the systems presented here are “switch OFF”.

Sequences of modules to assemble IMPs (switch-off receptors) are obtained from the SEQ ID NO. 61-64 (extracellular CID binding domain) or 83-98 (intracellular CID binding domain) and from engineered mcCAR with SEQ ID NO.65-76 (extracellular CID binding domain) or 99-106 (intracellular CID binding domain). Their configuration is depicted in the Figures 2A and 2B.

Table 5: Switch OFF receptors and engineered CARs

Component	Architecture: Signal sequenc / Interacting partr / Hinge / transmembrane / intracellular	SEQ ID NO.	Aminoacid sequence
EXTRA CELLULAR CID BINDING DOMAINS			
IMP complex (switch OFF receptor)	CD8a-ss FKBP CD8a CD8a PD1	61	MALPVTALLLPLALLLHAARPGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRG WEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPTTTPAPRPPTPAPTIASQPLSLRPEACR PAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCRSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWRE KTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE		
	CD8a-ss FRB IgG1 4-1BB PD1	62	MALPVTALLLPLALLLHAARPGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAY GRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIART PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGKKDIISFFLALTSTALLFLLFFLTLRFSVVKRGRSRAARGTIGARRTGQPLKEDPS AVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE
	CD8a-ss FKBP EpoR_D2 DAP1	63	MALPVTALLLPLALLLHAARPGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRG WEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPAPVGLVARLADESGHVVLRWLPPPETPM TSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSDILLAGLVAA DAVASLLIVGAVFLCARRSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSG MGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE

	0 PD1		
	CD8a-ss FRB CD8a CD28 PD1	64	MALPVTALLLPLALLLHAARPGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAY GRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGL DFACDIFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRRSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPE PPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE
CID binding domain on γ chain of multi chain CAR	Gamma-ss FKBP CD8a Gamma-CD3z	65	MAIPAVVLLLLLLVEQAAAGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWE EGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDFACDIGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRE
	Gamma-ss FRB IgG1 Gamma-CD3z	66	MAIPAVVLLLLLLVEQAAAGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGKKDIGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPA YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLS TATKDTYDALHMQALPPRE
	Gamma-ss FKBP EpoR_D2 Gamma-CD3z	67	MAIPAVVLLLLLLVEQAAAGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWE EGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPAPVGLVARLADESGHVVLRWLPPPETPMTS HIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSDIGEPQLCYILD AILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPR RKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	Gamma-ss	68	MAIPAVVLLLLLLVEQAAAGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF

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	FRB CD8a Gamma-CD3z		ACDIGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLD KRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	Gamma-ss FKBP - Gamma-CD3z	69	MAIPAVVLLLLLLVEQAAAGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWE EGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPLGEPQLCYILDAILFLYGIVLTLLYCRLKI QVRKAAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAE AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	Gamma-ss FRB - Gamma-CD3z	70	MAIPAVVLLLLLLVEQAAAGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPLGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEK SRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR RGKGHDGLYQGLSTATKDTYDALHMQALPPRE
CID binding domain on α chain of multi chain CAR	VH-4G7-scFv FKBP EpoR_D2 Alpha	71	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDG TKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIV MTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEA EDVGVYYCMQHLEYPFTFGAGTKLELKRSDPGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKF MLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPAPVGLVARLADESGHVVL RWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPS DIFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNE
	VH-4G7-scFv FRB EpoR_D2 Alpha	72	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDG TKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIV MTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEA EDVGVYYCMQHLEYPFTFGAGTKLELKRSDPGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQT LKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPAPVGLVARLADESGHVVLRWLPPPETPMTS

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CID binding domain on α chain of multi chain CAR			HIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSDIFIPLLVVILF AVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNE
	VH-4G7-scFv FKBP IgG1 Alpha	73	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDG TKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIV MTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEA EDVGVYYCMQHLEYPFTFGAGTKLELKRSDPGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKF MLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPEPKSPDKTHTCPPCPAPP VAGPSVFLFPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDIFIPLLVVILFAVDTGLFISTQQQVTFLLKI KRTRKGFRLLNPHPKPNPKNNE
	VH-4G7-scFv FRB IgG1 Alpha	74	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDG TKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIV MTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEA EDVGVYYCMQHLEYPFTFGAGTKLELKRSDPGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQT LKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPEPKSPDKTHTCPPCPAPPVAGPSVFLFPPK PKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKL TVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDIFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNP HPKPNPKNNE
	VH-4G7-scFv FKBP CD8a Alpha	75	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDG TKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIV MTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEA EDVGVYYCMQHLEYPFTFGAGTKLELKRSDPGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKF MLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPTTTPAPRPPTPAPTIASQ

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			PLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNE
	VH-4G7-scFv FRB CD8a Alpha	76	MAPAMESPTLLCVALLFFAPDGVLAEVQLQQSGPELIKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGYINPYNDG TKYNEKFKGKATLTSDKSSSTAYMELSSLTSEDSAVYYCARGTYYYGSRVFDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIV MTQAAPSIPVTPGESVSISCRSSKSLLNSNGNTYLYWFLQRPGQSPQLLIYRMSNLASGVPDRFSGSGSGTAFTLRISRVEA EDVGVYYCMQHLEYPFTFGAGTKLELKRSDPGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQT LKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDFACDFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNE				
CID binding domain on α chain of single chain CAR	sc_scFV_C D12 FKBP CD8a CD8a 4-1BB-CD3z	77	MALPVTALLLPLALLLHAARPQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGESTYS ADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPAS LAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYC QQSNEDPPTFGAGTKLELKRSDPGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVI RGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPTTTPAPRPPTPAPTIASQPLSLRPEA CRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCRRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGG CELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE RRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	sc_scFV_C D12 FRB CD8a CD8a 4-1BB-CD3z	78	MALPVTALLLPLALLLHAARPQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGESTYS ADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPAS LAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYC QQSNEDPPTFGAGTKLELKRSDPGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQ AYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR GLDFACDIYIWAPLAGTCGVLLLSLVITLYCRRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSAD APAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPRE
	sc_scFV_C D12 FKBP	79	MALPVTALLLPLALLLHAARPQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGESTYS ADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPAS

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CID binding domain on α chain of single chain CAR	IgG1 CD8a 4-1BB- CD3z		LAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYC QQSNEDPPTFGAGTKLELKRSDPGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVI RGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPEPKSPDKTHTCPPCPAPPVAGPSVFL FPPKPKDTLMIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDIYIWAPLAGTCGVLLLSLVITLYCRRGRKKLLYIFKQPF MRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQ EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	sc_scFV_C D12 FRB IgG1 CD8a 4-1BB- CD3z	80	MALPVTALLLPLALLLHAARPQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGESTYS ADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPAS LAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYC QQSNEDPPTFGAGTKLELKRSDPGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQ AYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIA RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ QGNVFSCSVMHEALHNHYTQKSLSLSPGKKDIYIWAPLAGTCGVLLLSLVITLYCRRGRKKLLYIFKQPFMRPVQTTQEEDG CSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	sc_scFV_C D12 FKBP EpoR_D2 CD8a 4-1BB- CD3z	81	MALPVTALLLPLALLLHAARPQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGESTYS ADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPAS LAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYC QQSNEDPPTFGAGTKLELKRSDPGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVI RGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGAPAPVGLVARLADESGHVVLRWLPPPET PMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSDIYIWAPL AGTCGVLLLSLVITLYCRRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNE

