

CAR T CELLS FOR TREATING CD19+, CD20+ OR CD22+ TUMORS OR B-CELL DERIVED AUTO-IMMUNE DISEASES

5 The present invention concerns CAR T cells for treating CD19+,
CD20+ OR CD22+ tumors or autoimmune diseases caused by B cells
producing auto-antibodies. In particular, the present invention concerns
powerful CAR T cells for treating CD19+, CD20+ OR CD22+ tumors, such
as leukemia and lymphoid malignancies, which provide increased safety in
the therapy of said tumors and prevent epitope masking in CAR+ B-cell
10 leukemia blasts, said CAR T cells being able to decrease the potential risk
of CD19-/CAR+, CD20-/CAR+ or CD22-/CAR+ leukemic relapse. In
addition, CAR T cells according to the present invention provides
increased safety also in the treatment of autoimmune diseases caused by
B cells producing auto-antibodies.

15 Patient-derived T cells genetically modified to express chimeric
antigen receptors (CARs) specific for CD19 represent a novel, effective
option for patients with relapsed/refractory B-cell precursor acute
lymphoblastic leukemia (Bcp-ALL)^{1,2}. Indeed, two recent shortcut FDA
approvals (the Novartis' CD19-targeting CAR T cell product Kymriah™
20 and the Kite's product Yescarta™, based on a lentiviral and retroviral
platform, respectively) have highlighted the rapid pace of progress made in
this field. In view of the exciting results reported in patients with CD19+
malignancies given CAR T-cells^{3,4}, it is expected that a continuously
growing number of patients will be considered for this treatment and, thus,
25 will be exposed to gene-modified products. Since the techniques of gene
manipulation are relatively new, some of the delayed side effects
associated with CAR T-cell therapy are still unpredictable and medical
researchers, institutions, and regulatory agencies are working to ensure
that gene therapy is as safe as possible.

30 In the case of B-ALL, CAR T-cell therapy causes rapid and
sustained clinical response, but a barrier to widespread is represented by
life-threatening reactions such as relapse, cytokine release syndrome
(CRS), and on-target off-tumor effect.

Relapse is a tough step to overcome for CAR T-cell anti-leukemia therapy, despite the high initial complete remission rates. Early relapses, CD19 positive (or CD20/CD22 positive for which early relapses are also expected), are related to the short in vivo persistence of CAR T-cells, or its inhibition induced by microenvironment. Over time, up to 30% of patients relapses are characterized by CD19-negative B-ALL progression, in which the loss of antigen occurs, due to an alternative exon splicing, or gene deletion. The mutation renders the tumor cells invisible again to the armored CAR-T⁵.

CRS is an immune-mediated disorder characterized by the activation of large number of T cells and an excessive secretion of inflammatory cytokines leading to visceral or vascular endothelial injury, heart failure and respiratory distress among other complications that can be fatal⁶.

On-target off-tumor is due to a reactivity of CAR-T cells against normal tissues, expressing the tumor associated antigens. So, the targeted antigens should be as specific as possible to reduce the off-tumor targeting.

In the contest of CAR.CD19 T cell treatment, the off-tumor action of T cells in patients is associated with long-term B cell aplasia⁷.

As a safety point, modification of the CAR construct to selectively eliminate adoptively transferred T cells is becoming a critical step for the successful clinical translation of this approach.

The inclusion of a suicide gene provides an additional safety measures in the event of serious toxicity.

Several switches have been developed, based on genetic integration of a transgenic enzyme to activate a cytotoxic pro-drug (HSV-TK), or surface molecules (CD20, EGFR) mediating an antibody-dependent depletion mechanisms of genetically modified T cell^{8,9}.

The clinical use of the suicide gene inducible caspase 9 (iC9) has also been reported in patients who received a haplo-identical hematopoietic stem cell transplant, in which donors' lymphocytes were

modified to improve GVHD or CRS control. The infusion of AP1903 drug initiates cell apoptosis via activation of iC9 dimerization^{10,11}.

5 In the field of CAR T cells, the presence of leukemic clonotypes in patient-derived Drug Products (DPs) obtained through a gene manipulation based on a lentiviral vector encoding for a second-generation CAR.CD19 was reported. In particular, two Bcp-ALL patients who relapsed with CD19-negative, CAR.CD19-expressing B leukemia have been reported. This observation being interpretable as inadvertent leukemic B cell transduction with second-generation CAR.CD19 lentivirus during CAR
10 T-cell manufacturing. This leukemic clone resulted to be resistant to CAR.CD19 T cell killing in a xenograft model¹². Basically, in the clinical practice of patients receiving standard CAR.CD19 T cell infusion, it was discovered the cases of patients that relapsed with a leukemia expressing the CAR.CD19. In the same CAR+ leukemia, the target antigen CD19 was not anymore detectable, for a masking effect of the CAR itself on the B cell
15 leukemia.

Next-generation immunoglobulin heavy chain sequencing (NGIS) analysis of 17 additional infusion products also identified the leukemic clonotypes in six additional products (35%).

20 *In vitro* and *in vivo* experiments proved that these CAR+ leukemia cell clones were not killed by CAR.CD19 T-cells¹². Therefore, CAR T-cell approaches revealed a potential risk of CD19-/CAR+ leukemic relapse. A study shows that CAR+ leukemia cell clones can be controlled by an anti-CAR.CD19 idiotype CAR¹³. According to this approach, both CAR-T and
25 anti-CAR cells should be generated for each patient with a consequent increasing of costs.

Current treatments for autoimmune disease mainly depend on the use of nonsteroidal anti-inflammatory drugs, antimalarial drugs, glucocorticoids, and immunosuppression for severe symptoms with organ
30 dysfunction. As B cells play a central role in the pathogenesis of these diseases, many B-cell-directed immunotherapies have been recently developed. However, these therapies have shown only modest success in the subgroup of SLE patients with serologically active disease. The anti-

BAFF (B-cell-activating factor) agent belimumab, which was the first targeted biological treatment for SLE, has obtained approval from the Food and Drug Administration and the European Medicines Agency but can only partially deplete naive B cells. In contrast, two other BAFF-blocking agents, tabalumab and blisibimod, showed negative results in clinical trials for SLE treatment. Rituximab, an antibody against CD20, can deplete B cells more efficiently, but response rates in SLE patients vary widely between studies. Disease relapse after treatment with rituximab remains a problem. B cell markers, as CD19, CD20 and CD22 expression is maintained at a high level throughout all stages of B-cell differentiation. Thus, they are considered a good target to achieve more efficient and long-lasting therapeutic responses in these patients. For this reason, the transfer of autologous T cells expressing anti-CD19 chimeric antigen receptors has been used to treat three patients with SLE.(DOI: 10.1056/NEJMc2107725).

As the number of patients that can benefit from CAR T infusion is increasing, it is becoming mandatory to increase the level of safety of the gene therapy approach.

In the light of the above, it is therefore apparent the need to provide for further CD19, CD20 and CD22 -targeting CAR T cells, which are able to overcome the disadvantages of the known CAR T cells.

In this contest, the aim of the present invention is to increase the safety of CD19, CD20 and CD22 -targeting CAR T cell gene therapy.

This aim, besides being relevant in cancer therapy, is also relevant when CAR T cells are used in patients with autoimmune diseases caused by B cells generating auto-antibodies, including but not only limited to systemic lupus erythematosus (SLE), Systemic sclerosis (SSc), ANCA-Associated Vasculitis (AAV), Dermatomyositis (DM).

According to the present invention, it has been now found that a CAR comprising a short linker is a molecule with an increased level of security respect to standard conformation of CAR, since it is able to recognize and kill unwanted CAR+ tumor B cells.

According to the present invention, it is now provided a new CAR CD19, CD20 or CD22 design able to provide protection in the unlikely case of CAR+ leukemic B cell generation, even when CAR T-cell production was started from patient-derived material rich in leukemic cells (peripheral blood with more than 45% of leukemic blasts, as well as bone marrow-derived cells).

Experimental results show that the CAR design of the present invention provides CAR.CD19 T cells able to recognize and kill the potential generation of unwanted CAR+ leukemia cells.

In particular, *in vitro* studies showed that CD19+ leukemic cells transduced with the CAR.CD19 (iCas9CAR.CD19SL-LH) of the present invention, which is characterized by a short linker (SL) vector, besides having a long hinge (LH), show a reduced but not null expression of the target antigen CD19, allowing to be recognized by CAR.CD19 T cells *in vitro* and *in vivo*.

The experimental results show that CAR.CD19 of the present invention allows to control CAR+ leukemic cells in both *in vitro* and *in vivo* experiments, even when the production starts from biological material characterized by heavy contamination of leukemia blasts.

According to the present invention, the impact of high percentage of leukemia contamination in patient-derived starting material (SM) on CAR T cell drug product (DP) has been also evaluated. The results showed that although the presence of large number of CD19+ cells in SM did not affect transduction level of DPs, as well as the yield of final recover when SM had more than 45% of CD19+ B leukemic cell contamination. DPs were deeply characterized by cytofluorimetric-assay and molecular biology for Immunoglobulin (Ig)-rearrangements, showing that level of B cell contamination in DPs did not correlate with the percentage of CD19+ cells in SM.

Therefore, evidence is provided that the use of patient derived material highly enriched in B leukemic cells led to the generation of CAR T-cell products with a significant contamination of leukemic cells.

According to the present invention, peripheral blood mononuclear cells isolated from patients with a high percentage of circulating blasts (at either diagnosis or relapse to increase the likelihood of high level of B cell contamination in the starting materials) were genetically modified with a γ -retroviral vector carrying a second-generation CAR.CD19.41bb molecule. By applying quantitative PCR for Ig rearrangements (molecular MRD), B-cell contamination in 50% of the CAR T-cell products has been observed, in the absence of any statistical correlation between MRD in the DPs and the percentage of CD19+ leukemic cells present in the starting material. In a CAR T-cell sample with a detection level of B cells below the sensitivity of the molecular assay, the EuroFlow flow-cytometry platform was indeed able to detect the presence of a significant percentage of contaminating B cells, that were also stained positive for the presence of CAR.CD19.

According to the present invention, as shown in the example, B leukemia cell lines genetically modified with CAR.CD19 vector with a short linker show a significant reduction in the CD19 MFI, with CD19 still detectable by cytofluorimetric assay, respect to what observed in B cell line expressing CAR.CD19 with the long liker, showing a complete negative binding of CD19 antibody.

Functional analysis confirmed these findings:

CAR.CD19 T cells of the invention were able to eliminate CAR+ leukemia cell lines in *in vitro* co-culture.

Finally, *in vivo* models corroborate the above-mentioned *in vitro* data.

CAR.CD19 according to the present invention can comprise also the suicide gene inducible caspase 9 (iC9) in the construct.

According to the present invention, a γ -retroviral vector coding for iC9.CAR.CD19 has been used, which is a bicistronic vector cloned in frame with the suicide gene iC9.

Therefore, CAR+ B cell leukemia could be *in vivo* controlled by either the systemic infusion of CAR.CD19 T cells or by the administration of AP1903 to activate the suicide gene inducible caspase 9 (iC9).

In fact, AP1903 (Rimiducid) is an inert small bio-molecule, which is able to activate the iC9-mediated caspase cascade by inducing dimerization of the FK-binding protein domain of the construct¹⁰. In a pivotal study conducted in patients given a T-cell depleted allograft followed by post-transplant infusion of donor T cells transduced with iC9, it was shown that AP1903 administration can trigger chemically-induced dimerization and eliminate genetically modified T cells from both peripheral blood and central nervous system (CNS), leading to rapid resolution of GVHD and CRS. Thus, iC9 activation by a single dose of AP1903 produce both rapid and long-term control of T cells carrying the suicide gene¹¹.

As described in the example below, CD19⁺ tumor cell lines were genetically modified with the bicistronic vector coding for iC9.CAR.CD19 to reproduce a CAR⁺ leukemic clonotype. *In vitro* experiments showed that the activation of iC9 by exposition to AP1903 was able to eliminate CAR⁺ leukemic cells. In particular, cytofluorimetric analysis of iC9.CAR⁺ leukemic cell lines treated with AP1903 shows that the remaining cells surviving in culture were characterized by a strongly reduced expression of the CAR respect to un-treated cells, but still with a detectable CAR MFI threshold. A more sensitive molecular analysis reveals that iC9 activation was associated with a significant reduction of transgene detection among the residual cells after the treatment, with leukemia B cells surviving after the AP1903 treatment showing a residual low VCN threshold.

These data confirmed several both *in vitro*^{14,15} and *in vivo* evidences¹⁶ showing that AP1903 treatment is highly efficient in eliminating the great majority of iC9⁺ cells, while sparing cells with a lower level of iC9 expression. Indeed, in the context of genetically modified T cells, patients who received a haploidentical iC9-T cells infusion after HSCT to treat GVHD, showed 1% of residual iC9-T cells after AP1903 administration. The residual iC9⁺ T cells were characterized by a remarkable dim expression of the transgene, possibly related to a low level of T cell activation. Indeed, iC9⁺ T cell elimination could be enhanced by T-cell activation during repeated AP1903 administrations¹⁶. By contrast,

when iC9 is expressed in tumor cells, AP1903 is able to induce massive and rapid apoptosis, leading to a significant *in vivo* tumor control^{14,15}.

In the context of the present invention, in case of an unlikely event of genetic modification of leukemia cells by retroviral vector encoding a iC9.CAR.CD19 gene, the leukemia cells surviving to AP1903 exposure will lack high CAR expression on their surface, resulting in the possibility to target the CD19 antigen with high efficiency by both CAR.CD19 allogenic NK cells and CAR.CD19 T-cells, at the same extend of WT leukemia/lymphoma cells.

In addition, in the context of the present invention, CAR design is also a relevant factor regulating the ability of CAR.CD19 T-cells to recognize and kill CAR+ leukemia cells, without substantially modify the anti-leukemic activity of CAR.CD19 T cells. Indeed, CAR.CD19SL/LH or SL/SH T cells exert a significant anti-leukemia activity in both in vitro and in vivo models, at the same extent of the more conventional CAR.CD19LL/SH T cells. Nevertheless, when the CD19 and the short-linker CAR.CD19 are expressed in CIS on the same cellular membrane of leukemia B-cell lines, these latter showed significantly reduced CD19 MFI respect to wild-type B cells, with CD19 being still detectable by flow-cytometry, suggesting a non complete CIS masking of the antigen.

It is therefore specific object of the present invention a chimeric antigen receptor comprising or consisting of, from the N-terminus to the C-terminus:

- a) a signal peptide,
- b) a single chain antibody domain chosen from the group consisting of anti CD19 single chain antibody domain, anti CD20 single chain antibody domain or anti CD22 single chain antibody domain, said single chain antibody domain comprising or consisting of VL and VH sequences linked each other by a linker,
- c) a hinge,
- d) a trans membrane domain,
- e) a co-stimulatory signaling domain, and
- f) CD3Zeta chain sequence,

wherein said linker is a short flexible linker with a length from 7 to 14 (i.e., with a length of 7, 8, 9, 10, 11, 12, 13 or 14 amino acids), for example from 7 to 12 or from 7 to 10 or 8 amino acids.

Unless specified, as used herein, an scFv may have the VL and VH variable regions in either order, e.g. with respect to the N-terminal and C-terminal ends of the polypeptide, the scFv may comprise VL-linker-VH or may comprise VH-linker-VL.

According to the present invention, anti CD19 single chain antibody domain can comprise anti CD19 FMC63 hybridoma VL and VH sequences, in either order, wherein anti CD19 FMC63 hybridoma VL sequence comprises CDR1 sequence QDISKY (SEQ ID NO:1), CDR2 sequence HTS and CDR3 sequence GNTLP (SEQ ID NO:2), whereas anti CD19 FMC63 hybridoma VH sequence comprises CDR1 sequence GVSLPDYG (SEQ ID NO:3), CDR2 sequence IWGSETT (SEQ ID NO:4) and CDR3 sequence AKHYYYGGSYAMDY (SEQ ID NO:5);

anti CD20 single chain antibody domain can comprise anti CD20 VL and VH sequences, wherein anti CD20 VL sequence²² comprises CDR1 sequence SSVSY (SEQ ID NO:6), CDR2 sequence ATS and CDR3 sequence QQWTSNPPT (SEQ ID NO:7), whereas anti CD20 VH sequence comprises CDR1 sequence GYTFTSYN (SEQ ID NO:8), CDR2 sequence IYPGNGDT (SEQ ID NO:9) and CDR3 sequence ARSTYYGGDWYFNV (SEQ ID NO:10);

anti CD22 single chain antibody domain can comprise anti CD22 VL and VH sequences, wherein anti CD22 VL sequence comprises CDR1 sequence QSLANSYGNTF (SEQ ID NO:11), CDR2 sequence GIS and CDR3 sequence LQGTHQP (SEQ ID NO:12), whereas anti CD 22 VH sequence comprises CDR1 sequence GYRFTNYWIH (SEQ ID NO:13), CDR2 sequence INPGNNYA (SEQ ID NO:14) and CDR3 sequence TR.