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			LNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH MQALPPRE
	sc_scFV_C D12 FRB EpoR_D2 CD8a 4-1BB- CD3z	82	MALPVTALLLPLALLLHAARPQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGESTYS ADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPAS LAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYC QQSNEDPPTFGAGTKLELKRSDPGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQ AYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGAPAPVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDV SAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSDIYIWAPLAGTCGVLLLSLV ITLYCRRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
INTRA CELLULAR CID BINDING DOMAINS			
IMP complex (switch OFF receptor)	VH- 4G7-ss CD8a FKBP PD1	83	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLE DGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGSSS RAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQ PLRPEDGHCSWPLE
	VH- 4G7-ss CD8a FRB PD1	84	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRSGGRVAILWHEMWHEGLEEASRLYFGERNVKGM FEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGSSSRAARGTIGARRT GQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWP LE
	VH- 4G7-ss CD8a OX40 FKBP PD1	85	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQA DAHSTLAKIGSGSGSGSSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGV AQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGSGSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGE LDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE
	VH- 4G7-ss	86	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRPRRSPAQEDGKVYINMPGRGGSGSGSGSSGGVQ

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IMP complex (switch OFF receptor)	CD8a DAP10 FKBP PD1		VETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGAT GHPGIIPPHATLVFDVELLKLEGSGSGSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTE YATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE
	VH-4G7-ss CD8a HVEM FKBP PD1	87	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRCVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIE ALQAPPDVTTVAVEETIPSFTGRSPNHGSGSGSGSSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKP FKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGSGSRAARGTIGARRTGQ PLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE
	VH-4G7-ss CD8a OX40 FRB PD1	88	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQA DAHSTLAKIGSGSGSGSSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLM EAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGSGSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEP PVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE
	VH-4G7-ss CD8a DAP10 FRB PD1	89	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRPRRSPAQEDGKVYINMPGRGGSGSGSGSSGGRV AILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAW DLYYHVFRRIGSGSGSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGT SSPARRGSADGPRSAQPLRPEDGHCSWPLE
	VH-4G7-ss CD8a HVEM FRB PD1	90	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRCVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIE ALQAPPDVTTVAVEETIPSFTGRSPNHGSGSGSGSSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERG PQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGSGSRAARGTIGARRTGQPLKEDPSAVPVF SVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLE
	VH-4G7-ss CD8a PD1	91	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGE LDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLGSGSSSGGVQVETISPGDG RTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPH

IMP complex (switch OFF receptor)	FKBP		ATLVFDVELLKLEE
	VH-4G7-ss CD8a PD1 FRB	92	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLGSGSSSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE
	VH-4G7-ss CD8a OX40 PD1 FKBPr	93	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGSGSGSGSSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLGSGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
	VH-4G7-ss CD8a DAP10 PD1 FKBP	94	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRPRRSPAQEDGKVYINMPGRGGSGSGSGSSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLGSGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
	VH-4G7-ss CD8a HVEM PD1 FKBP	95	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRCVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIPSFTGRSPNHGSGSGSGSSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLGSGSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
	VH-4G7-ss CD8a OX40 PD1 FRB	96	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIGSGSGSGSSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLGSGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE
	VH-	97	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRPRRSPAQEDGKVYINMPGRGGSGSGSGSSRAAR

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CID binding domain on single-chain CAR	4G7-ss CD8a DAP10 PD1 FRB		GTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRP EDGHCSWPLGSGSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEW CRKYMKSGNVKDLTQAWDLYYHVFRRIE
	VH-4G7-ss CD8a HVEM PD1 FRB	98	MALPVTALLLPLALLLHAARPGSDIYIWAPLAGTCGVLLLSLVITLYCRCVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIE ALQAPPDVTTVAVEETIPSFTGRSPNHGSGSGSGSSRAARGTIGARRTGQPLKEDPSAVPVFSVDYGELDFQWREKTPEPPV PCVPEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPLGSGSGGRVAILWHEMWHEGLEEASRLYFGERNV KGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE
	CD123-scFv CD8a FKBP	99	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGE STYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQ SPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVA TYYCQQSNEDPPTFGAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVIL FAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNRSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFD SSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
	CD123-scFv CD8a FRB	100	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGE STYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQ SPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVA TYYCQQSNEDPPTFGAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVIL FAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNRSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEP LHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE
	CD123-scFv CD8a OX40 FKBP	101	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGE STYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQ SPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVA TYYCQQSNEDPPTFGAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVIL

			FAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNRALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTL AKIGSGSGSGSSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVG QRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
CID binding domain on single-chain CAR	CD123-scFv CD8a DAP10 FKBP	102	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGE STYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQ SPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVA TYYCQQSNEDPPTFGAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVIL FAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNRPRRSPAQEDGKVYINMPGRGGSGSGSGSSGGVQVETISP GDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGII PPHATLVFDVELLKLEE	
	CD123-scFv CD8a HVEM FKBP	103	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGE STYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQ SPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVA TYYCQQSNEDPPTFGAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVIL FAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNRCVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPP DVTTVAVEETIPSFTGRSPNHGSGSGSGSSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLG KQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
	CD123-scFv CD8a OX40 FRB	104	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGE STYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQ SPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVA TYYCQQSNEDPPTFGAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVIL FAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNRALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTL AKIGSGSGSGSSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWC RKYMKSGNVKDLTQAWDLYYHVFRRIE
	CD123-	105	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGE

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	scFv CD8a DAP10 FRB		STYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQ SPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVA TYYCQQSNEDPPTFGAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVIL FAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNRPRRSPAQEDGKVYINMPGRGGSGSGSGSSGGRVAILWHE MWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHV FRRIE	
	CD123-scFv CD8a HVEM FRB	106	MAPAMESPTLLCVALLFFAPDGVLAQIQLVQSGPELKKPGETVKISCKASGYIFTNYGMNWVKQAPGKSFKWMGWINTYTGE STYSADFKGRFAFSLETSASTAYLHINDLKNEDTATYFCARSGGYDPMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQ SPASLAVSLGQRATISCRASESVDNYGNTFMHWYQQKPGQPPKLLIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVA TYYCQQSNEDPPTFGAGTKLELKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDFFIPLLVVIL FAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNNRCVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPP DVTTVAVEETIPSFTGRSPNHGSGSGSGSSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKE TSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIE
CID binding domain on single-chain CAR	klo43_scFV CD8a CD8a 41BB-CD3z FKBP	107	MALPVTALLLPLALLLHAARPEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPGKALEWLALIRSKADGYTTE YSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEGAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSM ADYKDIVMTQSHKFMSTSVGDRVNITCKASQNVDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSV QAEDLAVYYCQQYYSTPWTFGGGTKLEIKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW APLAGTCGVLLLSLVITLYCRRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPRSSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVG QRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEE
	klo43_scFV CD8a CD8a 41BB-	108	MALPVTALLLPLALLLHAARPEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPGKALEWLALIRSKADGYTTE YSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEGAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSM ADYKDIVMTQSHKFMSTSVGDRVNITCKASQNVDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSV QAEDLAVYYCQQYYSTPWTFGGGTKLEIKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW

CID binding domain on single-chain CAR	CD3z FRB		APLAGTCGVLLLSLVITLYCRRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD ALHMQALPPRSSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWC RKYMKSGNVKDLTQAWDLYYHVFRRIE
	klo43_s cFV CD8a CD8a 41BB FKBP CD3z	109	MALPVTALLLPLALLLHAARPEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPGKALEWLALIRSKADGYTTE YSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEGAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSM ADYKDIVMTQSHKFMSTSVGDRVNITCKASQNVDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSV QAEDLAVYYCQQYYSTPWTFGGGTKLEIKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW APLAGTCGVLLLSLVITLYCRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGSGSGSGSSGGVQVETISPGD GRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPP HATLVFDVELLKLEGSGSGRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQ KDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	klo43_s cFV CD8a CD8a 41BB FRB CD3z	110	MALPVTALLLPLALLLHAARPEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPGKALEWLALIRSKADGYTTE YSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEGAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSM ADYKDIVMTQSHKFMSTSVGDRVNITCKASQNVDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSV QAEDLAVYYCQQYYSTPWTFGGGTKLEIKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW APLAGTCGVLLLSLVITLYCRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGSGSGSGSSGGRVAILWHEMW HEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFR RIGSGSGRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	klo43_s cFV CD8a CD8a FKBP 41BB- CD3z	111	MALPVTALLLPLALLLHAARPEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPGKALEWLALIRSKADGYTTE YSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEGAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSM ADYKDIVMTQSHKFMSTSVGDRVNITCKASQNVDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSV QAEDLAVYYCQQYYSTPWTFGGGTKLEIKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW APLAGTCGVLLLSLVITLYCRSGGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGW

			EEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLEGSGSSRGRKKLLYIFKQPFMRPVQTTQEEDGCSC RFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEA YSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRE
	klo43_s cFV CD8a CD8a FRB 41BB-CD3z	112	MALPVTALLLPLALLLHAARPEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPGKALEWLALIRSKADGYTTE YSASVKGRFTLSRDDSQSILYLQMNALRPEDSATYYCARDAAYYSYYSPEGAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSM ADYKDIVMTQSHKFMSTSVGDRVNITCKASQNVDSAVAWYQQKPGQSPKALIYSASYRYSGVPDRFTGRGSGTDFTLTISSV QAEDLAVYYCQQYYSTPWTFGGGTKLEIKRSDPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW APLAGTCGVLLLSLVITLYCRSGGRVAILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYG RDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRIGSGSSRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRR GKGHDGLYQGLSTATKDTYDALHMQALPPRE

Switch-off receptors and engineered CARs, including additional adequate chains of the mcCAR (beta and gamma or alpha), are further subcloned in a lentiviral production plasmid, possibly upstream a 2A cis-acting hydrolase element (GSG-P2A and GSG-T2A ribosomal skip peptides) followed by a reporter marker (e.g. fluorescent proteins), or membranar protein (such as RQR8) either separately or together separated by 2A cis-acting hydrolase elements. Standard molecular biology technics such as PCR, enzymatic restriction digestion and ligation are applied to create all constructions.

Example 2. Lentiviral delivery of switch-off receptors and engineered mcCARs in primary T-cells

Viral vectors are either produced according to standard protocols or by an external contractor. Primary T-cells are transduced simultaneously or sequentially using switch-off receptor and engineered mcCAR lentiviral particles. The transduced T-cells are purified for positive engineered mcCAR and switch-off receptor expression using bulk FACS sorting or magnetic separation. The whole bulk engineered mcCAR / switch-off receptor positive population is then assessed for engineered mcCAR driven activation (degranulation/cytotoxicity), proliferation, and cytokine release using a model cell line expressing the engineered CAR target antigens in absence of small molecule (Rapamycin, rapalogs or synthetic rapalogs). The same experiment is then reproduced in presence of the small molecule to assess the inhibition properties of the system.

Example 3. Assembly of switch-off receptors and engineered scCARs (single chain CARs)

Sequences of modules to assemble IMPs (switch-off receptors) are obtained from the SEQ ID NO. 61-64 (extracellular CID binding domain) or 83-98 (intracellular CID binding domain) and from engineered scCAR with SEQ ID NO.77-82 (extracellular CID binding domain) or 107-112 (intracellular CID binding domain) such as depicted in the above Table 5. Their configuration is depicted in the Figures 4 (extracellular CID binding domain) and Figure 5 (intracellular CID binding domain).

Assembly of constructs are done using Golden Gate assembly strategies according to published protocols. Switch-off receptors and engineered CARs are thus cloned in separated shuttle cloning plasmid. Switch-off receptors and engineered scCARs are further subcloned in a lentiviral production plasmid, possibly upstream a 2A cis-acting hydrolase element followed by a reporter

marker (e.g. fluorescent proteins) either separately or together separated by 2A cis-acting hydrolase elements. Standard molecular biology technics such as PCR, enzymatic restriction digestion and ligation are applied to create all constructions.

Example 4. Lentiviral delivery of switch-off receptors and engineered scCARs in primary T-cells

Viral vectors were either produced according to standard protocols or by an external contractor. Primary T-cells are transduced simultaneously or sequentially using switch-off receptor and engineered scCAR lentiviral particles. The transduced T-cells are purified for positive engineered scCAR and switch-off receptor expression using bulk FACS sorting or magnetic separation. The whole bulk engineered scCAR / switch-off receptor positive population is then assessed for engineered scCAR driven activation (degranulation/cytotoxicity), proliferation, and cytokine release using a model cell line expressing the engineered CAR target antigens in absence of small molecule (Rapamycin, rapalogs or synthetic rapalogs). The same experiment is then reproduced in presence of the small molecule to assess the inhibition properties of the system.

REFERENCES

Belshaw, PJ; Ho, SN; Crabtree, GR; Schreiber, SL (1996). "Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins.". *Proceedings of the National Academy of Sciences of the United States of America* 93 (10): 4604–7

Bierer, B. E., G. Hollander, et al. (1993). "Cyclosporin A and FK506: molecular mechanisms of immunosuppression and probes for transplantation biology." Curr Opin Immunol 5(5): 763-73.

Boch, J., H. Scholze, et al. (2009). "Breaking the code of DNA binding specificity of TAL-type III effectors." Science 326(5959): 1509-12.

Carsberg, C.J., Myers, K.A., Evans, G.S., Allen, T.D., Stern, P.L (1995) "Metastasis-associated 5T4 oncofoetal antigen is concentrated at microvillus projections of the plasma membrane" *J. Cell. Sci.* 108 (8):2905-16Castro, F. V., McGinn, O. J., Krishnan, S., Marinov, G., Rutkowski, A. J., Elkord, E., Burt, D. J., Holland, M., Vaghjiani, R., Gallego, A., Saha, V. and Stern, P. L. (2012). "5T4 oncofetal antigen is expressed in high risk of relapse childhood pre-B acute lymphoblastic leukemia and is associated with a more invasive and chemotactic phenotype." Leukemia 26(7):1487-98

Chu J., Deng, Y. Benson, D. M., Hughes T., Zhang, J., Peng, Y., Mao, H., Yi, L., Ghoshal, K., He, X., Devine, S. M., Zhang, X., Caligiuri, M. A., Hofmeister, C. C. and Yu, J. (2013).”CS1-specific chimeric antigen receptor (CAR)-engineered natural killer cells enhance in vitro and in vivo antitumor activity against human multiple myeloma”. Leukemia

Deaglio S, Mehta K, Malavasi F (2001). "Human CD38: a (r)evolutionary story of enzymes and receptors". Leuk. Res. 25 (1): 1–12.)

Dolan, D.E., and Gupta S. (2014). “PD-1 Pathway Inhibitors: Changing the Landscape of Cancer Immunotherapy”, Cancer Control, Vol. 21 (3):231-237

Doronina, V. A., C. Wu, et al. (2008). "Site-specific release of nascent chains from ribosomes at a sense codon." Mol Cell Biol 28(13): 4227-39.

Farrar, MA; Alberol-Ila, J; Perlmutter, RM (Sep 12, 1996). "Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization.". *Nature* 383 (6596): 178–81.

Fedorov, V.D., Themeli, M., Sadelain, M. (2013). “PD-1– and CTLA-4–Based Inhibitory Chimeric Antigen Receptors (iCARs) Divert Off-Target Immunotherapy Responses”. *Sci Transl Med 5 (215).* Finbloom, D. S., and Winestock, K. D. (1995). "IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes." The Journal of Immunology 155(3): 1079-1090

Henderson, D. J., I. Naya, et al. (1991). "Comparison of the effects of FK-506, cyclosporin A and rapamycin on IL-2 production." Immunology 73(3): 316-21.

Ho, SN; Biggar, SR; Spencer, DM; Schreiber, SL; Crabtree, GR (Aug 29, 1996). "Dimeric ligands define a role for transcriptional activation domains in reinitiation.". *Nature* 382 (6594): 822–6.

Hole, N., and Stern, P.L., (1988). "A 72 kD trophoblast glycoprotein defined by a monoclonal antibody." Br.J. Cancer 54: 239-246.Jena, B., G. Dotti, et al. (2010). "Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor."Blood 116(7):1035-44

Ito, S., Ansari, P., Sakatsume, M., Dickensheets, H., Vazquez, N., Donnelly, R.P., Larner, A.C., and Finbloom D.S. (1999). “*I*nterleukin-10 Inhibits Expression of Both Interferon – and Interferon γ–Induced Genes by Suppressing Tyrosine Phosphorylation of STAT1”. Blood: 93 (5).

Jena, B., G. Dotti, et al. (2010). "Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor." Blood 116(7): 1035-44.

Liu, J., M. W. Albers, et al. (1992). "Inhibition of T cell signaling by immunophilin-ligand complexes correlates with loss of calcineurin phosphatase activity." Biochemistry 31(16): 3896-901.

Miyamoto, T; DeRose, R; Suarez, A; Ueno, T; Chen, M; Sun, TP; Wolfgang, MJ; Mukherjee, C; Meyers, DJ; Inoue, T (2012). "Rapid and orthogonal logic gating with a gibberellin-induced dimerization system.". *Nature chemical biology* 8 (5): 465–70.

Moscou, M. J. and A. J. Bogdanove (2009). "A simple cipher governs DNA recognition by TAL effectors." Science 326(5959): 1501.

Park, T. S., S. A. Rosenberg, et al. (2011). "Treating cancer with genetically engineered T cells." Trends Biotechnol 29(11): 550-7.

Rivera, VM; Clackson, T; Natesan, S; Pollock, R; Amara, JF; Keenan, T; Magari, SR; Phillips, T; Courage, NL; Cerasoli F, Jr; Holt, DA; Gilman, M (September 1996). "A humanized system for pharmacologic control of gene expression." *Nature Medicine* 2 (9): 1028–32.

Sheppard, K. A., Fitz, L.J., Lee, J.M., Benander, C., George, J.A., Wooters, J., Qiu, Y., Jussif. J.M., Carter ,L.L., Wood, C.R., Chaudhary, D. (2004) "PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta". FEBS Lett. 10, 574(1-3):37-41.

Spencer, D.M., Wandless, T.J., Schreiber, S.L., Crabtree, G.R. (1993). "Controlling signal transduction with synthetic ligands". *Science* 262 (5136): 1019–24.

Springer, T.A. (1985) "Hybridoma technology in biosciences and medecine", Plenum Press .

Starzynska, T., Wiechowska-Kozlowska, A., Marlicz, K., et al. (1998). "5T4 oncofetal antigen in gastric carcinoma and its clinical significance". Eur J Gastroenterol Hepatol 10 (6): 479–84.

Wrigley, E. McGown A.T., Rennison J., Swindell R. Crowther, D. Starzynska T. and (1995). “Stern P.L. 5T4 oncofetal antigen expression in ovarian carcinoma”. International Journal of Gynecological Cancer. 5 (4) :269–274.

CLAIMS

1. Method for modulating the level of activation of an engineered immune cell, said method comprising the following steps of:

(a) transfecting an immune cell with at least a first polynucleotide encoding a Chimeric Antigen Receptor (CAR) , which can be activated in-vivo and/or in vitro by an external ligand; said CAR comprising a first binding domain and ;

(b) further transfecting said immune cell with at least a second polynucleotide encoding an engineered inhibitory membrane protein (IMP) complex, comprising at least one intracellular inhibitory signaling domain and one second binding domain,

such that said immune cell co-expresses, said CAR, and said inhibitory membrane protein (IMP) complex, and;

(c) contacting said engineered immune cell with said external ligand, so that a signal is transduced by the CAR, and then;

(d) reducing the level of said signal transduction of said CAR by adding a soluble compound that binds the binding domain of said IMP and the binding domain of the CAR molecule to co-localize said IMP and said CAR,

thereby modulating the level of activation of the engineered immune cell.