According to the present invention, anti-CD19 FMC63 hybridoma VL sequence can comprise or consist of sequence

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVK
LLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLP
YTFGGGKLEIT (SEQ ID NO:15) and

10

anti-CD19 FMC63 hybridoma VH sequence can comprise or consist of sequence

EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKG
LEWLGVIWGSETTYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYC
5 AKHYYYGGSYAMDYWGQGTSTVTVSS (SEQ ID NO:16);

anti-CD20 VL sequence can comprise or consist of sequence

QIVLSQSPAILSASPGEKVTMTCRASSSVSYIHWFQQKPGSSPKP
WIYATSNLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNP
PTFGGGTKLEIK (SEQ ID NO:17) and

10 anti-CD20 VH sequence can comprise or consist of sequence

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGR
GLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSA
VYYCARSTYYGGDWYFNWVGAGTTVTVSA (SEQ ID NO:18);

anti-CD22 VL sequence can comprise or consist of sequence

15 DVQVTQSPSSLSASVGDRVITCRSSQSLANSYGNTFLSWYLHK
PGKAPQLLIYGISNRFSGVPDRFSGSGSGTDFTLTISLQPEDFATYYCL
QGTHQPYTFGQGTKVEIK (SEQ ID NO:19) and

anti-CD22 VH sequence can comprise or consist of sequence

EVQLVQSGAEVKKPGASVKVSCKASGYRFTNYWIHWVRQAPGQ
20 GLEWIGGINPGNNYATYRRKFQGRVTMTADTSTSTVYMELSSLRSEDTA
VYYCTREGYGNYGAWFAYWGQGLTVTVSS (SEQ ID NO:20).

The linker which links VL and VH sequences can be chosen from the group consisting of a short and flexible aminoacid glycines-rich sequence, such as (G4S)₂ linker GGGGSGGGG (SEQ ID NO:35), G4SG2 linker GGGGSGG (SEQ ID NO:37) or G3SG4 linker GGGSGGGG (SEQ ID NO:38), SG4SG3 linker SGGGGSGGG (SEQ ID NO:186), (SG4)₂ S linker SGGGGSGGGGS (SEQ ID NO:187), (SG4)₂ SG linker SGGGGSGGGGSG (SEQ ID NO:188), (SG4)₂ SG3 linker SGGGGSGGGGSGGG linker (SEQ ID NO:189), (SG4)₂ SGGGGSGGGG (SEQ ID NO:190), (SG4)₂ SG2 SGGGGSGGGGSGG (SEQ ID NO:191), preferably G3SG4 linker.

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According to specific embodiments, said hinge can comprise or

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consist of one or more of the following hinges:

CD8stalk

TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD
(SEQ ID NO:21) (nucleotide ID NO: M12828.1 and Protein ID NO:
5 AAB04637.1);

Hinge

CD28

EVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFLPGPSKP (SEQ ID NO:22)
(nucleotide ID NO: AJ517504.1 and Protein ID NO: CAD57003.1);

hinge CH2-CH3 (UNIPROTKB:P01861)

10 ESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVV
VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKGLPSSIEKTKAKGQPREPQVYTLPPSQEEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSR
LTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLK (SEQ ID
15 NO:23); or

hinge CH3 (UNIPROTKB:P01861):

ESKYGPPCPSCPGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGF
YPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSRLTVDKSRWQEG
NVFSCSVMHEALHNHYTQKSLSLGLK (SEQ ID NO:24), preferably
20 CD8stalk.

According to the present invention, the hinge can be linked to the
single chain antibody domain by a second linker (or adapter).

According to the present invention, said hinge can be linked, at the
N terminus, to a trackable marker, said trackable marker being linked,
25 optionally by a second linker (or adapter), to the single chain antibody
domain, i.e. the second linker links the single chain antibody domain and
the trackable marker. For example, the second linker (or adapter) can be a
dipeptide, such as for example GS.

According to the present invention the trackable marker can
30 be chosen from the group consisting of:

ΔCD34 ELPTQGTFSNVSTNVS (SEQ ID NO:25) (nucleotide ID NO
AB238231.1 and Protein ID NO: BAE46748.1); or

NGFR

12

KEACPTGLYTHSGECCACNLGEGVAQPCGANQTVCEPCLDSV
 TFSDVVSATEPCKPCTECVGLQSMSAPCVEADDAVCRCAYGYQDETT
 GRCEACRVCEAGSGLVFSCQDKQNTVCEECPDGTYSDEANHVDPCLP
 CTVCEDTERQLRECTRWADAEECEIPGRWITRSTPPEGSDSTAPSTQEP
 5 EAPPEQDLIASTVAGVVTTVMGSSQPVVTRGTTDN (SEQ ID NO:26)
 (nucleotide ID NO:AK313654.1 and Protein ID NO:BAG36408.1),
 preferably Δ CD34.

According to the present invention, the hinge CD8stalk can be
 linked to the trackable marker Δ CD34.

10 The trans membrane domain can be chosen from the group
 consisting of CD28TM FWVLVVVGVLACYSLLVTVAFIIFWV (SEQ ID
 NO:27) (nucleotide ID NO: BC112085.1 and Protein ID NO: AAI12086.1)
 or CD8aTM IYIWAPLAGTCGVLLLSLVIT (SEQ ID NO:28) (nucleotide ID
 NO NM_001768.6 and Protein ID NO: NP_001759.3), preferably CD8aTM.

15 According to the present invention the co-stimulatory signaling
 domain can be chosen from the group consisting of

CD28 cytoplasmic sequence
 RSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRS (SEQ ID
 NO:29) (nucleotide ID NO: AF222341.1 and Protein ID NO: AAF33792.1),
 20 CD137 (4-1BB) sequence
 KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEEGGCEL (SEQ ID
 NO:30) (nucleotide ID NO: U03397.1 and Protein NO: AAA53133.1),

OX40 sequence
 RDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI (SEQ ID NO:31)
 25 (nucleotide ID NO: NM_003327.3 and Protein NO: NP_003318.1), or

a sequence obtained by linking:

CD28 cytoplasmic sequence (SEQ ID NO:29) with CD137 (4-1BB)
 sequence (SEQ ID NO:30),

CD137 (4-1BB) sequence (SEQ ID NO:30) with CD28 cytoplasmic
 30 sequence (SEQ ID NO:29),

CD28 cytoplasmic sequence (SEQ ID NO:29) with OX40 sequence
 (SEQ ID NO:31),

OX40 sequence (SEQ ID NO:31) with CD28 cytoplasmic sequence

(SEQ ID NO:29),

OX40 sequence (SEQ ID NO:31) with CD137 (4-1BB) sequence
(SEQ ID NO:30),

5 CD137 (4-1BB) sequence (SEQ ID NO:30) with OX40 sequence
(SEQ ID NO:31)

According to the present invention, CD3Zeta chain sequence is
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG
KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLS
TATKDTYDALHMQALPPR* (SEQ ID NO:32) (nucleotide ID NO: J04132.1
10 and Protein ID NO:AAA60394.1).

According to the present invention, the chimeric antigen receptor
can further comprise cytoplasmic moiety of CD8cyt:
LYCNHRNRRRVCKCPR (SEQ ID NO:40) (nucleotide ID NO
NM_001768.6 and Protein ID NO: NP_001759.3) between the
15 transmembrane domain and the co-stimulatory signaling domain.

The cytoplasmic moiety of CD8cyt can be linked to the co-
stimulatory signaling domain by a linker, such as a dipeptide, for example
VD.

According to the present invention, the signal peptide can comprise
20 or consist of MEFGLSWLFLVAILKGVQC (SEQ ID NO:41) (nucleotide ID
NO:AB776838.1 and Protein ID NO: BAN63131.1).

According to the present invention, the chimeric antigen receptor
comprises or consists of
MEFGLSWLFLVAILKGVQCSRDIQMTQTSSLSASLGDRVTISCRASQDI
25 SKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL
EQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGGEVKLQESGPGL
VAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVWGSSETYY
NSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDY
WGQGTSTVTVSSGSELPTQGTFSTNVSTNVSPAPRPPTPAPTIASQPLSLR
30 PEACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLVITLYCNHRN
RRRVCKCPRVDKRGKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEE
GGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRD
PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGL

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YQGLSTATKDTYDALHMQALPPR (SEQ ID NO:72) or
 MEFGLSWLFLVAILKGVQCSRDIQMTQTSSLSASLGDRVTISCRASQDI
 SKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL
 EQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGGGEVKLQESGPGL
 5 VAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVWGETTYY
 NSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYGGSYAMDY
 WGQGTSTVTVSSGSPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR
 GLDFACDIYWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRVDKRRGR
 KLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAP
 10 AYQQGQNQLYNELNLGRREEYDVLDRRRGRDPEMGGKPRRKNPQEG
 YNELQKDKMAEAYSEIGMKGERRRRGKGHDGLYQGLSTATKDTYDALHM
 QALPPR (SEQ ID NO:33).

Namely, anti-CD19 chimeric antigen receptor according to the present invention can consist of:

15 signal peptide MEFGLSWLFLVAILKGVQC (SEQ ID NO:41), which is linked by a SR linker to

anti CD19 single chain antibody domain from FMC63 hybridoma consisting of FMC63 VL sequence

20 DIQMTQTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYH
 TSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFG
 GGTKEIT (SEQ ID NO:15) linked by Flex Linker (short linker-SL), preferably G3SG4 linker GGGSGGGG (SEQ ID NO:38), to FMC63 VH sequence

25 EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWL
 GVIWGETTYYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY
 YYGGSYAMDYWGQGTSTVTVSS (SEQ ID NO:16), FMC63 VH sequence being linked by the GS linker (i.e. the second linker or adapter) to a:

Trackable marker Δ CD34 ELPTQGTFSNVSTNVS (SEQ ID NO:25)

and CD8stalk

30 PAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:21) (Long hinge-LH: Δ CD34 + CD8stalk) or CD8stalk PAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:21) (Short Hinge-SH: CD8stalk), which is linked to

15

CD8aTM IYIWAPLAGTCGVLLLSLVIT (SEQ ID NO:28), which is linked to

cytoplasmic moiety of CD8cyt LYCNHRNRRRVCKCPR (SEQ ID NO:40), which is linked by linker VD to

5 co-stimulatory signaling domain CD137 (4-1BB) sequence: KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEEGGCEL (SEQ ID NO:30), which is linked to CD3Zeta chain RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLS
10 TATKDTYDALHMQALPPR* (SEQ ID NO:32).

The present invention concerns also a nucleotide sequence comprising or consisting of a nucleotide sequence which encodes a chimeric antigen receptor as defined above.

In particular, anti CD19 FMC63 hybridoma VL sequence can be
15 encoded by the nucleotide sequence

GACATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTC
TGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGT
AAATATTTAAATTGGTATCAGCAGAAACCAGATGGAAGTGTAACTC
CTGATCTACCATACATCAAGATTACACTCAGGAGTCCCATCAAGGTTC
20 AGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCT
GGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAGGGTAATACGC
TTCCGTACACGTTCCGAGGGGGGACTAAGTTGGAAATAACA (SEQ ID
NO:52) and

anti CD19 FMC63 hybridoma VH sequence can be encoded by the
25 nucleotide sequence

GAGGTGAAACTGCAGGAGTCAGGACCTGGCCTGGTGGCGCC
CTCACAGAGCCTGTCCGTCACATGCACTGTCTCAGGGGTCTCATTAC
CCGACTATGGTGTAAAGCTGGATTGCCAGCCTCCACGAAAGGGTCTG
GAGTGGCTGGGAGTAATATGGGGTAGTGAAACCACATACTATAATTC
30 AGCTCTCAAATCCAGACTGACCATCATCAAGGACAACTCCAAGAGCC
AAGTTTTCTTAAAAATGAACAGTCTGCAAAGTATGACACAGCCATTT
ACTACTGTGCCAAACATTATTACTACGGTGGTAGCTATGCTATGGACT
ACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA (SEQ ID NO:53);

16

anti CD20 VL sequence can be encoded by the nucleotide sequence

CAGATCGTGCTGAGCCAGAGCCCCGCCATCCTGAGCGCCAGC
CCCGGCCGAGAAGGTGACCATGACCTGCAGGGCCAGCAGCAGCGTG
5 AGCTACATCCACTGGTTCCAGCAGAAGCCCGGCAGCAGCCCCAAGC
CCTGGATCTACGCCACCAGCAACCTGGCCAGCGGCGTGCCCGTGAG
GTTTCAGCGGCAGCGGCAGCGGCACCAGCTACAGCCTGACCATCAGC
AGGGTGGAGGCCGAGGACGCCGCCACCTACTACTGCCAGCAGTGGA
CCAGCAACCCCCCACCTTCGGCGGCGGCACCAAGCTGGAGATCAA
10 G (SEQ ID NO:54) and

anti CD20 VH sequence can be encoded by the nucleotide sequence

CAGGTGCAGCTGCAGCAGCCCGGCCGAGCTGGTGAAGCC
CGGCGCCAGCGTGAAGATGAGCTGCAAGGCCAGCGGCTACACCTTC
15 ACCAGCTACAACATGCACTGGGTGAAGCAGACCCCGGCAGGGGCC
TGGAGTGGATCGGCGCCATCTACCCGGCAACGGCGACACCAGCTA
CAACCAGAAGTTCAAGGGCAAGGCCACCCTGACCGCCGACAAGAGC
AGCAGCACCGCCTACATGCAGCTGAGCAGCCTGACCAGCGAGGACA
GCGCCGTGTACTACTGCGCCAGGAGCACCTACTACGGCGGCGACTG
20 GTACTTCAACGTGTGGGGCGCCGGCACCAACCGTGACCGTGAGC
(SEQ ID NO:55);

anti CD22 VL sequence can be encoded by the nucleotide sequence

GACGTGCAGGTGACCCAGAGCCCCAGCAGCCTGAGCGCCAGCGTG
25 GGCGACAGGGTGACCATCACCTGCAGGAGCAGCCAGAGCCTGGCCA
ACAGCTACGGCAACACCTTCCTGAGCTGGTACCTGCACAAGCCCGG
CAAGGCCCCCAGCTGCTGATCTACGGCATCAGCAACAGGTTTCAGC
GGCGTGCCCGACAGGTTTCAGCGGCAGCGGCAGCGGCACCGACTTC
ACCCTGACCATCAGCAGCCTGCAGCCCGAGGACTTCGCCACCTACTA
30 CTGCCTGCAGGGCACCCACCAGCCCTACACCTTCGGCCAGGGCACC
AAGGTGGAGATCAAG(SEQ ID NO:56) and

anti CD22 VH sequence can be encoded by the nucleotide sequence

17

GAGGTGCAGCTGGTGCAGAGCGGCGCCGAGGTGAAGAAGCCCGGC
GCCAGCGTGAAGGTGAGCTGCAAGGCCAGCGGCTACAGGTTACCA
ACTACTGGATCCACTGGGTGAGGCAGGCCCCCGGCCAGGGCCTGGA
GTGGATCGGCGGCATCAACCCCGGCAACAACCTACGCCACCTACAGG
5 AGGAAGTTCCAGGGCAGGGTGACCATGACCGCCGACACCAGCACCA
GCACCGTGTACATGGAGCTGAGCAGCCTGAGGAGCGAGGACACCGC
CGTGTACTACTGCACCAGGGAGGGCTACGGCAACTACGGCGCCTGG
TTCGCCTACTGGGGCCAGGGCACCTGGTGACCGTGAGCAGC (SEQ
ID NO:57).