2. Method for modulating the level of activation according to claim 1, wherein said signal of transduction of the CAR is an activation of the immune cell and said immune cell is activated.

3. Method for modulating the level of activation according to claim 1 or claim 2, wherein the co-localization of the IMP and CAR has the effect of switching off the CAR signal transduction.

4. Method for modulating the level of activation of an engineered immune cell according to any one of claim 1 to 3, wherein said IMP complex comprises at least two transmembrane polypeptides, each comprising a binding domain that is bound by said soluble compound.

5. Method for modulating the level of activation of an engineered immune cell according to any one of claim 1 to 4, wherein first and second binding domains are CID binding domains.

6. Method according to any one of claim 1 to 5, wherein the step d) of reduction of the level of activation is performed by co-localization of two inhibitory signaling domains IL10RA and IL10B born on two different polypeptides of the inhibitory membrane protein (IMP) complex.

7. Method for modulating the level of activation of an engineered immune cell according to one of claim 1 to 5, wherein the step d) of reduction of the level of activation is performed by co-localization of the IMP and the CAR structures through said soluble compound.

8. Method for modulating the level of activation of an engineered immune cell according to one of claim 1-5 or claim 7, wherein the CAR comprises a second binding domain, so that the same soluble compound can bind together the CAR and the IMP

9. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-8, wherein said method further comprises the step of infusing the immune cell into a patient upon step b).

10. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-9, wherein said immune cell is a T-cell.

11. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-10, wherein said CAR is a single chain CAR.

12. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-10, wherein said CAR is a multi-chain CAR.

13. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-12, wherein said binding domain(s) is (are) extracellular.

14. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-12, wherein both said binding domain(s) is (are) intracellular.

15. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-5 or claim 7-14, wherein said inhibitory signaling domain is from PD1.

16. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-15, wherein said soluble compound is a small molecule

17. Method for modulating the level of activation of an engineered immune cell according to claim 16, wherein said soluble compound is a rapalog.

18. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 17, wherein said small molecule is the rapamycin.

19. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-18, wherein said first and second binding domain have at least 80% identity with SEQ ID NO 14 and SEQ ID NO 15.

20. Method for modulating the level of activation of an engineered immune cell according to anyone of claim 1-19, wherein said soluble compound a bi-specific antibody.

21. Method for producing an engineered immune cell, which level of activation by a CAR is controlled by an inhibitory membrane protein (IMP) complex, said method comprising the step of:

(a) transfecting an immune cell with at least a first polynucleotide encoding a Chimeric Antigen Receptor (CAR); and

(b) transfecting said immune cell with at least a second polynucleotide encoding engineered inhibitory membrane protein (IMP) complex, said IMP complex comprising at least one inhibitory signaling domain and one binding domain to be contacted with a soluble compound that translocates the inhibitory domain

(c) selecting the immune cells where the first and second polypeptides are co-expressed into their membrane.

22. Method for producing an engineered immune cell according to claim 21, wherein the transfection is performed by the use of a retroviral vector.

23. Method for producing an engineered immune cell according to claim 21, wherein the transfection is performed by the use of polycistronic CAR/IMP-encoding mRNA.

24. Method for producing an engineered immune cell according to anyone of claim 21-23, comprising further a step of producing allogeneic immune cells by inactivation of at least one gene encoding a T-cell receptor (TCR) component.

25. Method for producing an engineered immune cell according to claim 24, wherein the step of allogeneic immune cells production is performed by the use of TALE nuclease.

26. Inhibitory dual CAR/IMP complex, comprising at least two transmembrane chimeric polypeptides:

- a first one encoding a Chimeric Antigen Receptor which comprises one dimerization binding domain;

-the second one encoding an engineered inhibitory membrane protein (IMP) complex, said IMP complex comprising at least one intracellular inhibitory signaling domain and one dimerization binding domain.

27. Inhibitory dual CAR/IMP complex according to claim 26, wherein both dimerization binding domains are located extracellularly.

28. Inhibitory dual CAR/IMP complex according to anyone of claim 26 or claim 27, wherein the CAR is a multi-chain CAR.

29. Inhibitory dual CAR/IMP complex according to claim 26 or claim 27, wherein the CAR is a single-chain CAR.

30. Inhibitory dual CAR/IMP complex according to anyone of claim 26 to 29, wherein the inhibitory domain is PD-1.

31. Inhibitory dual CAR/IMP complex according to anyone of claim 26 to 30, wherein the dimerization domain is FKBP or FRB.

32. Inhibitory dual CAR/IMP complex according to anyone of claim 31, wherein said first and second binding domains have at least 80 % identity with SEQ ID NO 14 and SEQ ID NO 15.

33. An inhibitory membrane protein (IMP) complex , comprising at least two transmembrane chimeric polypeptides, each of them comprising at least, an inhibitory domain and a binding domain, both binding domains interacting with a soluble compound, so that the respective inhibitory domains co-localize upon addition of said soluble compound.

34. An inhibitory membrane protein (IMP) complex according to claim 33 wherein the inhibitory domains are IL-10Rα/IL-10Rβ, TGF-β, VEGF, TNFR or DR3.

35. An inhibitory membrane protein (IMP) complex according to claim 33 or claim 34, wherein the dimerization domain is FKBP or FRB.

36. An inhibitory membrane protein (IMP) complex according to claim 35, wherein said first and second binding domains have at least 80 % identity with SEQ ID NO 14 and SEQ ID NO 15.

37. A polynucleotide encoding an inhibitory membrane complex according to anyone of claims 33-36.

38. A retroviral vector comprising the polynucleotide according to anyone of claims 33-37.

39. An engineered immune cell comprising an inhibitory membrane complex according to claim 33-36 or transfected with a polynucleotide according to claim 37 or with the retroviral vector according to claim 38.

40. An engineered immune cell according to claim 39, wherein it further expresses a Chimeric Antigen Receptor (CAR).

41. An engineered immune cell that expresses a CAR on its surface along with an inhibitory membrane protein (IMP), wherein said CAR and inhibitory transmembrane polypeptides each comprises a binding domain, both binding domains interacting with the same soluble compound.

42. An engineered immune cell according to claim 41, wherein said inhibitory domain of the IMP chimeric polypeptide is chosen amongst PD-1, CTLA-4, LAG3, Tim3, BTLA, BY55, TIGIT, LAIR1, SIGLEC10, 2B4, PPP2CA, PPP2CB, PTPN6, PTPN22, CD96, CRTAM, SIGLEC7, SIGLEC9, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFRBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3,.

43. An engineered immune cell according to claim 41 or claim 42, wherein both binding domains are extracellular.

44. An engineered immune cell according to claim 41 or claim 42, wherein both binding domains are intracellular.

45. An engineered immune cell according to anyone of claims 39-44, wherein the chimeric antigen receptor (CAR) is a multichain CAR (mcCAR).