10 According to the present invention, the nucleotide sequence
encoding anti-CD19 chimeric antigen receptor is:

ATGGAGTTTGGACTTTCTTGGTTGTTTTGGTGGCAATTCTGAAGGGT
GTCCAGTGTAGCAGGGACATCCAGATGACACAGACTACATCCTCCCT
GTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTC
15 AGGACATTAGTAAATATTTAAATTGGTATCAGCAGAAACCAGATGGAA
CTGTAAACTCCTGATCTACCATACATCAAGATTACACTCAGGAGTCC
CATCAAGGTTCAAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACC
ATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAG
GGTAATACGCTTCCGTACACGTTCCGAGGGGGGACTAAGTTGGAAAT
20 AACAGGCGGAGGAAGCGGAGGTGGGGGCGAGGTGAAACTGCAGGA
GTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCGTCACA
TGCACTGTCTCAGGGGTCTCATTACCCGACTATGGTGTAAGCTGGAT
TCGCCAGCCTCCACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGG
GGTAGTGAAACCACATACTATAATTCAGCTCTCAAATCCAGACTGACC
25 ATCATCAAGGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGT
CTGCAAACTGATGACACAGCCATTTACTACTGTGCCAAACATTATTAC
TACGGTGGTAGCTATGCTATGGACTACTGGGGTCAAGGAACCTCAGT
CACCGTCTCCTCAGGATCCGAACCTCCTACTCAGGGGACTTTCTCAA
ACGTTAGCACAAACGTAAGTCCCGCCCCAAGACCCCCCACACCTGC
30 GCCGACCATTGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCCTGCC
GGCCAGCTGCCGGCGGGGGCCGTGCATACAAGAGGACTCGATTTTCG
TTGCGACATCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCC
TTCTGCTCAGCCTGGTTATTACTCTGTACTGTAATCACCGGAATCGCC

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GCCGCGTTTGTAAGTGTCCCAGGGTCGACAAACGGGGCAGAAAGAA
ACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACT
CAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAG
GAGGATGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCC
5 CGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAG
GACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGA
CCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGC
CTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCGCTACAGTGA
GATTGGGATGAAAGGCGAGCGCCGGAGGGGGCAAGGGGCACGATGG
10 CCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCC
TTCACATGCAGGCCCTGCCCCCTCGCTAA (SEQ ID NO:58)or
ATGGAGTTTGGACTTTCTTGGTTGTTTTTGGTGGCAATTCTGAAGGGT
GTCCAGTGTAGCAGGGACATCCAGATGACACAGACTACATCCTCCCT
GTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTC
15 AGGACATTAGTAAATATTTAAATTGGTATCAGCAGAAACCAGATGGAA
CTGTTAACTCCTGATCTACCATACATCAAGATTACACTCAGGAGTCC
CATCAAGGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACC
ATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAG
GGTAATACGCTTCCGTACACGTTCCGAGGGGGGACTAAGTTGGAAAT
20 AACAGGCGGAGGAAGCGGAGGTGGGGGCGAGGTGAACTGCAGGA
GTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCGTCACA
TGCACTGTCTCAGGGGTCTCATTACCCGACTATGGTGTAAGCTGGAT
TCGCCAGCCTCCACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGG
GGTAGTGAAACCACATACTATAATTCACTCTCAAATCCAGACTGACC
25 ATCATCAAGGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGT
CTGCAAACTGATGACACAGCCATTTACTACTGTGCCAAACATTATTAC
TACGGTGGTAGCTATGCTATGGACTACTGGGGTCAAGGAACCTCAGT
CACCGTCTCCTCAGGATCCCCCGCCCCAAGACCCCCCACACCTGCG
CCGACCATTGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCCTGCCG
30 GCCAGCTGCCGGCGGGGCCGTGCATACAAGAGGACTCGATTTGCT
TGCGACATCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCCT
TCTGCTCAGCCTGGTTATTACTCTGTACTGTAATCACCGGAATCGCCG
CCGCGTTTGTAAGTGTCCCAGGGTCGACAAACGGGGCAGAAAGAAA

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CTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACTC
AAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAGGA
GGATGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCG
CGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGA
5 CGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACC
CTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCT
GTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGA
TTGGGATGAAAGGCGAGCGCCGGAGGGGGCAAGGGGCACGATGGCC
TTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTT
10 CACATGCAGGCCCTGCCCCCTCGCTAAA (SEQ ID NO:34).

According to the present invention, the nucleotide sequence can further comprise a nucleotide sequence encoding a suicide gene inducible amino acid sequence linked to the nucleotide sequence encoding said chimeric antigen receptor by a nucleotide sequence encoding a 2A self-cleaving peptide. The suicide gene inducible amino acid sequence can be a chimeric Caspase-9 polypeptide or comprises a herpes simplex virus thymidine kinase.

Therefore, in the cell, the polynucleotide 2A self-cleaving peptide cut the peptide comprising the suicide gene inducible amino acid sequence and the chimeric antigen receptor and in two separate peptides, i.e. the suicide gene inducible and the chimeric antigen receptor amino acid sequences.

According to an embodiment, the nucleotide sequence can be:

ATGCTCGAGATGCTGGAGGGAGTGCAGGTGGAGACTATTAGC
25 CCCGGAGATGGCAGAACATTCCCCAAAAGAGGACAGACTTGCGTCG
TGCATTATACTGGAATGCTGGAAGACGGCAAGAAGGTGGACAGCAG
CCGGGACCGAAACAAGCCCTTCAAGTTCATGCTGGGGAAAGCAGGAA
GTGATCCGGGGCTGGGAGGAAGGAGTCGCACAGATGTCAGTGGGAC
AGAGGGCCAACTGACTATTAGCCCAGACTACGCTTATGGAGCAACC
30 GGCCACCCCGGGATCATTCCCCCTCATGCTACACTGGTCTTCGATGT
GGAGCTGCTGAAGCTGGAAAGCGGAGGAGGATCCGGAGTGGACGG
GTTTGGAGATGTGGGAGCCCTGGAATCCCTGCGGGGCAATGCCGAT
CTGGCTTACATCCTGTCTATGGAGCCTTGCGGCCACTGTCTGATCAT

20

TAACAATGTGAACTTCTGCAGAGAGAGCGGGCTGCGGACCAGAACAA
GGATCCAATATTGACTGTGAAAAGCTGCGGAGAAGGTTCTCTAGTCT
GCACTTTATGGTCGAGGTGAAAGGCGATCTGACCGCTAAGAAAATGG
TGCTGGCCCTGCTGGAAGTGGCTCGGCAGGACCATGGGGCACTGGA
5 TTGCTGCGTGGTCGTGATCCTGAGTCACGGCTGCCAGGCTTCACATC
TGCAGTTCCTGGGGCAGTCTATGGAAGTACGGCTGTCCAGTCAG
CGTGGAGAAGATCGTGAACATCTTCAACGGCACCTCTTGCCCAAGTC
TGGGCGGGAAGCCCAAAGTGTCTTTATTTCAGGCCTGTGGAGGCGA
GCAGAAAGATCACGGCTTCGAAGTGGCTAGCACCTCCCCGAGGAC
10 GAATCACCTGGAAGCAACCCTGAGCCAGATGCAACCCCTTCCAGGA
AGGCCTGAGGACATTTGACCAGCTGGATGCCATCTCAAGCCTGCCCA
CACCTTCTGACATTTTCGTCTCTTACAGTACTTTCCCTGGATTTGTGA
GCTGGCGCGATCCAAAGTCAGGCAGCTGGTACGTGGAGACACTGGA
CGATATCTTTGAGCAGTGGGCCATTCTGAAGACCTGCAGAGTCTGC
15 TGCTGCGAGTGGCCAATGCTGTCTCTGTGAAGGGGATCTACAAACAG
ATGCCAGGATGCTTCAACTTTCTGAGAAAGAACTGTTCTTTAAGACC
TCCGCATCTAGGGCCCCGCGGAAGGCCGAGGGAGCCTGCTGACAT
GTGGCGATGTGGAGGAAAACCCAGGACCACCATGGATGGAGTTTGG
ACTTTCTTGTTGTTTTTGGTGGCAATTCTGAAGGGTGTCCAGTGTAG
20 CAGGGACATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTC
TGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGT
AAATATTTAAATTGGTATCAGCAGAAACCAGATGGAAGTGTAAACTC
CTGATCTACCATACATCAAGATTACACTCAGGAGTCCCATCAAGGTTT
AGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCT
25 GGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAGGGTAATACGC
TTCCGTACACGTTCCGAGGGGGGACTAAGTTGGAAATAACAGGCGG
AGGAAGCGGAGGTGGGGGCGAGGTGAAACTGCAGGAGTCAGGACC
TGGCCTGGTGGCGCCCTCACAGAGCCTGTCCGTACATGCACTGTC
TCAGGGGTCTCATTACCCGACTATGGTGTAAAGCTGGATTGCCAGCC
30 TCCACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGGTAGTGAA
ACCACATACTATAATTCAGCTCTCAAATCCAGACTGACCATCATCAAG
GACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCAAACT
GATGACACAGCCATTTACTACTGTGCCAAACATTATTACTACGGTGGT

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AGCTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTC
 CTCAGGATCCGAACTTCCTACTCAGGGGACTTTCTCAAACGTTAGCA
 CAAACGTAAGTCCCGCCCCAAGACCCCCACACCTGCGCCGACCAT
 TGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCCTGCCGGCCAGCT
 5 GCCGGCGGGGCCGTGCATACAAGAGGACTCGATTTGCTTGCGACA
 TCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCCTTCTGCTC
 AGCCTGGTTATTACTCTGTACTGTAATCACCGGAATCGCCGCCGCGT
 TTGTAAGTGTCCCAGGGTCGACAAACGGGGCAGAAAGAACTCCTGT
 ATATATTCAAACAACCATTTATGAGACCAGTACAACTACTCAAGAGG
 10 AAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGGAGGATGT
 GAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACC
 AGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGA
 GAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGA
 TGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAA
 15 TGAAGTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGG
 ATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTAC
 CAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACAT
 GCAGGCCCTGCCCCCTCGCTAA (SEQ ID NO:180).

Namely, this nucleotide sequence, which encodes the sequence
 20 named also as iCas9CAR.CD19SL-LH, comprises the following
 sequences:

iCas9 chimeric protein (FKBP12wt binding region-linker-Caspase-9 polypeptide):

FKBP12wt binding region:

25 ATGCTCGAGATGCTGGAGGGAGTGCAGGTGGAGACTATTAGC
 CCCGGAGATGGCAGAACATTCCCCAAAAGAGGACAGACTTGCGTCG
 TGCATTATACTGGAATGCTGGAAGACGGCAAGAAGGTGGACAGCAG
 CCGGGACCGAAACAAGCCCTTCAAGTTCATGCTGGGGAAGCAGGAA
 GTGATCCGGGGCTGGGAGGAAGGAGTCGCACAGATGTCAGTGGGAC
 30 AGAGGGCCAACTGACTATTAGCCAGACTACGCTTATGGAGCAACC
 GGCCACCCCGGGATCATTCCCCTCATGCTACACTGGTCTTCGATGT
 GGAGCTGCTGAAGCTGGAA (SEQ ID NO:59) (nucleotide ID NO:
 BT007066.1)

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Link of connection

AGCGGAGGAGGATCCGGA (SEQ ID NO:60)

Caspase-9 polypeptide

GTGGACGGGTTTGGAGATGTGGGAGCCCTGGAATCCCTGCGG

5 GGCAATGCCGATCTGGCTTACATCCTGTCTATGGAGCCTTGCGGCCA
 CTGTCTGATCATTAAACAATGTGAACCTTCTGCAGAGAGAGCGGGCTGC
 GGACCAGAACAGGATCCAATATTGACTGTGAAAAGCTGCGGAGAAGG
 TTCTCTAGTCTGCACTTTATGGTCGAGGTGAAAGGCGATCTGACCGC
 TAAGAAAATGGTGCTGGCCCTGCTGGAACCTGGCTCGGCAGGACCAT
 10 GGGGCACTGGATTGCTGCGTGGTCGTGATCCTGAGTCACGGCTGCC
 AGGCTTCACATCTGCAGTTCCTGGGGCAGTCTATGGAACCTGACGGC
 TGTCCAGTCAGCGTGGAGAAGATCGTGAACATCTTCAACGGCACCTC
 TTGCCCCAAGTCTGGGCGGGAAGCCCAAACCTGTTCTTTATTCAGGCCT
 GTGGAGGCGAGCAGAAAGATCACGGCTTCGAAGTGGCTAGCACCTC
 15 CCCCAGGACGAATCACCTGGAAGCAACCCTGAGCCAGATGCAACC
 CCCTTCCAGGAAGGCCTGAGGACATTTGACCAGCTGGATGCCATCTC
 AAGCCTGCCCACACCTTCTGACATTTTCGTCTCTTACAGTACTTTCCC
 TGGATTTGTGAGCTGGCGCGATCCAAAGTCAGGCAGCTGGTACGTG
 GAGACACTGGACGATATCTTTGAGCAGTGGGCCCATTCTGAAGACCT
 20 GCAGAGTCTGCTGCTGCGAGTGGCCAATGCTGTCTCTGTGAAGGGG
 ATCTACAAACAGATGCCAGGATGCTTCAACTTTCTGAGAAAGAACTG
 TTCTTTAAGACCTCC (SEQ ID NO:61) (nucleotide ID NO: AK292111.1)

Link of connection

GCATCTAGGGCCCCGCGG (SEQ ID NO:62)

25 T2A self-cleaving peptides

GAAGGCCGAGGGAGCCTGCTGACATGTGGCGATGTGGAGGA

AAACCCAGGACCA (SEQ ID NO:63) (nucleotide ID NO: AF062037.1)

Short linker of connection

CCATGG

30 Signal peptide

ATGGAGTTTGGACTTTCTTGGTTGTTTTTGGTGGCAATTCTGAA

GGGTGTCCAGTGTAGCAGG (SEQ ID NO:64) (nucleotide ID NO:AB776838.1)

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VL sequence (FMC63)

GACATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTC
 TGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGT
 AAATATTTAAATTGGTATCAGCAGAAACCAGATGGAAGTGTAACTC
 5 CTGATCTACCATACATCAAGATTACACTCAGGAGTCCCATCAAGGTTC
 AGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCT
 GGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAGGGTAATACGC
 TTCCGTACACGTTCGGAGGGGGGACTAAGTTGGAAATAACA (SEQ ID
 NO:52)

10 Flex short Linker

GGCGGAGGAAGCGGAGGTGGGGGC (SEQ ID NO:65)

VH sequence (FMC63)

GAGGTGAAACTGCAGGAGTCAGGACCTGGCCTGGTGGCGCC
 CTCACAGAGCCTGTCCGTCACATGCACTGTCTCAGGGGTCTCATTAC
 15 CCGACTATGGTGTAAGCTGGATTCGCCAGCCTCCACGAAAGGGTCTG
 GAGTGGCTGGGAGTAATATGGGGTAGTGAAACCACATACTATAATTC
 AGCTCTCAAATCCAGACTGACCATCATCAAGGACAACTCCAAGAGCC
 AAGTTTTCTTAAAAATGAACAGTCTGCAAACTGATGACACAGCCATTT
 ACTACTGTGCCAAACATTATTACTACGGTGGTAGCTATGCTATGGACT
 20 ACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA (SEQ ID NO:53)

LH (Long Hinge): (short linker, i.e. the second linker or adapter) +
 (trackable marker: Δ CD34 extracellular + hinge: CD8stalk extracellular):

Short adapter

GGATCC

25 Δ CD34 extracellular

GAACTTCCTACTCAGGGGACTTTCTCAAACGTTAGCACAAACG
 TAAGT (SEQ ID NO:66) (nucleotide ID NO AB238231.1)

CD8stalk extracellular

CCCGCCCCAAGACCCCCACACCTGCGCCGACCATTGCTTCT
 30 CAACCCCTGAGTTTGAGACCCGAGGCCTGCCGGCCAGCTGCCGGCG
 GGGCCGTGCATACAAGAGGACTCGATTTGCTTGCAC (SEQ ID
 NO:67) (nucleotide ID NO: M12828.1);

CD8aTM (transmembrane)

24

ATCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCCTTC
TGCTCAGCCTGGTTATTACT (SEQ ID NO:68) (nucleotide ID NO
NM_001768.6)

CD8cyt (Cytoplasmic)

5 CTGTACTGTAATCACCGGAATCGCCGCCGCGTTTGTAAGTGTC
CCAGG (SEQ ID NO:69) (nucleotide ID NO NM_001768)

Short linker (containing Sal I site)

GTCGAC

4-1BB

10 AAACGGGGCAGAAAGAACTCCTGTATATATTCAAACAACCATT
TATGAGACCAGTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCC
GATTTCCAGAAGAAGAAGAAGGAGGATGTGAACTG (SEQ ID NO:70)
(nucleotide ID NO: U03397.1)

CD3Zeta chain

15 AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACCAG
CAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGA
GGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATG
GGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATG
AACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGAT
20 GAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCA
GGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACATGC
AGGCCCTGCCCCCTCGCTAA (SEQ ID NO:71) (nucleotide ID NO:
J04132.1).

25 According to the present invention iCas9CAR.CD19SL-SH consists
of the sequence of iCas9CAR.CD19SL-LH with the exception of the fact
that it comprises a short hinge (SH): (short adapter) + (Hinge: CD8stalk
extracellular).

The present invention concerns also a vector comprising the
nucleotide sequence as defined above, wherein said vector is a DNA
30 vector, a RNA vector, a plasmid, a lentivirus vector, adenoviral vector,
retrovirus vector, such as γ -retroviral vector, or non viral vector.

In addition, the present invention concerns a cell, such as T cell,
such as alfa/beta and gamma/delta T cell, NK cells, NK-T cells, comprising

25

the chimeric antigen receptor as defined above and/or the vector or plasmid as defined above.

The cell according to the present invention can further comprise a suicide gene inducible amino acid sequence such as a chimeric Caspase-9 polypeptide or comprises a herpes simplex virus thymidine kinase (HSVTK SEQ ID NO:42).

The suicide gene inducible amino acid sequence can be a chimeric Caspase-9 polypeptide or comprise a herpes simplex virus thymidine kinase (HSVTK SEQ ID NO:42).

10 The chimeric Caspase-9 polypeptide can comprise or consist of:

FKBP12 binding region comprising or consisting of a short 5' leader peptide MLEMLE (SEQ ID NO:43) and the mutant of human FKBP12(V36F) of sequence

15 GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDSSRDNRN
KPFKFM LGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIP
PHATLVFDVELLKLE (SEQ ID NO:44) (nucleotide ID NO: BT007066.1
and Protein ID NO: AAP35729.1), which is linked by a linker, such as
SGGGSG (SEQ ID NO:45) linker, to Caspase-9 polypeptide

20 VDGFGDVGALESLRGNADLAYILSMEPCGHCLIINN VNFCRESGL
RTRTGSNIDCEKLRRRFSSLHFMVEVKGDLTAKKMVLALLELARQDHGA
LDCCVVVILSHGCCQASHLQFPGAVYGTGCPVSV EKIVNIFNGTSCPSLG
GKPKLFFIQACGGEQKDHGF EVASTSPEDESPGSNPEPDATPFQEGLRT
FDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDIFEQWA
HSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (SEQ ID
25 NO:46) (nucleotide ID NO: AK292111.1 and Protein ID NO: BAF84800.1),
which is linked by a linker, such as ASRAPR (SEQ ID NO:47) linker
(containing SacII enzyme site), to

a Polynucleotide 2A self-cleaving peptide chosen from the group
consisting of T2A (derived from thosea asigna virus 2)
30 AEGRGSLLTCGDVEENPGP (SEQ ID NO:48) (nucleotide ID
NO: AF062037.1 and Protein ID NO: YP_009665206.1), P2A (derived
from porcine teschovirus-1 2A) ATNFSLLKQAGDVEENPGP (SEQ ID
NO:49) (nucleotide ID NO: AB038528.1 and Protein ID NO: BAB32828.1),

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E2A (derived from equine rhinitis A virus) QCTNYALLKLAGDVESNPGP (SEQ ID NO:50) (nucleotide ID NO: NC_039209.1 and Protein ID NO: "YP_009513027.1") or F2A (derived from foot-and-mouth disease virus: VKQTLNFDLLKLAGDVESNPGP (SEQ ID NO:51) (nucleotide ID NO: AY593825.1 and Protein ID NO: AAT01768.1), preferably T2A.

According to a specific embodiment of the present invention, the chimeric Caspase-9 polypeptide consists of:

- the leader peptide MLEMLE (SEQ ID NO:43),
- FKBP12 binding region

GVQVETISPGDGRTPKRGQTCVVHYTGMLEDGKKVDSSRDRNK
PFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPP
HATLVFDVELLKLE (SEQ ID NO:44), which is linked by SGGGSG (SEQ ID NO:45) linker to

- Caspase-9 polypeptide

VDGFGDVGALESLRGNADLAYILSMEPCGHCLIINNWNFCRESGLR
TRTGSNIDCEKLRRRFSSSLHFMVEVKGDLTAKKMLALLELARQDHGAL
DCCVVVILSHGCQASHLQFPGAVYGTGCPVSVEKIVNIFNGTSCPSLG
GKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSNPEPDATPFQEGLRT
FDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDIFEQWA
HSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (SEQ ID NO:46), which is linked by a linker ASRAPR (SEQ ID NO:47) to

- T2A self-cleaving peptides encoding AEGRGSLTTCGDVEENPGP (SEQ ID NO:48).

According to the present invention, the cell can be obtained in culture conditions wherein both IL-7 and IL-15 are present, for example in the culture conditions of the activation step, transduction step and/or expansion step of the process for the preparation of said cell.

The present invention concerns also a pharmaceutical composition comprising the nucleotide sequence as defined above, or the vector as defined above, or the cell as defined above, together with one or more excipients and/or adjuvants.

In addition, the present invention concerns a chimeric antigen receptor as defined above, nucleotide sequence as defined above, vector

as defined above, cell as defined above, pharmaceutical composition as defined above, for medical use.

It is also an object of the present invention chimeric antigen receptor as defined above, nucleotide sequence as defined above, vector as defined above, cell as defined above, pharmaceutical composition as defined above, for use in the treatment of CD19+, CD20+ or CD22+ cancers, for example B cell lymphomas (Non-Hodgkin's Lymphoma (NHL)), acute lymphoblastic leukemia (ALL), myeloid leukemia and chronic lymphocytic leukemia (CLL).

It is also an object of the present invention chimeric antigen receptor as defined above, nucleotide sequence as defined above, vector as defined above, cell as defined above, pharmaceutical composition as defined above, for use in the treatment of autoimmune diseases caused by B cells generating auto-antibodies, including but not only limited to systemic lupus erythematosus (SLE), Systemic sclerosis (SSc), ANCA-Associated Vasculitis (AAV), Dermatomyositis (DM).

The present invention now will be described by an illustrative, but not limitative way, according to the preferred embodiments thereof, with particular reference to the examples and the enclosed drawings, wherein:

- **Figure 1** shows CAR.CD19 T-cells generated from Bcp-ALL patients' derived PBMCs at diagnosis. **(A)** The scFv of α -CD19 is cloned in frame with the iC9 suicide gene, Δ CD34 trackable marker and both 4.1BB and the CD3 ζ signaling endodomains. PBMCs of Bcp-ALL patients at diagnosis are activated with soluble α -hCD3/ α -hCD28 mAbs and rh-IL7/rh-IL15 and then transduced with the CAR.CD19 γ -retroviral supernatant. **(B)** Flow-cytometry analysis of a representative donor showing CAR expression by Δ CD34 detection in un-transduced (NT) T-cells (negative control; left panel) and CAR.CD19 genetically modified T-cells (right panel). **(C)** Percentage of CAR+ T-cells at end-production (Day+14) in DPs with more than 45% (n=8) or equal to/less than 45% (n=7) of CD19+ leukemia/lymphoma cells in the starting raw material used for CAR T-cell manufacturing. The median value of 45% was used as cutoff. **(D)** Correlation matrix between percentage of CAR+ T-cells in the DPs at the

end of production and the percentage of CD19+ leukemic cells in the starting raw materials from patients. **(E)** Histograms representing the total fold expansion from Day +3 to the end of production of CAR T cells in the two subgroups of patients with <45% or >45% of CD19+ B cells in the SM.

5 **- Figure 2** shows BM Patient-derived CAR T cell proliferation and transduction. **(A)** Flow-cytometry analysis in a representative DP generated from BM mononuclear cells of a patient at diagnosis. Upper Panel A shows flow-cytometry analysis of CAR+ T cells in the negative control sample of NT T cells, whereas bottom panel shows the analysis in
10 iC9.CAR.CD19 genetically modified T cells. **(B)** Percentage of CD19+ leukemic blasts and CAR+ T cells in DPs generated from BM Bcp-ALL patients (n=10). **(C)** Fold expansion of NT and iC9.CAR.CD19 BM-derived T cells (black bars) compared to PB-derived T cells (white bars) of 10 Bcp-ALL patients, at the end of production. Data are expressed as average $\pm$
15 SD;

- Figure 3 shows the MRD analysis in DPs generated from raw materials of patients at diagnosis highly contaminated by leukemia cells. **(A)** Time-course experiments have been designed to evaluate the impact of manufacturing time on MRD value in DPs. T-cells were generated from
20 5 different patients (n= 5) and cultured for 8, 14 or 30 days before collection of the cells for MRD analysis, performed by qPCR analysis on Ig rearrangement with detection limit specified in Table1, and represented in the figure as negative range between 10⁻⁴ and 10⁻⁵ (dotted area). **(B)** Flow-cytometry analysis of two DPs: ALL#12 with high MRD values and
25 ALL#14 for which the detection of MRD was below the PCR sensitivity. Panels show the presence of leukaemia cells (black dots) positive for CAR expression, detected with both anti-CAR.CD19 FITC antibody (anti-CARTCD19, Cytognos SL, Salamanca, Spain) and anti-CD34 APC (CD34QBEND10), targeting the CD34 epitope in the CAR construct. B cell
30 precursors were identified by the use of EuroFlow standard operating procedures (SOP) for staining of surface markers (www.EuroFlow.org) (26) by EuroFlow Bcp-ALL MRD tubes, previously described for high-sensitive MRD measurements in Bcp-ALL by flow-cytometry.(27-29)

- **Figure 4** shows Flow-cytometry analysis of control un-transduced T cells and iC9.CAR.CD19LH T cells from one representative Bcp-ALL patient. Upper panels show flow-cytometric analysis of CD19 and CD10 B cell markers in control un-transduced T cells from ALL#14 patient revealing 1.5% of leukemic cells, whereas the contamination was significantly reduced in the iC9.CAR.CD19LH T cell sample manufactured from the same patient ALL#14 (0.0036% of leukemic cells).

- **Figure 5** shows the MRD analysis of DPs generated from BM raw materials of Bcp-ALL patients at diagnosis highly contaminated by leukaemia cells. Flow-cytometry analysis of B cell markers in two BM derived DPs from ALL#1 and ALL#2 patients. Panels show the presence of leukaemia cells (black dots);

- **Figure 6** shows that CAR.CD19 structure impacts on CD19 antigen engagement when they are both expressed on the same plasma membrane. **(A)** Cartoons representing CAR.CD19LL/SH (a), CAR.CD19LL/LH (b), CAR.CD19SL/SH (c) and CAR.CD19SL/LH (d). **(B)** CD19 expression detected by flow-cytometry in NALM-6 cells genetically modified by CAR.CD19LL/SH. Matched isotype staining histogram and the specific CD19 staining for CAR.CD19LL/SH cells histogram are shown (see arrows). **(C-E)** CD19 expression detected by flow-cytometry in NALM-6 cells genetically modified by CAR.CD19LL/LH **(C)**, by CAR.CD19SL/SH **(D)** and by CAR.CD19SL/LH **(E)** is shown by histograms, in comparison to the histogram of CD19 expression on CAR.CD19LL/SH.

- **Figure 7** shows the CD19 mRNA expression in both WT and CAR.CD19 Bcp-ALL cell lines. **(A)** Quantitative Real Time PCR (qRT-PCR) of CD19 mRNA expression in WT, CAR.CD19_{LH} and CAR.CD19_{SH} Bcp-ALL cell lines. Karpas cell line has been used as negative control. mRNA levels are shown as relative expression of a target gene versus ACT-B mRNA expression. Reactions were performed in triplicates; **(B)** MFI analysis of CAR.CD19 expression in leukaemia and lymphoma cell lines. MFI values of CAR.CD19 expression levels in WT and CAR.CD19SL/LH DAUDI (top panels) and RAJI (bottom panels). Data shows one representative experiment. **(C)** Long-term in vitro assay to evaluate anti-

tumor activity of CAR.CD19 T-cells on both WT and CAR.CD19 NALM-6 cell lines. The percentage of WT (black bars), NALM-6 CAR.CD19SL/LH (white bars) and NALM-6 CAR.CD19 UPenn (also named as CAR.CD19 LL/SH) leukemic cells (striped bars) after 7 days of in vitro co-culture. The assay was performed at decreasing effector target ratios, from 1:1 to 1:32 on NALM-6 Bcp-ALL tumor cell lines. Experiments were performed in triplicates. Data are expressed as average $\pm$ SD. * p-value= <0.05 , ** p-value= <0.01 , *** p-value= <0.001 .

- **Figure 8** shows long-term in vitro assays to evaluate the activity of CAR.CD19 T-cells and iC9 controlling CAR.CD19 positive leukemia or lymphoma cell lines. **(A-B)** 7 days co-culture assay of WT (black bars) and CAR.CD19SL/LH (white bars) with CAR.CD19 T-cells (E:T ratio is shown in the x axis of the graph as percentage of CAR+ T-cells in the culture). **(C)** 7 days co-culture assay of NALM-6 WT and CAR.CD19 genetically modified NALM-6 with NT (black bars), CAR.CD19SL/LH (white bars) and CAR.CD19LL/SH (striped bars). All experiments were performed in triplicate (n=6). Data are expressed as mean $\pm$ SD. * p-value= <0.05 , ** p-value= <0.01 , *** p-value= <0.001 , **** p-value= <0.0001 . **(D-E)** CAR.CD19 DAUDI cells were treated with 0 nM **(D)** and 20 nM **(E)** AP1903; both CAR and CD19 expression were monitored over time by flow-cytometry. **(F)** Detection of CAR.CD19 vector in tumor cells by qRT-PCR after AP1903 exposure. Reactions were performed in triplicate. Black histograms represent the positive control of reference (0nM AP1903) and white histograms represent results after one drug exposure (20nM of AP1903). * p-value= <0.05 , ** p-value= <0.01 , *** p-value= <0.001 .

- **Figure 9** shows CAR.CD19 T cells activation profile that is similar beside the CAR configuration. **(A)** IFN- γ production was measured after 24h of co-culture of Effector T cells and NALM-6 WT, or NALM-6 genetically modified with CAR.CD19 constructs. Data from 6 different CAR T products generated from HDs are expressed as mean $\pm$ SD. **(B)** CFSE Proliferation analysis representing the overlays of CAR T-cells unstimulated and stimulated with WT or CAR.CD19 modified NALM-6 cells.

- **Figure 10** shows the Effect of AP1903 administration on iC9.CAR.CD19 Bcp-ALL cell lines. iC9.CAR.CD19 (CAR.CD19LH) RAJI and NALM-6 cell lines were treated with 0 nM (**A-D**) and 20 nM (B-D) AP1903; both CAR and CD19 expression was monitored over time by FACS. AP1903 treatment (20nM) results in a prompt reduction of CAR+ cells starting from 6 hours after drug exposure. The reduction of CAR positivity after AP1903 exposure is associated with a gradual detection of CD19 antigen on cell surface. (E) iC9.CAR.CD19 DAUDI cell line were treated with 0 nM (black line) and 20 nM AP1903 (short dotted line); CAR.CD19 MFI was monitored over time by flow-cytometry analysis from Day 0 to Day 15 after treatment and compared to control WT NALM-6 cell line (long dotted line). (F) Graph reporting the vector copy number (VCN) of transgene in WT and gene-modified DAUDI, RAJI and NALM-6 cell lines either untreated (black bars) or exposed to 20 nM (white bars) AP1903. Data are expressed as mean $\pm$ SD.