46. An engineered immune cell according to claim 45, wherein the multichain chimeric antigen receptor (mcCAR) comprises at least an alpha chain (FCεRα), a beta chain (FCεRβ) and a gamma chain (FCεRγ).

47. An engineered immune cell according to claim 46, wherein the binding domain is part of the alpha chain (FCεRα).

48. An engineered immune cell according to claim 46 or claim 47, wherein the multi-chain based chimeric antigen receptor (CAR) comprises:

- an alpha chain (FCεRα) containing at least:

(a) a signal sequence and an antigen-specific targeting region;

(b) a binding domain;

(c) an extracellular spacer domain (hinge);

(d) a transmembrane domain;

- a beta chain (FCεRβ) contains at least:

(a) a signal sequence;

(b) an extracellular spacer domain (hinge);

(c) a transmembrane domain;

(d) a co-stimulatory ligand;

- a gamma chain (FCεRγ) contains at least:

(a) a signal sequence;

(b) an extracellular spacer domain (hinge);

(c) a transmembrane domain;

(d) a signal transducing domain.

49. A method for treating a patient in need thereof comprising:

(a) providing a immune cell according to any one of the claims 39 to 48.

(b) administrating said immune cell to said patient,

(c) administrating a chemical inducer of dimerization (CID) to modulate the activation of said immune cell in the patient.

50. The method for treating a patient of claim 49, wherein said chemical inducer of dimerization (CID) is rapamycin.

51. The method for treating a patient of claim 50, wherein said immune cells are recovered from donors.

52. The method for treating a patient of anyone of claim 48, wherein said immune cell is recovered from the patient himself.

53. A kit or vector for engineering immune cells with modulable level of activation, which comprises:

- a first polynucleotide encoding a Chimeric Antigen Receptor (CAR); and;

- a second polynucleotide encoding an engineered inhibitory membrane protein (IMP) complex according to anyone of claims 27-32.