- **Figure 11** shows that iC9.CAR.CD19 leukemia and lymphoma cells spared after iC9 activation could be efficiently recognized and eliminated by CAR.CD19 T cells as well as by allogenic CAR.CD19 NK cells. **(A)** 7-days co-culture assay was carried out between un-transduced T cells or CAR.CD19 T cells and wt DAUDI cells, iC9.CAR.CD19LH DAUDI cells never exposed to AP1903, and iC9.CAR.CD19LH DAUDI residual after AP1903 exposure and further re-expanded (at E:T ratio of 1:1). **(B)** 7-days co-culture assay was carried out between un-transduced T cells or CAR.CD19 T cells and wt NALM-6 cells, iC9.CAR.CD19LH NALM-6 cells never exposed to AP1903, and iC9.CAR.CD19LH NALM-6 residual after AP1903 exposure and further re-expanded (at E:T ratio of 1:1). **(C)** 7-days co-culture assay was carried out between un-transduced NK cells or CAR.CD19 NK cells and wt DAUDI cells, iC9.CAR.CD19LH DAUDI cells never exposed to AP1903, and iC9.CAR.CD19LH DAUDI residual after AP1903 exposure and further re-expanded (at E:T ratio of 1:1). **(D)** 7-days co-culture assay was carried out between un-transduced NK cells or CAR.CD19 NK cells and wt NALM-6 cells, iC9.CAR.CD19LH NALM-6 cells never exposed to AP1903, and iC9.CAR.CD19LH NALM-6

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residual after AP1903 exposure and further re-expanded (at E:T ratio of 1:1). ** p-value= <0.01 , *** p-value= <0.001 .

- **Figure 12** shows that T-cells genetically modified with different CAR.CD19 constructs control in vivo expansion of CAR positive leukemia in a xenograft mouse model. **(A)** Schematic representation of the experimental design, with FF-Luciferase positive NALM-6 WT cells, infused at Day -3. At Day 0, mice were evaluated for leukemia engraftment and treated with 10×10^6 un-transduced (NT) or CAR.CD19SL/LH or CAR.CD19LL/SH T-cells/mouse (top panel). Bioluminescence imaging of each treated mouse (middle panel). Mean $\pm$ SD of bioluminescence values of the three mice cohorts, receiving NT (black line) or CAR.CD19SL/LH T-cells (short-dotted line) or CAR.CD19LL/SH T cells (long-dotted line)(bottom panel). **(B)** Schematic representation of the experimental design, with CAR.CD19SL/LH positive/FF-Luciferase positive NALM-6 cells, infused at Day -3. At Day 0, mice were evaluated for leukemia engraftment and treated with 10×10^6 un-transduced (NT) or CAR.CD19SL/LH (top panel). Bioluminescence imaging of each treated mouse (middle panel). Mean $\pm$ SD of bioluminescence values of the two mice cohorts, receiving NT (black line) or CAR.CD19SL/LH T-cells (short-dotted line) (bottom panel). **(C)** Schematic representation of the experimental design, with CAR.CD19LL/SH positive/FF-Luciferase positive NALM-6 cells, infused at Day -3. At Day 0, mice were evaluated for leukemia engraftment and treated with 10×10^6 un-transduced (NT) or CAR.CD19LL/SH (top panel). Bioluminescence imaging of each treated mouse (middle panel). Mean $\pm$ SD of bioluminescence values of the two mice cohorts, receiving NT (black line) or CAR.CD19LL/SH T-cells (long-dotted line) (bottom panel). * p-value= <0.05 , ** p-value= <0.01 , *** p-value= <0.001 , **** p-value= <0.0001 .

(D) Schematic representation of the experimental design, with iC9.CAR.CD19_{LH} positive FF-Luciferase positive DAUDI cells infused at day-3. At Day 0, mice were evaluated for leukemia engraftment and treated with 10×10^6 un-transduced (NT) or CAR.CD19 T-cells/mouse. Bioluminescence images of each treated mice. Average plus standard

deviation of bioluminescence values of the two mice cohorts, receiving NT (black line) or CAR.CD19 T-cells (dotted line). * p-value= ≤ 0.05 . (E) Histogram representing tumor bioluminescence differences at Day 16 between mice bearing NALM-6 CAR.CD19SL/LH and CAR.CD19LL/SH NALM-6 cells. Data are shown as Mean $\pm$ SD of bioluminescence increase of the two mice cohorts at Day 16 compared to Day0.

- **Figure 13** shows that iC9 activation is able to control *in vivo* expansion of iC9.CAR positive leukemia in a xenograft mouse model. (A) Schematic representation of the experimental design, with iC9.CAR.CD19LH positive FF-Luciferase positive DAUDI cells infused at day-3. At Day 0, mice were evaluated for leukemia engraftment and treated with 100 μ g/mouse of AP1903 from day 0 to day 28. (B) Bioluminescence images of each control untreated mouse and each AP1903 treated mouse. Mice were monitored for more than 30 days after AP1903 withdrawal (C) Bioluminescence values over time for each treated mouse in the two cohorts, un-treated (black lines) or AP1903 treated (dotted line) mice. (D) Kaplan-Meier survival curve analysis of leukaemia-bearing mice untreated (black line) or AP1903 treated (blue line). **** p-value= ≤ 0.00001 . The only mouse (#20) showing a persisting positive signal at IVIS analysis after AP1903 administration was sacrificed at day 35 together with a negative control (#11) and a positive control (mouse not exposed to AP1903 administration, #6), to characterize the leukemia cells.

- **Figure 14** shows in silico modeling data concerning CAR.CD19 15aa linker (SG4)₃ SEQ ID NO:39 vs CAR.CD19 8aa linker G3SG4 SEQ ID NO:38.

- **Figure 15** shows in silico modeling data concerning CAR.CD19 15aa linker (SG4)₃ SEQ ID NO:39 vs CAR.CD19 9aa linker G4SG3 SEQ ID NO:186.

- **Figure 16** shows in silico modeling data concerning CAR.CD19 15aa linker (SG4)₃ SEQ ID NO:39 vs CAR.CD19 10 aa linker (SG4)₂ SEQ ID NO:190.

- **Figure 17** shows in silico modeling data concerning CAR.CD19 15aa linker (SG4)₃ SEQ ID NO:39 vs CAR.CD19 11aa linker (SG4)₂ S

SEQ ID NO:187.

- **Figure 18** shows in silico modeling data concerning CAR.CD19 15aa linker (SG4)3 SEQ ID NO:39 vs CAR.CD19 12aa linker (SG4)2 SG SEQ ID NO:188.

5 - **Figure 19** shows in silico modeling data concerning CAR.CD19 15aa linker (SG4)3 SEQ ID NO:39 vs CAR.CD19 13aa linker (SG4)2 SG2 (SEQ ID NO:191).

10 - **Figure 20** shows in silico modeling data concerning CAR.CD19 15aa linker (SG4)3 SEQ ID NO:39 vs CAR.CD19 14aa linker (SG4)2 SG3 SEQ ID NO:189.

 - **Figure 21** shows in silico modeling data concerning CAR.CD19 15aa linker (SG4)3 SEQ ID NO:39 vs CAR.CD19 15aa linker (SG4)3 SEQ ID NO:39.

15 **EXAMPLE 1:** *CAR vector design according to the present invention and study of the safety thereof in case of CAR+ leukaemia relapse*

Materials and Methods

20 *The biological material of human origin used in these experiments has been sampled after that both parents and healthy donors signed a written informed consent, in accordance with rules set by the Institutional Review Board (IRB) of Bambino Gesù Children's Hospital of Rome (OPBG; Approval of Ethical Committee N°969/2015 prot. N°669LB, and N°1422/2017 prot.N°810).*

25 *The OGMs described in the experiments were prepared in compliance with the obligations regarding OGMs, deriving from national or community regulations, and in particular from the provisions of paragraph 6 and of the legislative decrees of 12 April 2001, n. 206, and 8 July 2003, n. 224*

Cell cultures.

30 CD19 positive human Burkitt's lymphoma cell lines Daudi, NALM-6 and Raji (American Type Culture Collection Company (ATCC)), and CD19 negative Non-Hodgkin's Large Cell Lymphoma cell line Karpas-299 (Sigma Aldrich) were maintained in RPMI 1640 (EuroClone, Italy) supplemented with 10% heat-inactivated fetal bovine serum (EuroClone,

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Italy), 2mM L-glutamine (GIBCO, USA), 25 IU/mL of penicillin, and 25 mg/mL of streptomycin (EuroClone, Italy), in a humidified atmosphere containing 5% CO₂ at 37°C. All cell lines were authenticated by PCR-single-locus-technology (Promega, PowerPlex 21 PCR) analysis in "BMR Genomics s.r.l.", and were periodically checked for mycoplasma and surface markers expression.

Effector cells generation and expansion.

Buffy coats (BC) from healthy donors (HDs), peripheral blood (PB) and bone marrow (BM) derived from children with Bcp-ALL were used to isolate unfractionated mononuclear cells using Lympholyte Cell Separation Media (Cedarlane, Canada). T cells were activated with soluble OKT3 and anti-CD28 (1 µg/ml, Miltenyi, Germany) monoclonal antibody (mAb) with a combination of recombinant human interleukin-7 (IL7, 10 ng/ml; R&D; USA) and interleukin-15 (IL15, 5 ng/ml; R&D; USA). NK cells were generated from BC of HDs following previously described method¹⁷. Then T and NK cells were transduced with retroviral supernatant, after three/four days, in 24-well plates pre-coated with recombinant human RetroNectin (Takara-Bio. Inc; Japan). T lymphocytes were expanded in the presence of cytokines, in TexMacs complete medium (Miltenyi, Germany) and replenished twice a week.

CAR constructs.

Four different retroviral CAR constructs were used to carry out the experiments:

1)CAR construct carrying anti-human CD19-scFv from FMC63 clone in which VL and VH fragments were joined by a linker represented by three GSSSS repetitions (3xG4S, long linker, LL), in frame with CD8 stalk domain (short hinge, SH), CD8 transmembrane domain, 4.1bb and CD3ζ cytoplasmic domain (CAR.CD19 VL-3GS-VH-CD8-4.1bb.ζ, i.e. LL/SH);

CAR.CD19 LL/SH nt (SEQ ID NO:73)

ATGGAGTTTGGACTTTCTTGGTTGTTTTTGGTGGCAATTCTGAA
GGGTGTCCAGTGTAGCAGGGACATCCAGATGACACAGACTACATCCT
CCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCA

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AGTCAGGACATTAGTAAATATTTAAATTGGTATCAGCAGAAACCAGAT
GGAAGTGTAACTCCTGATCTACCATACATCAAGATTACACTCAGGA
GTCCCATCAAGGTTCAAGTGGCAGTGGGTCTGGAACAGATTATTCTCT
CACCATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCA
5 ACAGGGTAATACGCTTCCGTACACGTTCCGAGGGGGGACTAAGTTG
GAAATAACAAGCGGAGGTGGGGGCAGCGGAGGTGGGGGCAGCGGA
GGTGGGGGCGAGGTGAACTGCAGGAGTCAGGACCTGGCCTGGTG
GCGCCCTCACAGAGCCTGTCCGTACATGCACTGTCTCAGGGGTCT
CATTACCCGACTATGGTGTAAAGCTGGATTCGCCAGCCTCCACGAAAG
10 GGTCTGGAGTGGCTGGGAGTAATATGGGGTAGTGAAACCACATACTA
TAATTCAGCTCTCAAATCCAGACTGACCATCATCAAGGACAACTCCAA
GAGCCAAGTTTTCTTAAAAATGAACAGTCTGCAAACTGATGACACAGC
CATTTACTACTGTGCCAAACATTATTACTACGGTGGTAGCTATGCTAT
GGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGGATCCC
15 CCGCCCCAAGACCCCCCACACCTGCGCCGACCATTGCTTCTCAACC
CCTGAGTTTGAGACCCGAGGCCTGCGGGCCAGCTGCGGGCGGGGC
CGTGCCATACAAGAGGACTCGATTTGCTTGCGACATCTATATCTGGG
CACCTCTCGCTGGCACCTGTGGAGTCCTTCTGCTCAGCCTGGTTATT
ACTCTGTACTGTAATCACCGGAATCGCCGCCGCGTTTGTAAGTGTCC
20 CAGGGTCGACAAACGGGGCAGAAAGAACTCCTGTATATATTCAAAC
AACCATTTATGAGACCAGTACAACTACTCAAGAGGAAGATGGCTGTA
GCTGCCGATTTCCAGAAGAAGAAGAAGGAGGATGTGAACTGAGAGT
GAAGTTCAGCAGGAGCGCAGACGCCCCCGGTACCAGCAGGGCCA
GAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTACG
25 ATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGGGAAA
GCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAATGAACTGCAG
AAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATGAAAGGCG
AGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTCTCA
GTACAGCCACCAAGGACACCTACGACGCCCTTCACATGCAGGCCCT
30 GCCCCCTCGCTAA

CAR.CD19 LL/SH aa (SEQ ID NO:74)

MEFGLSWLFLVAILKGVQCSRDIQMTQTTSSLSASLGDRVITSCR
ASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSL

37

TISNLEQEDIATYFCQQGNTLPYTFGGGKLEITSGGGGSGGGGSGGGG
 EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWL
 GVIWGSETTYYN SALKSRLTIKDNSKSQVFLKMNSLQTD DTAIYYCAKHY
 YYGGSYAMDYWGQGSTVTVSSGSPAPRPPTPAPTIASQPLSLRPEACR
 5 PAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRRVC
 KCPRVDKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEEGGCEL
 RVKF SRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG
 KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRRGK GHDGLYQGLS
 TATKDTYDALHMQALPPR-

10 2) CAR construct carrying anti-human CD19-scFv from FMC63
 clone in which VL and VH fragments were joined by a linker represented
 by three GSSSS repetitions (3xG4S, long linker, LL), in frame with 16aa
 sequence derived from human CD34 antigen (Δ CD34, long hinge, LH),
 CD8 stalk domain, CD8 transmembrane domain, 4.1bb and CD3 ζ
 15 cytoplasmic domain (CAR.CD19 VL-3GS-VH-CD34-CD8-4.1bb. ζ , i.e.
 LL/LH);

CAR.CD19LL/LH nt sequence (SEQ ID NO:36):

ATGGAGTTTGGACTTTCTTGGTTGTTTTTGGTGGCAATTCTGAA
 GGGTGTCCAGTGTAGCAGGGACATCCAGATGACACAGACTACATCCT
 20 CCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCA
 AGTCAGGACATTAGTAAATATTTAAATTGGTATCAGCAGAAACCAGAT
 GGAAGTGTAAACTCCTGATCTACCATACATCAAGATTACACTCAGGA
 GTCCCATCAAGGTTCAAGTGGCAGTGGGTCTGGAACAGATTATTCTCT
 CACCATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCA
 25 ACAGGGTAATACGCTTCCGTACACGTTCCGAGGGGGGACTAAGTTG
 GAAATAACAAGCGGAGGTGGGGGCAGCGGAGGTGGGGGCAGCGGA
 GGTGGGGGCGAGGTGAAACTGCAGGAGTCAGGACCTGGCCTGGTG
 GCGCCCTCACAGAGCCTGTCCGTACATGCACTGTCTCAGGGGTCT
 CATTACCCGACTATGGTGTAAGCTGGATTCGCCAGCCTCCACGAAAG
 30 GGTCTGGAGTGGCTGGGAGTAATATGGGGTAGTGAAACCACATACTA
 TAATTCAGCTCTCAAATCCAGACTGACCATCATCAAGGACAACCTCAA
 GAGCCAAGTTTTCTTAAAAATGAACAGTCTGCAAAGTATGACACAGC
 CATTACTACTGTGCCAAACATTATTACTACGGTGGTAGCTATGCTAT

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GGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGGATCC
 GCATGCGAACTTCCTACTCAGGGGACTTTCTCAAACGTTAGCACAAA
 CGTAAGTGCGGCCGCcCCCGCCCCAAGACCCCCCACACCTGCGCCG
 ACCATTGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCCTGCCGGCC
 5 AGCTGCCGGCGGGGCCGTGCATACAAGAGGACTCGATTTGCTTGC
 GACATCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCCTTCT
 GCTCAGCCTGGTTATTACTCTGTAATCACCAGGAATCGCCGCC
 GCGTTTGTAAGTGTCCAGGGTCGACAAACGGGGCAGAAAGAACT
 CCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACTCAA
 10 GAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGGAG
 GATGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGC
 GTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGAC
 GAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCC
 TGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTG
 15 TACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGAT
 TGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCT
 TTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTC
 ACATGCAGGCCCTGCCCCCTCGCTAA