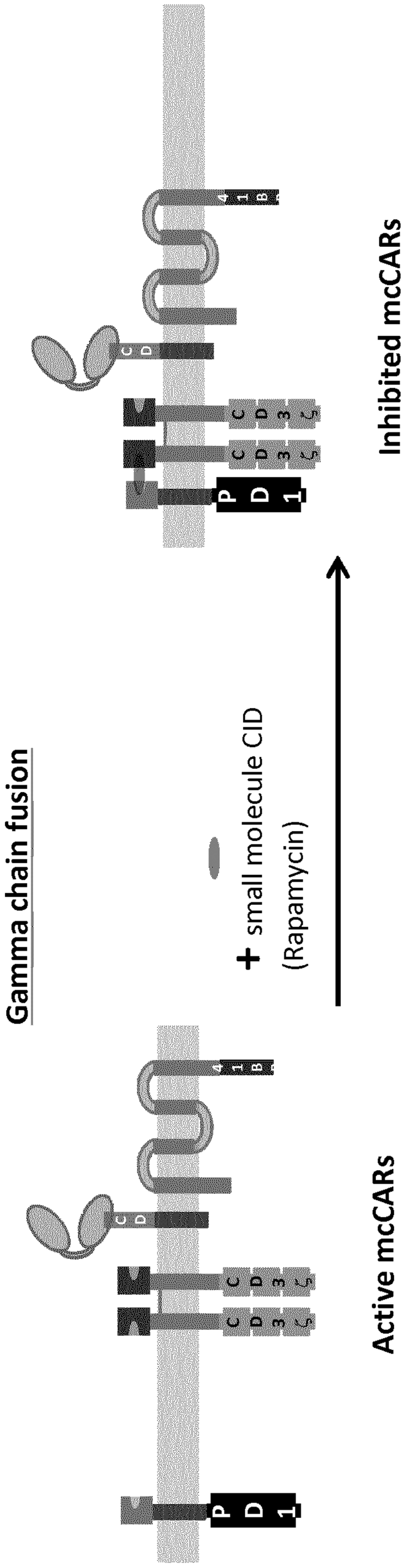

Figure 1A

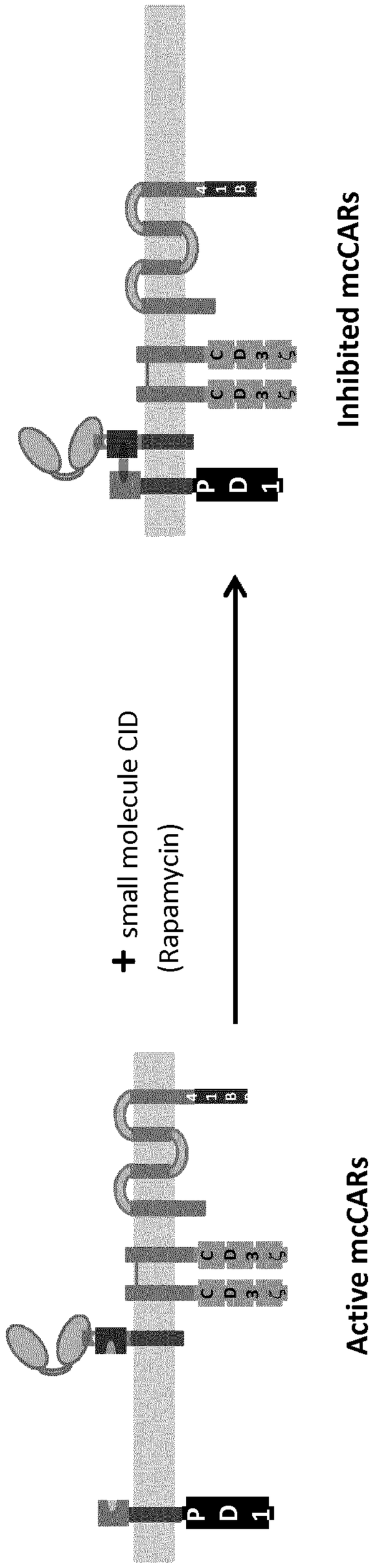

Figure 1B

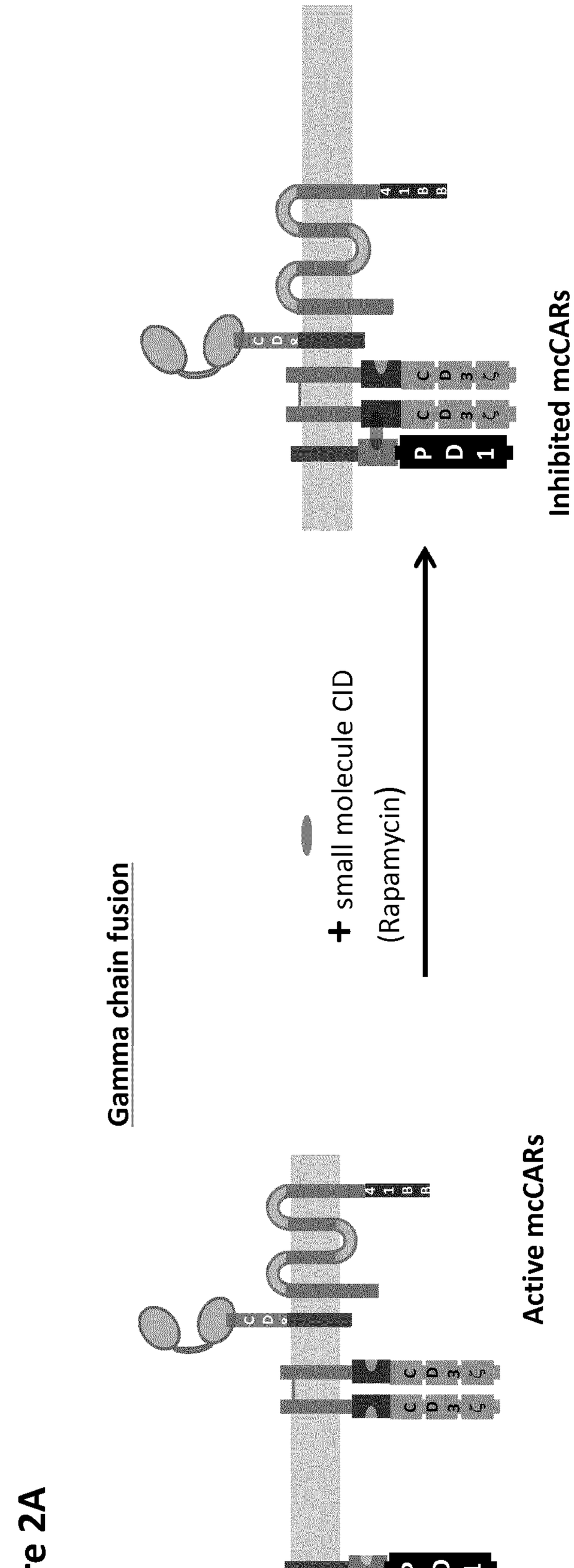

Figure 2A

Figure 2B

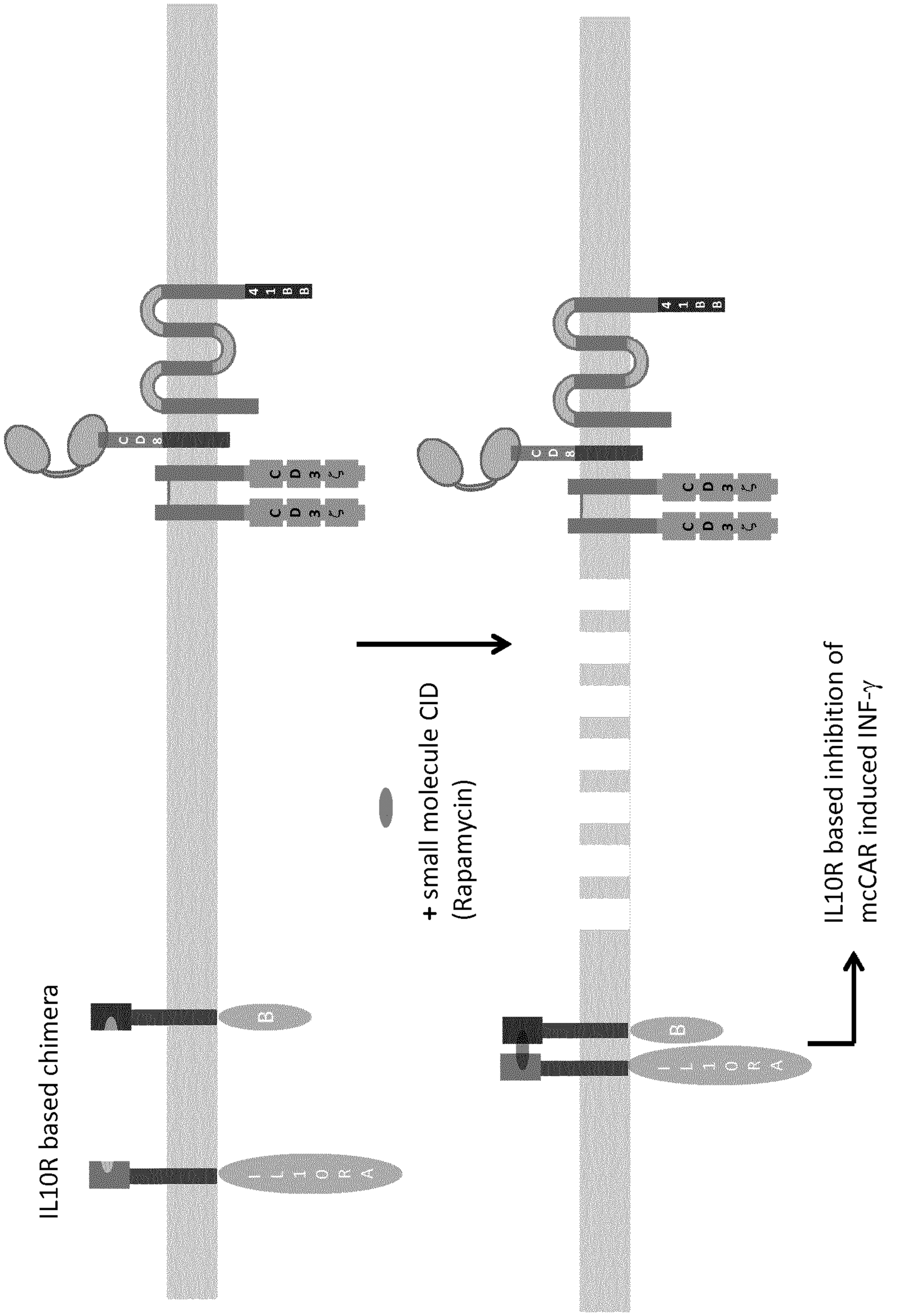
IL10R based chimera
+ small molecule CID
(Rapamycin)
IL10R based inhibition of
mcCAR induced INF-γ

Figure 3

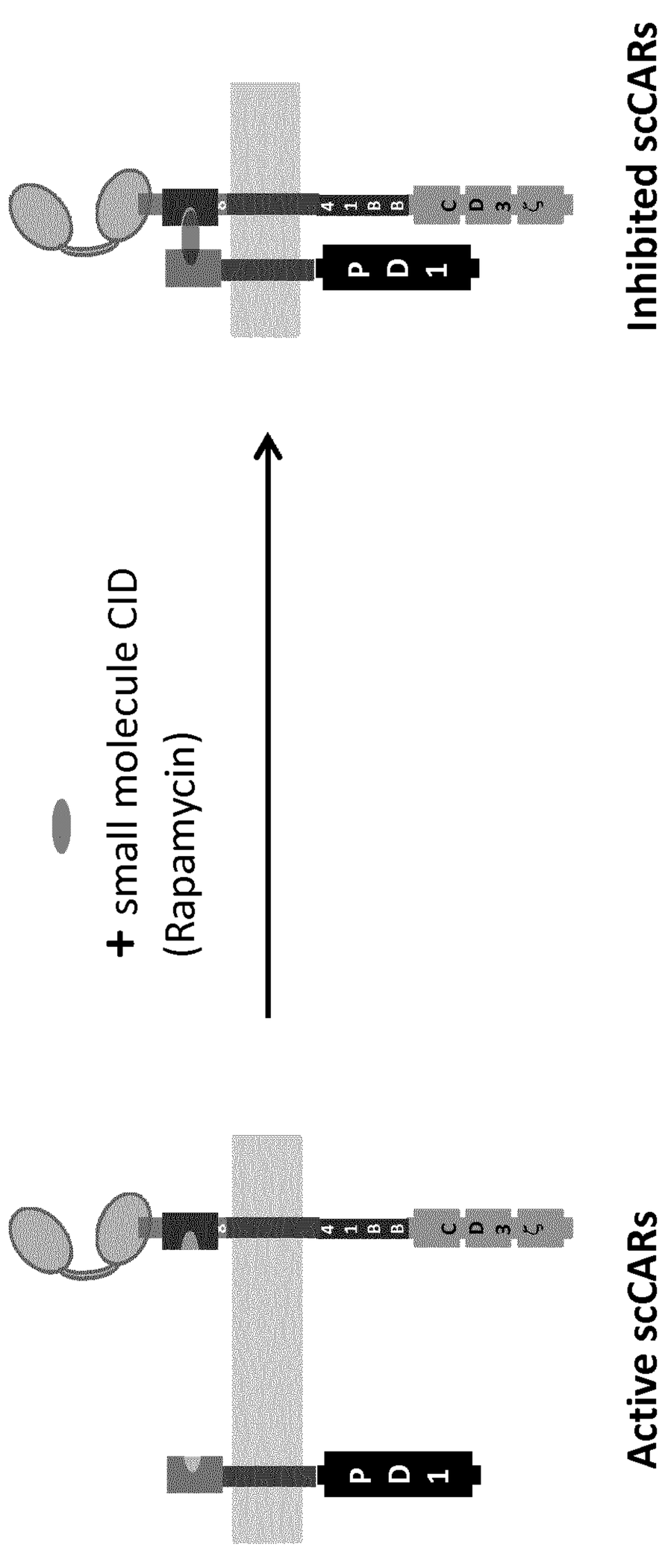
Inhibited scCARs
4 1 B B C D 3 ζ
P D 1
+ small molecule CID
(Rapamycin)
Active scCARs
4 1 B B C D 3 ζ
P D 1

Figure 4

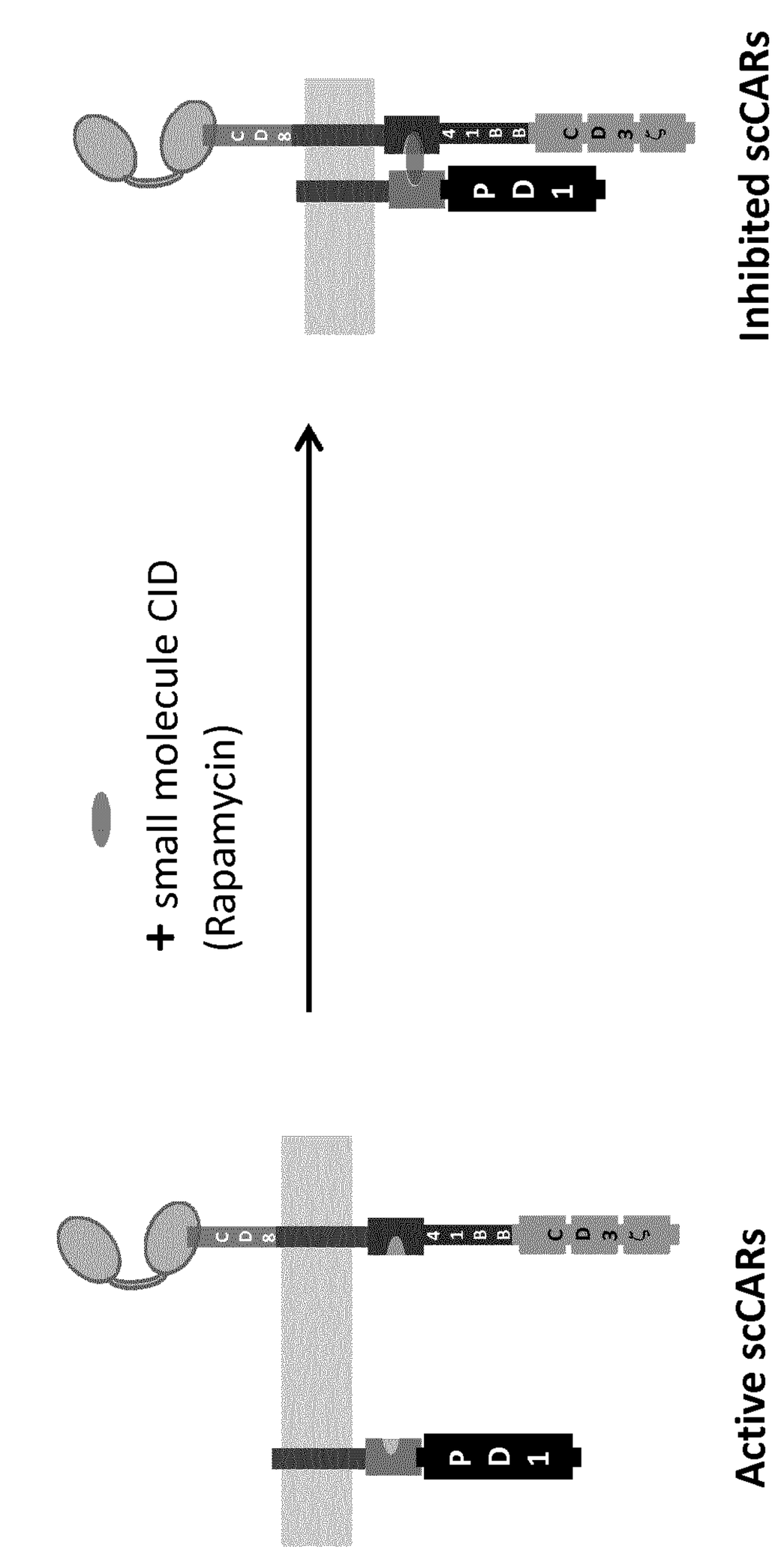
Inhibited scCARs
CD8
4-1BB
CD3ζ
PD1
+ small molecule CID
(Rapamycin)
Active scCARs
CD8
4-1BB
CD3ζ
PD1

Figure 5