CAR.CD19LL/LH aa sequence (SEQ ID NO:39):

20 MEFGLSWFLVAILKGVQCSRDIQMTQTSSLSASLGDRVTISCRASQDI
 SKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL
 EQEDIATYFCQQGNTLPYTFGGGKLEITSGGGGSGGGGSGGGGEVKL
 QESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKLEWLGVW
 GSETTYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYG
 25 GSYAMDYWGQGTSTVSSGSACELPTQGTFSNVSTNVSAAPAPRPPT
 PAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLL
 LSLVITLYCNHRNRRRVCKCPRVDKRGKLLYIFKQPFMRPVQTTQEE
 DGCSCRFEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRRE
 EYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMK
 30 GERRRGKGHGGLYQGLSTATKDTYDALHMQALPPR-

3) CAR construct carrying anti-human CD19-scFv from FMC63 clone in which VL and VH fragments were joined by a linker represented by one GGGSGGGG repetition (SEQ ID NO:38) (G3SG4, short linker, SL),

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in frame with CD8 stalk domain (short hinge, SH), CD8 transmembrane domain, 4.1bb and CD3 ζ cytoplasmic domain (CAR.CD19 VL-1GS-VH-CD8-4.1bb. ζ , i.e. SL/SH);

CAR.CD19 SL/SH nt sequence(SEQ ID NO:34):

```
5      ATGGAGTTTGGACTTTCTTGGTTGTTTTTGGTGGCAATTCTGAA
      GGGTGTCCAGTGTAGCAGGGACATCCAGATGACACAGACTACATCCT
      CCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCA
      AGTCAGGACATTAGTAAATATTTAAATTGGTATCAGCAGAAACCAGAT
      GGAAGTGTAAACTCCTGATCTACCATACATCAAGATTACACTCAGGA
10     GTCCCATCAAGGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCT
      CACCATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCA
      ACAGGGTAATACGCTTCCGTACACGTTCCGAGGGGGGACTAAGTTG
      GAAATAACAGGCGGAGGAAGCGGAGGTGGGGGCGAGGTGAAACTG
      CAGGAGTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCG
15     TCACATGCACTGTCTCAGGGGTCTCATTACCCGACTATGGTGTAAGC
      TGGATTGCGCCAGCCTCCACGAAAGGGTCTGGAGTGGCTGGGAGTAA
      TATGGGGTAGTGAAACCACATACTATAATTCAGCTCTCAAATCCAGAC
      TGACCATCATCAAGGACAACCTCCAAGAGCCAAGTTTTCTTAAAAATGA
      ACAGTCTGCAAACTGATGACACAGCCATTTACTACTGTGCCAAACATT
20     ATTACTACGGTGGTAGCTATGCTATGGACTIONTGGGGTCAAGGAACC
      TCAGTCACCGTCTCCTCAGGATCCCCCGCCCCAAGACCCCCCACAC
      CTGCGCCGACCATTGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCC
      TGCCGGCCAGCTGCCGGCGGGGCCGTGCATACAAGAGGACTCGATT
      TCGCTTGCGACATCTATATCTGGGCACCTCTCGCTGGCACCTGTGGA
25     GTCCTTCTGCTCAGCCTGGTTACTCTGTACTGTAATCACCGGAAT
      CGCCGCCGCGTTTTGTAAGTGTCCCAGGGTCGACAAACGGGGCAGAA
      AGAAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAAA
      CTACTIONAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAA
      GAAGGAGGATGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACG
30     CCCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAA
      TCTAGGACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGC
      CGGGACCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAG
      GAAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTA
```

40

CAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCA
 CGATGGCCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACG
 ACGCCCTTCACATGCAGGCCCTGCCCCCTCGCTAAA

CAR.CD19 SL/SH aa sequence (SEQ ID NO:33):

5 MEFGLSWLFLVAILKGVQCSRDIQMTQTTSSLSASLGDRVTISCRASQDI
 SKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL
 EQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGGGEVKLQESGPGL
 VAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVWGSETYY
 NSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDY
 10 WGGGTSVTVSSGSPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR
 GLDFACDIYWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRVDKRRGR
 KLLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEEGGCELRVKFSRSADAP
 AYQQGQNQLYNELNLGRREEYDVLDRRRGRDPEMGGKPRRKNPQEGL
 YNELQKDKMAEAYSEIGMKGERRRRGKGHDGLYQGLSTATKDTYDALHM
 15 QALPPR

4) CAR construct carrying anti-human CD19-scFv from FMC63 clone in which VL and VH fragments were joined by a linker represented by one GGGSGGGG (SEQ ID NO:38) repetition (G3SG4, short linker, SL), in frame with 16aa sequence derived from human CD34 antigen (Δ CD34, long hinge, LH), CD8 stalk domain, CD8 transmembrane domain, 4.1bb and CD3 ζ cytoplasmic domain (CAR.CD19 VL-1GS-VH-CD34-CD8-4.1bb. ζ , i.e. SL/LH).

CAR.CD19SL/LH nt sequence (SEQ ID NO:58):

ATGGAGTTTGGACTTTCTTGGTTGTTTTTGGTGGCAATTCTGAAGGGT
 25 GTCCAGTGTAGCAGGGACATCCAGATGACACAGACTACATCCTCCCT
 GTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTC
 AGGACATTAGTAAATATTTAAATTGGTATCAGCAGAAACCAGATGGAA
 CTGTAAACTCCTGATCTACCATACATCAAGATTACACTCAGGAGTCC
 CATCAAGGTTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACC
 30 ATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAG
 GGTAATACGCTTCCGTACACGTTTCGGAGGGGGGACTAAGTTGGAAAT
 AACAGGCGGAGGAAGCGGAGGTGGGGGCGAGGTGAAACTGCAGGA
 GTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCGTCACA

41

TGCACTGTCTCAGGGGTCTCATTACCCGACTATGGTGTAAGCTGGAT
TCGCCAGCCTCCACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGG
GGTAGTGAAACACATACTATAATTCACTCTCAAATCCAGACTGACC
ATCATCAAGGACAACCTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGT
5 CTGCAAACCTGATGACACAGCCATTTACTACTGTGCCAAACATTATTAC
TACGGTGGTAGCTATGCTATGGACTACTGGGGTCAAGGAACCTCAGT
CACCGTCTCCTCAGGATCCGAACCTTCCTACTCAGGGGACTTTCTCAA
ACGTTAGCACAAACGTAAGTCCCGCCCCAAGACCCCCACACCTGC
GCCGACCATTGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCCTGCC
10 GGCCAGCTGCCGGCGGGGCGGTGCATACAAGAGGACTCGATTTTCGC
TTGCGACATCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCC
TTCTGCTCAGCCTGGTTATTACTCTGTACTGTAATCACCGGAATCGCC
GCCGCGTTTGTAAGTGTCCAGGGTCGACAAACGGGGCAGAAAGAA
ACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACT
15 CAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAG
GAGGATGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCC
CGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAG
GACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGA
CCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGC
20 CTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGA
GATTGGGATGAAAGGCGAGCGCCGAGGGGCAAGGGGCACGATGG
CCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCC
TTCACATGCAGGCCCTGCCCCCTCGCTAA

PROTEIN of CAR.CD19SL/LH (SEQ ID NO:72):

25 MEFGLSWLFLVAILKGVQCSRDIQMTQTTSSLSASLGDRVTISCR
ASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSL
TISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGGEVKLQES
GPNLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVWIGSE
TTYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSY
30 AMDYWGQGTSTVTSSGSELPTQGTFSNVSTNVSPAPRPPTPAPTASQ
PLSLRPEACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLVITLYC
NHRNRRRVCKCPRVDKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP
EEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDRR

42

GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH
DGLYQGLSTATKDTYDALHMQALPPR*

5 NALM-6 genetically modified with a lentiviral vector carrying CAR.CD19 already published (5) have been included in the experiments as NALM6 CAR UPenn (CAR.CD19 LL/SH in the lentivirus platform provided by Dr Ruella (5)).

10 NK cells from HDs, as well as T cells from HDs or Bcp-ALL patients have been genetically modified by retroviral construct carrying anti-human CD19-scFv from FMC63 clone in which V_H and V_L fragments were joined by a linker represented by one GGGSGGGG (**SEQ ID NO: 38**) (G3SG4, short linker), in frame with CD8 stalk domain, 16aa sequence derived from human CD34 antigen (Δ CD34; **long hinge**), CD8 transmembrane domain, 4.1bb and CD3 ζ cytoplasmic domain (CAR.CD19 long hinge, CAR.CD19_{LH}). The retroviral vector is a bicistronic construct in which the
15 above-described CAR construct is in frame with the gene cassette coding for the suicide gene inducible caspase 9 (iC9). **iC9-CAR.CD19SL/LH** retroviral construct has been used also to genetically modify B leukemic cell lines, including DAUDI, RAJI and NALM-6. CAR⁺ B cell lines were FACS sorted for CAR expression after transduction. NALM-6 cells were
20 also genetically modified with a lentiviral construct carrying anti-human CD19-scFv from FMC63 clone in which V_H and V_L fragments were joined by a linker represented by (S3G4)₃ Flex linker (SGGGGSGGGGSGGGG SEQ ID NO:39), long linker), in frame with CD8 stalk domain (**short hinge**), CD8 transmembrane domain, 4.1bb and CD3 ζ cytoplasmic
25 domain (NALM-6 CAR.CD19 short hinge, **CAR.CD19** UPenn; kindly provided by Dr Ruella).

Activation of the suicide gene.

30 To induce the *in vitro* activation of iC9, cells were treated once with 20 nM of AP1903 (Medchemexpress, Cat. HY-16046). The percentage of CAR⁺ cells after the AP1903 treatment was evaluated by FACS at the indicated time points. For *in vivo* experiments, to prove the ability of the suicide gene iC9 to be active in the control of CAR⁺ leukemia expansion upon activation by AP1903, NSG mice were infused with 0.25x10⁶

iC9.CAR.CD19_{LH}-DAUDI cells genetically modified with a retroviral construct for FF-Luciferase; after tumor engraftment monitored by IVIS imaging system, the dimerizing drug AP1903 was intraperitoneally administrated from day 1 to day 28 (100µg/mouse). Control cohort was
5 infused with sterile PBS as vehicle solution. Tumor was monitored by weekly IVIS imaging analysis.

Phenotypic analysis.

Flow cytometry analysis was performed to determine cell surface antigens expression; monoclonal antibodies for CD45, CD3, CD19, CD22,
10 CD10, CD34 (all from Becton Dickinson, USA) were combined with different fluorescence according to needs. iC9.CAR.CD19 expression was detected using a mAb directed to hCD34 epitope (anti-CD34 QBend-10 PE from R&D System, USA), or CD19 CAR Detection Reagent (Biotin; Miltenyi, Germany). Flow-cytometry analysis was performed using a BD
15 LSRFortessa X-20 cytometer (BD Biosciences, USA) and analyzed by FACSDiva software (BD Biosciences, USA). FACS-sorting on CAR-transduced tumor cell lines was performed on a FACSARIA (BD Biosciences, USA).

DPs were also characterized by either an 11-color or 16-color
20 combination of antibodies plus CD19-FITC human protein, using the EuroFlow standard operating procedures (SOP) for staining of surface markers only, available at www.EuroFlow.org¹⁸. Antibody combination used were both based on a backbone consisting of the EuroFlow BCP-ALL MRD tubes previously described for high-sensitive MRD
25 measurements in B-cell acute lymphoblastic leukemia by flow cytometry¹⁹⁻²¹.

to which the anti-CD3 and both anti-CD22 and anti-HLADR antibody reagents were added for staining of transfected and non-transfected T-cells and specific gating of CD19-negative B cell precursors and blasts,
30 respectively. Finally, the anti-CD34 Qbend10 clone (R&D Systems, Minneapolis, MN) and the CD19-FITC human protein (Cytognos SL, Salamanca, Spain) were also added to the reagent staining mix for identification of transfected CAR.CD19 cells.

All specific reagents are listed in Table 1 A, B and C reported below.

Table 1A

Patient	IG/TR	ASO primer
ALL#1	IGH VH4JH5	GTCCGCAATTTTTCATTGGTAGTA (SEQ ID NO:75)
	IGK VK2Kde	GCAAGCTACACAATTAAGGAGAAGATAGT (SEQ ID NO:76)
ALL#2	IGH VH3JH6	TAGAGATCCGGCCTTTAACTGGAAGT (SEQ ID NO:77)
	IGH VH3JH5	GCAGCACCCCTCAAGCA (SEQ ID NO:78)
ALL#3	IGH VH3JH4	TGTGCGAAAGATCTTTTTTATGGTGTATGCTATTTCTT (SEQ ID NO:79)
	TRD VD2DD3	CGTATCCCCCCCCCACA (SEQ ID NO:80)
ALL#4	TRB VB20 JB2.7	GCCCCGGACTAGCTAGTTTACGA (SEQ ID NO:81)
	IGH VH3JH5	ACTGTCCCCGAGGTTGTAATAATG (SEQ ID NO:82)
ALL#5	IGH VH3JH6	TGCTATACCGGGCGGGTG (SEQ ID NO:83)
	TRD VD2DD3	CCCAGTAAGGTCGGTGGAGTC (SEQ ID NO:84)
ALL#6	TRA VD2JA29	CGTATCCCCCAGGAGAAGCA (SEQ ID NO:85)
	IGH VH1JH4	ATAGATGTGTACTACTGTGCGAGCGTACTA (SEQ ID NO:86)
ALL#7	IGH VH4JH4	TCCGGTTGGTATCACCTATCCCCTAA (SEQ ID NO:87)
	TRD VD2DD3	TGTGCGTATCCCCCAGAGACA (SEQ ID NO:88)
ALL#8	IGH DH1JH5	TGGGTATAACTGGAAGTACGGCTGGTT (SEQ ID NO:89)
ALL#9	IGH VH1JH4	CGGATTAACTGGGGATCTCCCCTTA (SEQ ID NO:90)
	TRD VD2DD3	CCCCTCCACTCCCCG (SEQ ID NO:91)
ALL#10	IGK VK2Kde	CAAGGTACACACTGGCTGGGAA (SEQ ID NO:92)
	IGH VH3JH6	CTGCCGACCCACTACATGGA (SEQ ID NO:93)
ALL#11	IGH VH3JH6	TATAACAGCTCTACTTCTACCACACGACCTA (SEQ ID NO:94)
	TRD DD2DD3	CTACGTGGAACCGTGAGGCT (SEQ ID NO:95)
ALL#12	TRD VD2DD3	CGTATCCCCCAGTCGCACA (SEQ ID NO:96)
	IGH VH3JH4	CGGAGGGTAAATTACTATGATAGTAGTGGTTT (SEQ ID NO:97)

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ALL#13	IGH VH3JH4	AAAAGGGTCTTGGGCGTTTAGGA(SEQ ID NO:98)
	TRD VD2DD3	GTCCGTACCCCTTGCCG(SEQ ID NO:99)
ALL#14	IGH VH2JH4	AGTTCCTATCCGAGACCTCCAATT(SEQ ID NO:100)
	TRA VD2JA29	AATTCGGGAGTCGGGGGTAT(SEQ ID NO:101)
ALL#15	IGH VH4JH6	AGAGAGGAGAGCCTAGGGATATTTTGA (SEQ ID NO:102)
ALL#16	IGH VH1JH4	GCGAGCAACAACCTGGATTTTGA(SEQ ID NO:103)
	TRG VG9JG1.3	GAACCAACCTCCGAGGCCT(SEQ ID NO:104)
ALL#17	IGH VH2JH3	GTTAATATGGGGCCATCTGGG(SEQ ID NO:105)
ALL#18	IGH VH1JH3	AGAGGGGGCTCCCCTATGG(SEQ ID NO:106)
	IGH VH3JH4	AGCAGTGGCATGCCATTGA(SEQ ID NO:107)
ALL#19	TRD VD2DD3	CCTGCCCCCGCTACAA(SEQ ID NO:108)

Table 1B

Patient	IG/TR	RQ primer	
ALL#1	IGH VH4JH5	jh5 rp2	CAAGCTGAGTCTCCCTAAGTGGA(SEQ ID NO:109)
	IGK VK2Kde	kde rp2	ATATGGCAAAAATGCAGCTGC(SEQ ID NO:110)
ALL#2	IGH VH3JH6	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT(SEQ ID NO:111)
	IGH VH3JH5	jh5 rp2	CAAGCTGAGTCTCCCTAAGTGGA(SEQ ID NO:112)
ALL#3	IGH VH3JH4	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:113)
	TRD VD2DD3	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA(SEQ ID NO:114)
ALL#4	TRB VB20 JB2.7	jb2.7 rp	GCTGGAAGGTGGGGAGA(SEQ ID NO:115)
	IGH VH3JH5	jh5 rp2	CAAGCTGAGTCTCCCTAAGTGGA(SEQ ID NO:116)
ALL#5	IGH VH3JH6	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT(SEQ ID NO:117)
	TRD VD2DD3	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA(SEQ ID NO:118)
ALL#6	TRA VD2JA29	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA(SEQ ID NO:119)
	IGH	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:120)

46			
	VH1JH4		ID NO:120)
ALL#7	IGH VH4JH4	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:121)
	TRD VD2DD3	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA(SEQ ID NO:122)
ALL#8	IGH DH1JH5	jh5 rp2	CAAGCTGAGTCTCCCTAAGTGGA(SEQ ID NO:123)
ALL#9	IGH VH1JH4	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:124)
	TRD VD2DD3	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA(SEQ ID NO:125)
ALL#10	IGK VK2Kde	kde rp2	ATATGGCAAAAATGCAGCTGC(SEQ ID NO:126)
	IGH VH3JH6	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT(SEQ ID NO:127)
ALL#11	IGH VH3JH6	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT(SEQ ID NO:128)
	TRD DD2DD3	dd3 rp1	TTTGCCCCTGCAGTTTTTGT(SEQ ID NO:129)
ALL#12	TRD VD2DD3	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA(SEQ ID NO:130)
	IGH VH3JH4	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:131)
ALL#13	IGH VH3JH4	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:132)
	TRD VD2DD3	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA(SEQ ID NO:133)
ALL#14	IGH VH2JH4	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:134)
	TRA VD2JA29	vd2 fp	TGCAAAGAACCTGGCTGTACTTAA(SEQ ID NO:135)
ALL#15	IGH VH4JH6	jh6 rp	GCAGAAAACAAAGGCCCTAGAGT(SEQ ID NO:136)
ALL#16	IGH VH1JH4	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:137)
	TRG VG9JG1.3	vg9 fp	GGCATTCCGTCAGGCAAA(SEQ ID NO:138)
ALL#17	IGH VH2JH3	jh3 rp	AGGCAGAAGGAAAGCCATCTTAC(SEQ ID NO:139)
ALL#18	IGH VH1JH3	jh3 rp	AGGCAGAAGGAAAGCCATCTTAC(SEQ ID NO:140)
	IGH VH3JH4	jh4 rp	CAGAGTTAAAGCAGGAGAGAGGTTGT(SEQ ID NO:141)
ALL#19	TRD VD2DD3	dd3 rp3	CTGCTTGCTGTGTTTGTCTCCT(SEQ ID NO:142)

Table 1C

Patient	IG/TR	TaqMan Probe	
ALL#1	IGH VH4JH5	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:143)
	IGK VK2Kde	kde tp1	AGCCCAGGGCGACTCCTCATGAGT(SEQ ID NO:144)
ALL#2	IGH VH3JH6	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA(SEQ ID NO:145)
	IGH VH3JH5	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:146)
ALL#3	IGH VH3JH4	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:147)
	TRD VD2DD3	vd2 tp	AGACCCTTCATCTCTCTGATGGTGCAAGTA(SEQ ID NO:148)
ALL#4	TRB VB20 JB2.7	jb2.7 tp	CGGGCACCAGGCTCACGGTC(SEQ ID NO:149)
	IGH VH3JH5	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:150)
ALL#5	IGH VH3JH6	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA(SEQ ID NO:151)
	TRD VD2DD3	vd2 tp	AGACCCTTCATCTCTCTGATGGTGCAAGTA(SEQ ID NO:152)
ALL#6	TRA VD2JA29	vd2 tp	AGACCCTTCATCTCTCTGATGGTGCAAGTA(SEQ ID NO:153)
	IGH VH1JH4	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:154)
ALL#7	IGH VH4JH4	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:155)
	TRD VD2DD3	vd2 tp	AGACCCTTCATCTCTCTGATGGTGCAAGTA(SEQ ID NO:156)
ALL#8	IGH DH1JH5	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:157)
ALL#9	IGH VH1JH4	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:158)
	TRD VD2DD3	vd2 tp	AGACCCTTCATCTCTCTGATGGTGCAAGTA(SEQ ID NO:159)
ALL#10	IGK VK2Kde	kde tp1	AGCCCAGGGCGACTCCTCATGAGT(SEQ ID NO:160)
	IGH VH3JH6	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA(SEQ ID NO:161)
ALL#11	IGH VH3JH6	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA(SEQ ID NO:162)
	TRD DD2DD3	dd3 tp1	CGCACAGTGCTACAAAACCTACAGAGACCTG(SEQ ID NO:163)
ALL#12	TRD VD2DD3	vd2 tp	AGACCCTTCATCTCTCTGATGGTGCAAGTA(SEQ ID NO:164)
	IGH	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:165)

	VH3JH4		
ALL#13	IGH VH3JH4	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:166)
	TRD VD2DD3	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA(SEQ ID NO:167)
ALL#14	IGH VH2JH4	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:168)
	TRA VD2JA29	vd2 tp	AGACCCTTCATCTCTCTCTGATGGTGCAAGTA(SEQ ID NO:169)
ALL#15	IGH VH4JH6	jh6 tp	CACGGTCACCGTCTCCTCAGGTAAGAA(SEQ ID NO:170)
ALL#16	IGH VH1JH4	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:171)
	TRG VG9JG1.3	vg9 tp	TAGGATACCTGAAACGTCTACATCCACTCTCACC(SEQ ID NO:172)
ALL#17	IGH VH2JH3	jh3 tp	CAAGGGACAATGGTCACCGTCTCTTCA(SEQ ID NO:173)
ALL#18	IGH VH1JH3	jh3 tp	CAAGGGACAATGGTCACCGTCTCTTCA(SEQ ID NO:174)
	IGH VH3JH4	jh1.2.4.5 tp	CCCTGGTCACCGTCTCCTCAGGTG(SEQ ID NO:175)
ALL#19	TRD VD2DD3	dd3 tp2	ATATCCTCACCCCTGGGTCCCATGCC(SEQ ID NO:176)

Sample acquisition was performed immediately after sample preparation was completed, $>1.5 \times 10^6$ cells (range: $1.6 - 7.5 \times 10^6$ cells) were measured per sample using an LSRFortessa X-20 [Becton Dickinson Biosciences (BD), San Jose, CA] flow cytometer and the FACSDiva software (BD) or a 3-laser Aurora (Cytek Biosciences, Fremont, CA) spectral flow cytometer equipped with the SpectroFlo software (Cytek). For instrument setup and data acquisition, the EuroFlow SOP for instrument setup and calibration available at www.euroflow.org was strictly followed DOI: 10.1038/leu.2012.122 The Infinicyt software (Cytognos SL, Salamanca, Spain) was used for data analysis.

Quantitative Real-Time PCR.

Total DNA was purified by QIAamp DNA Mini Kit (Qiagen, USA) according to manufacturer.

Quantitative Real-Time PCR

The average of vector copy number (VCN) per cell was determined by real-time PCR, using a TaqMan probe designed on the retroviral

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construct using the Primer Express® software (Applied Biosystems) and reported in Table 2 (iC9 Probe and primers iC9).

Table 2

Gene	iC9
Forward primer	5'-ACCAGCTGGATGCCATCTC-3'(SEQ ID NO:177)
Reverse primer	5'-CAGCTGCCTGACTTTGGATC-3'(SEQ ID NO:178)
TaqMan Probe	5'AGCCTGCCC/ZEN/ACACCTTCTGACAT 3' (SEQ ID NO:179) having 5HEX in 5' and 3IABkFQ in 3'

5 TaqMan primer/probes were designed for each specific Immunoglobulin (IG) or T-cell Receptor (TR) clonal target by Primer Express® software (Applied Biosystems, Italy).

For VCN, each sample was analyzed in triplicate and the average of threshold cycles was used to quantify DNA copies in relation to the mean values of negative control samples. Relative gene expression was calculated using the house-keeping gene ACT1N1 (Hs_02249516 ACT1N1, ThermoFisher Scientific) qPCR was performed employing QuantStudio 12K Flex Real-Time PCR System (ThermoFisher Scientific). For IG/TR PCR-MRD of the DPs, each MRD value was calculated from the corresponding standard curve, and the results were normalized for values of the housekeeping Albumin gene. Quantitative range (QR) and sensitive range (SR), positive value, reproducibility of replicates, were interpreted following the Euro MRD guidelines, in order to assign the appropriate MRD value to each sample analyzed. qPCR was performed by using the 7900 HT fast-Real Time-PCR System and ViiA7 system (ThermoFisher Scientific) and TaqMan Gene Expression Master Mix (ThermoFisher Scientific).

***In vivo* CAR+ leukemia mouse model.**

Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) female mice were provided by Charles River and maintained in the *Plaisant* animal facility in *Castel Romano*, Rome Italy. All procedures were performed in accordance with the Guidelines for Animal Care and Use of the National Institutes of Health (Ethical committee for animal experimentation Prot. N 088/2016-PR). To test CAR T activity on CAR+ leukemia, the NSG mouse model was

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intravenously engrafted with 0.25×10^6 NALM-6 WT or NALM-6 CAR.CD19^{SL/LH} or NALM-6 CAR.CD19^{LL/SH} or DAUDI CAR.CD19^{SL/LH} cells genetically modified with firefly luciferase (FF-Luc). On day +3 mice were treated with 10×10^6 CAR.CD19 T-cells or control untransduced (NT) T-cells.

- 5 Tumor growth was monitored weekly by IVIS Imaging System, after D-Luciferin (PerkinElmer, D-Luciferin potassium salt) intraperitoneally administration.

Statistical analysis.

Unless otherwise noted, data are summarized as average $\pm$ standard deviation (SD). Student *t*-test (two-sided) was used to determine statistically significant differences between samples, with *p* value <0.05 indicating a significant difference. The mouse survival data were analyzed using the Kaplan-Meier survival curve and Fisher's exact test was used to measure statistically significant differences. No valuable samples were excluded from the analyses. Animals were excluded only in the event of their death after tumor implant but before treatment. Neither randomization nor blinding was done during the *in vivo* study. However, mice were matched based on the tumor signal for control and treatment groups before infusion of control or specific. To compare the growth of tumors over time, bioluminescence signal intensity was collected in a blind fashion. Bioluminescence signal intensity was log transformed and then compared using a two-sample *t*-test. The sample size was estimated considering no significant variation within each group of data. A conclusion using as small a sample size as possible was tried to be reached. The sample size to detect a difference in averages of 2 standard deviation at the 0.05 level of significance with an 80% power was estimated. Graphic representations and statistical analysis were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Results

- 30 ***Generation of CAR.CD19 T-cells from PB mononuclear cells of patients affected by Bcp-ALL.***

The un-fractionated population of PB mononuclear cells was isolated from patients at diagnosis having median $45.05 \pm 28.12\%$ circulating blasts

(n=10. Range, 5.5-86.4%). Mononuclear cells derived from PB of the enrolled patients were transduced with a second-generation iC9.CAR.CD19 long hinge (iC9.CAR.CD19_{LH}) according to the method detailed in Figure 1A. After 5 days from transduction process, T-cell products showed a transduction efficiency of 55,15±16,54% (Figure 1B shows an exemplificative analysis, whereas Figure 1C shows the average of 15 leukemic patients subdivided in two groups based on the %CD19⁺ cells in the starting material, considering the 45% median value cut-off). It was evaluated whether the leukemic blast contamination in the patient's sample has any impact on the production of CAR T-cells, especially in terms of CAR transduction level in the DPs as well as the total number of generated CAR T-cells. No correlation was noticed between CAR T-cell percentage at the end of the manufacturing procedure and the level of CD19⁺ B cell contaminating the starting material (Figure 1C and 1D), as well as on the CAR T-cell yield observed when the manufacturing started from patient's material characterized by more than 45% CD19⁺ cells (cut-off media value; Figure 1E). To consider patient's samples with an increased percentage of leukemic blast cells, as well as leukemic cells with a more immature stage, CAR T-cells were generated starting from BM aspirate samples of Bcp-ALL patients at diagnosis (n=6. Figure 2A) in which the average of CD19⁺ cells in the starting material was 73.1±17,80% (range, 40.5-83.5%). Although the transduction level in BM-derived samples was significantly lower than in PB-samples (Figure 2 A-B; 36,04±19,83% vs 55,15±16,54% CAR⁺ T cells, respectively; p=0.03), no differences were observed in terms of total yield of DP recovery from PB or BM samples (Figure 2C).

Deep characterization of patient-derived CAR T-cell products.

Real-time quantitative PCR was performed on CAR T-cell DPs (day 14, end-production) for the amplification of patient-specific Ig rearrangements, observed at diagnosis of each patient. Positivity of MRD in 7 out of 14 tested CAR T cell DPs was observed, with a median MRD of 6.01E-3±1.00E-2 (Table 3).

Table 3

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ALL Patients (PB)	%CD19+ Blasts	Sample	MRD#1	MRD#2
ALL#7	86,4	NT CAR	1,00E-04 1,20E-04	NEG NEG
ALL#8	28,5	NT CAR	NEG NEG	ND ND
ALL#9	5,5	NT CAR	NEG NEG	NEG NEG
ALL#4	28,7	NT CAR	ND NEG	ND NEG
ALL#10	44,8	NT CAR	9,40E-03 8,40E-03	2,30E-03 1,90E-03
ALL#6	75,3	NT CAR	NEG NEG	NEG NEG
ALL#11	13,9	NT CAR	1,10E-03 NEG	6,00E-04 NEG
ALL#12	79,8	NT CAR	1,50E-04 3,50E-04	1,00E-04 1,30E-04
ALL#13	33,6	NT CAR	NEG NEG	NEG ND
ALL#14	36,8	NT CAR	1.2E-04 NEG	NEG NEG
ALL#15	48.3	NT CAR	1.9E-04 6.4E-04	ND ND
ALL#16	75.2	NT CAR	3.4E-03 2.7E-03	3.5E-03 2.1E-02
ALL#18	36.4	NT CAR	3.4E-04 3.0E-04	6.2E-04 NEG
ALL#19	94.1	NT CAR	3.8E-03 2.1E-03	ND ND

Table 3 shows data from each enrolled patient as regarding the percentage of CD19+ leukemia cells in the row starting material considered for the CAR T manufacturing, and the value of MRD for two different Ig markers (MRD#1 and MRD#2) identified at the time of

diagnosis in each single patient. MRD data were reported for both control un-transduced T cell samples (NT) and CAR.CD19 T cell samples (CAR).

Moreover, it was observed that the leukemic blast cells contamination was also present in 9 out of 13 tested un-transduced T cell samples (NT, Table 3), proving that leukemic cells survive in culture independently from transduction process. More in depth, a time-course experiments have been performed, in which DPs were analyzed for MRD at very early time point of Day+8 after activation, Day+14 as standard procedures for CAR T manufacturing, and Day+30 as much extended culture for a CAR T-cell DP (data obtained from 5 different patient's productions, n=5). As shown in Figure 3A, an inverse correlation was observed between MRD levels and the time-course of *in vitro* culture, with a significant reduction of MRD as the time of culture progressively increase ($p=0.02$ considering MRD at Day+8 vs Day+14), reaching a level behind the sensitivity at the last time-point of Day+30. For those samples with enough available materials, DP analysis have also been performed by applying the high sensitivity EuroFlow cytometry platform (Leukemia. 2012 Sep; 26(9): 1899–1907). As clearly shown in Figure 3B reporting data from one exemplificative CAR T-cell DP at Day+14 of manufacturing process and control culture of NT T-cells, the applied cytofluorimetric analysis was sensitive enough to detect positive MRD. B cell precursors contaminating CAR products resulted to be CD19 very dull (Figure 4) but preserved other B cell markers, as CD10 (Figure 4). Nevertheless, the percentage of B cells contaminating *in vitro* expanded NT T-cells, resulting CD19+ CD10+, was significantly higher than those observed in CAR samples (Figure 4, MRD 1.5% vs 0.00036%, respectively). It was verified whether B cells were characterized by CAR transduction. As shown in Figure 5, for two samples with positive MRD in PCR and then analyzed by cytofluorimetric assay, CAR positive leukemia cells were detected, showing a double positivity to two different staining for CAR molecule (anti-CD34 detecting epitope on the hinge region of iC9.CAR.CD19_{LH} as well as CD19 epitope recognized by CAR.CD19 scFv). Data were also confirmed on BM-derived

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DPs, in which RT-qPCR show positivity of MRD in 6 out of 6 CAR T productions (Table 4).

Table 4

ALL Patients (BM)	%CD19+ Blasts	Sample	MRD#1	MRD#2
ALL#1	80,7	NT	6,03E-03	7,50E-03
		CAR	3,60E-03	5,20E-03
ALL#2	40,5	NT	7,60E-03	5,20E-03
		CAR	9,90E-03	7,00E-03
ALL#3	50,9	NT	2,60E-03	4,20E-03
		CAR	5,40E-02	5,40E-02
ALL#4	75,6	NT	2,80E-03	2,60E-03
		CAR	2,30E-03	1,90E-03
ALL#5	83,9	NT	1,20E-03	ND
		CAR	1,20E-03	1,90E-04
ALL#6	83,5	NT	ND	ND
		CAR	POS<QR	NEG

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Table 4 shows data of each enrolled patient as regarding the percentage of CD19+ leukaemia cells in the patient derived BM mononuclear cells used as starting row material for the CAR T cell manufacturing, and the value of MRD for two different Ig markers identified at the time of diagnosis in each single patient. MRD data were reported for both control un-transduced T cell samples (NT) and iC9.CAR.CD19LH T cell samples (CAR).

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For two BM-derived DPs the MRD was also confirmed by EuroFlow cytometry platform (Figure 5). In these cases, the sensitivity of the assay and the quality of frozen samples did not allow the detection of CAR positive B cell precursors. Also in BM-derived CAR T cell products, B-cell precursors contaminating the DPs were CD19 dim/negative, whereas in

un-transduced T-cell products contaminating B cells showed a high expression of CD19.

In summary, the proof has been provided that if a deep characterization of the drug product is carried out, the occurrence of leukemia CAR+ B cells is often detectable, underlined an urgent need to improve the safety profile of the CAR construct as high number of patients will soon treated with CAR T cells.

The length of the CAR linker influences epitope masking

Whether CAR.CD19 design in the linker and the hinge regions could have any impact on the CD19 masking when CAR.CD19 is co-expressed with CD19 on the same cellular membrane has been evaluated. In particular, four specific conformations, summarized in Figure 6A, were considered to demonstrate which configuration conformation in the CAR construct is responsible for the CD19 antigen masking in CAR+ leukemia cells. To this end, the 4 different CAR constructs were used to genetically modify NALM-6 cell line. Although the cells maintained CD19 positivity at the mRNA level (Figure 7A), the pattern of CD19-associated fluorescence levels in NALM-6 CAR.CD19LL/SH (light gray histogram) was superimposable to the control isotype (dark gray histogram) (Figure 6B), confirming previously published data (5). Then, a different second-generation CAR.CD19 configuration has been considered characterized by a long linker (as the one before), but in the presence of a long hinge that includes Δ CD34 (CAR.CD19LL/LH). In this case, masking flow-cytometry analysis showed no difference compared to the reference CAR.CD19LL/SH (Figure 6C). Then the contribution of the linker length between VL and VH regions has been evaluated, considering the MFI detection of CD19 in NALM-6 carrying CAR.CD19SL/SH. As shown in Figure 6D, CD19 MFI was higher in NALM-6 CAR.CD19SL/SH with respect to the reference CAR.CD19LL/SH. Finally, NALM-6 genetically modified with the CAR construct of the present invention has been considered in which the short linker between VL and VH is associated to the long hinge that includes Δ CD34, as trackable marker for CAR T cells. Also in the case of a LH, the SH is associated to a different CD19 MFI

respect to the reference structure (Figure 6E). The same data were also observed in other two different B cell lines, DAUDI and RAJI cells, in which an un-complete CD19 masking has been shown when lymphoma cells were genetically modified with CAR.CD19SL/LH (Figure 7B). Based on these observations, it has been speculated that the linker length could be the factor driving a complete or un-complete CD19 antigen CIS masking on CAR+ leukemic cells in the retroviral platform. These results were also corroborated by functional analysis. Indeed, very low level of CD19 expression on DAUDI, RAJI and NALM-6 CAR.CD19SL/LH cells was sufficient to elicit CAR.CD19 T-cell response, although to a lower extent if compared to wild-type cell lines (Figure 8A-C), particularly at low effector/target ratios. As shown in Figure 8C, CAR.CD19 T cells exert a complete leukaemia control against NALM-6 WT, with no significant differences compared to the anti-leukaemia activity observed against NALM-6 CAR.CD19SL/SH and NALM-6 CAR.CD19SL/LH. Of note, whereas CAR.CD19 T cells were completely unable to recognize NALM-6 CAR+ applied by Ruella et al in the previous publication (5) (Figure 7C), some activity of CAR.CD19 T cells against NALM-6 CAR.CD19LL/SH has been observed, although to a lower extent compared to NALM-6 CAR.CD19SL/SH and NALM-6 CAR.CD19SL/LH (Figure 8C). In line with these findings, it has been also observed that NALM-6 cells genetically modified with both CAR.CD19 with SL or LL were able to induce a significant amount of interferon-gamma (IFN-g) by CAR T cells (Figure 9A), as well as to induce their proliferation (Figure 9B).

Short Linker and Long Hinge in CAR.CD19 construct did not impact on CAR functionality and immunogenicity.

Notably, whereas CAR.CD19SL/LH is leading to the un-complete CD19 antigen CIS masking, when expressed on T cells, it was able to exert a significant leukemia/lymphoma control. In particular, co-culture assay was used to demonstrate the cytotoxic effect of CAR.CD19SL/LH T cells against DAUDI (Figure 8A), Raji (Figure 8B) and NALM-6 (Figure 8C) cell line. As shown in Figure 8A and 8B, CAR.CD19SL/LH T cells are able to eliminate tumor cells from the culture even when used at low

effector/target ratio. For the NALM-6 model, also the anti-leukemia activity of CAR.CD19SL/LH T cells have been compared with that of the more standard CAR.CD19LL/SH T cells, showing no substantial differences in terms of cytotoxicity (Figure 8C, NALM-6 WT), interferon gamma (IFN-g) production (Figure 9A), or proliferation index after antigen stimulation (Figure 9B). This last assay was performed by stimulating CFSE loaded CAR T cells with NALM-6 WT cells, and observing that irrespectively of the CAR construct, both CAR.CD19 T cells were able to reach comparable level of high proliferating cells (light grey histograms) respect to unstimulated cells (dark grey histograms). Moreover, since the trackable marker CD34 has been included in the CAR configuration, in silico analysis has been also performed to predict its immunogenicity. In particular, the peptide sequences that are confidently foreseen to be presented by MHC molecules in the CAR region that include CD34 domain have been studied. "STNVSPAPR" (SEQ ID NO:181 peptide is predicted as potentially immunogenic for CD34-including construct (Table5, presented by the MHC molecules HLA-A11:01 and HLA-A33:03. The peptide "GSELPTQGTF" (SEQ ID NO:182) also matches the selection criteria, but in this case, its binding core is "ELPTQGTF" (SEQ ID NO:183) and is entirely part of the CD34 epitope region; therefore, it is unlikely to be highly immunogenic. For the CAR construct in which CD34 domain was not considered, the peptides "SVTVSSPAPR" (SEQ ID NO:184) and its shorter version "VTVSSPAPR" (SEQ ID NO:185) are both predicted to be immunogenic, presented by the same alleles HLA-A11:01 and HLA-A33:03. In light of these data, we predict that the inclusion of CD34 domain in the construct did not substantially impact on the immunogenic profile of the CAR.

Table 5

Peptide	Binding alleles	CAR construct
VTVSSPAPR (SEQ ID NO:185)	HLA-A11:01, HLA-A33:03	Short hinge no CD34

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SVTVSSPAPR (SEQ ID NO:184)	HLA-A33:03	Short hinge no CD34
GSELPTQGTF (SEQ ID NO:182)	HLA-B40:01	Long hinge including CD34
STNVSPAPR (SEQ ID NO:181)	HLA-A11:01,HLA-A33:03	Long hinge including CD34

The activation of the suicide gene iC9 controls expansion of CAR+ leukemic cells.

The possibility to promptly eliminating CAR+ leukemic cells was demonstrated, through the exposure of DAUDI, RAJI and NALM-6 iC9.CAR+ cells to 20 nM of AP1903. Indeed, very early activation (6 hours) of the suicide gene iC9 corresponded to a significant reduction in the percentage of CAR+ leukemic cells (Figure 8D and 8E for DAUDI cells and Figure 10 for RAJI and NALM6 cells). Prolonged culture of AP1903-treated iC9.CAR.CD19 DAUDI cells was not associated to re-expansion of iC9.CAR+ leukemic cells (Figure 8E). In particular, the MFI of CAR expression in AP1903 treated cells was equal to 142 ± 22 (threshold value; Figure 10E), a value significantly inferior as compared to un-treated cells, but higher than the CAR staining of DAUDI WT cells (125.8 ± 20.6 , Figure 10E). The same results were also confirmed in RAJI (Figure 10A-B) and NALM-6 (Figure 10C-D) cellular models. While the presence of leukemic cells with high CAR expression MFI was undetectable by flow-cytometry analysis in AP1903 treated cells, the presence of leukemic cells was observed with very dim (i.e. moderate) expression of CAR.CD19, but a completed re-established detection of CD19 antigen, as in wild-type cell lines (Figure 8E, 10B and 10D). Indeed, qPCR analysis reveals the detection of the transgene (TG) in the remaining cells after AP1903 exposure (Figure 9F), although significantly decreased respect to untreated CAR+ cells (TG positivity was observed in 22.8% of DAUDI cells, 18.6 % of RAJI cells, and 0.6% of NALM-6 cells). Vector Copy Number analysis reveals that AP1903 treated residual cells had a significantly lower number of inserted vector compared to the untreated one (5.3 ± 4.2 and 0.1 ± 0.1 average of VCN threshold in untreated and

AP1903 treated B cells, respectively, across all the considered cellular models; Figure 10F). Since CD19 detection was completely re-established in iC9.CAR+ B cells rescued after AP1903 exposure, whether they could be targeted by CAR.CD19 T-cells has been verified. As shown in Figure 11, CAR.CD19 T-cells (Figure 11A and 11B) were able to eliminate CAR+ leukemic cells spared by AP1903 treatment. Moreover, because of the clinical unfeasibility to generate an autologous CAR T-cell product from patients with a CAR+ B cell relapse, it has been also proved that healthy donor-derived CAR.CD19 NK-cells are able to significantly control iC9.CAR+ B cells rescued after AP1903 exposure (Figure 11C and 11D).

A double strategy for the *in vivo* control of a CAR+ leukemia.

The ability of CAR.CD19 T cells to target wild-type B cell leukemia beside CAR construct with a SL or a LL, has been proved in vivo in a B cell leukemia NSG xenograft model. In particular, mice were infused systemically with NALM-6 genetically modified with FF-luciferase to allow in vivo monitoring of the leukaemia burden overtime. Tumour engraftment was analysed by measuring the bioluminescence signal, and, on Day+0, mice were treated with both CAR.CD19SL/LH and CAR.CD19LL/SH T cells, as well as with control NT-T-cells derived from HDs (Figure 12A). CAR.CD19SL/LH and CAR.CD19LL/SH T cells were able to significantly control NALM-6 in vivo expansion, as clearly demonstrated by bioluminescence analysis. Then whether NALM-6 CAR.CD19SL/LH were also recognized by CAR T cells in the in vivo setting has been evaluated. As shown in Figure 12B, CAR.CD19SL/LH T cells were able to reduce significantly CAR+ NALM-6 cell in vivo expansion as compared to control NT T cells. The same data were also confirmed in a less aggressive lymphoma model of DAUDI cell line (Figure 12D). In this model, CAR.CD19SL/LH T cells were able to control and eliminate CAR+ lymphoma cells in all treated mice. The mouse cohort treated with CAR.CD19 T-cells reached 100% disease-free survival (DFS) at the end of the experimental procedure (day 21) vs 0% DFS for the control cohort of mice receiving NT T-cells. Moreover, the in vitro data relative to NALM-6 CAR.CD19LL/SH have been also corroborated. Also in the in vivo setting,

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CAR T cells were able to exert anti-leukemia control against NALM-6 CAR.CD19LL/SH (Figure 12C), although to a lower extent of those observed in CAR.CD19SL/LH model (Figure 12B and Figure 12E).

Finally, the ability of the suicide gene iC9 to be active in the control of CAR+ leukemia expansion upon activation by AP1903 was also proved in vivo. In particular, NSG mice were infused with iCas9.CAR.CD19_{LH} DAUDI cells; after tumor engraftment, the dimerizing drug AP1903 was intraperitoneally administrated from day 1 to day 28 (Figure 13A). The activation of iC9 by administration of AP1903 resulted in a complete CAR+ leukemia eradication in 9 out of 10 studied mice (Figure 13B and 13C). Moreover, AP1903 administration allow the survival of 100% of the treated mice even after drug administration suspension, with no mice showing leukemia recurrence until day 63 (endpoint of the experiment; Figure 13D). The only mouse showing a positive signaling in IVIS analysis after CID administration, was early sacrificed at day 35 without sign of suffering together with a negative control (mouse of the same cohort) and a positive control (mouse without CID administration), to characterize the leukemia cells. By applying citofluorimetric analysis on peripheral blood, spleen, and tibias BM (left flank), leukemia cells could not be detected in mice treated with CID with a positive expression of CAR molecule.

EXAMPLE 2: *Comparison of different CAR.CD19 molecules with short linker from 8 aa to 14 aa and long linker*

In silico model has been performed to demonstrate that CAR.CD19 molecules conceived with a short linker spanning from 8 to 14 aa are characterized by a 3D structure different compared to the classical CAR.CD19 with a long linker of 15aa, providing an additional proof that CAR.CD19s with a linker with a reduced length have a spatial configuration providing a different masking of the target respect to that observed in CAR.CD19 with a standard linker of 15aa.

The following linkers have been used in order to link VL sequence SEQ ID NO:15 and VH sequence SEQ ID NO:16:

G3SG4 GGGSGGGG short linker (SEQ ID NO:38)

SG4SG3 SGGGGSGGG (SEQ ID NO:186),

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(SG4)2 SGGGGSGGGG (SEQ ID NO:190)

(SG4)2 S SGGGGSGGGGS (SEQ ID NO:187)

(SG4)2 SG SGGGGSGGGGSG (SEQ ID NO:188)

(SG4)2 SG2 SGGGGSGGGGSGG (SEQ ID NO:191)

5 (SG4)2 SG3 SGGGGSGGGGSGGG (SEQ ID NO:189)

(SG4)3 SGGGGSGGGGSGGGG Long linker (SEQ ID NO:39)

For this model, Superpose tool has been used to calculate protein superposition using a modified quaternion approach. From a superposition of two or more structures, Superpose generates sequence alignments, structure alignments, PDB coordinates, RMSD statistics, Difference Distance Plots, and interactive images of the superimposed structures. The SuperPose web server supports the submission of either PDB-formatted files or PDB accession numbers. This tool has been used to compare the structures of CAR.CD19 that comprises linkers of different lengths spanning the entire repertoire from 8 aa to 15 aa.

The difference distance matrix is generated as a PNG image that may be used to visually identify regions where there are significant differences between any structures comprising a linker from 8 to 14 aa, versus the standard CAR.CD19 structure comprising a long linker of 15 aa. The lighter the region, the more similar the structures are (Figures 14-21). Likewise, the darker the region, the more different the structure are. The default display for SuperPose's difference distance plot shows 6 graded cutoffs.

Differences between 0 and 1,5 Angstroms (A) are white; Differences between 1,5 and 3,0 A are very light gray; Differences between 3,0 and 5,0 A are light gray; Differences between 5 and 7 A are gray; Differences between 7 and 9 A are dark gray; Differences between 9 and 12 A are very dark gray and those greater than 12 A are black.

Figures 14-21 show also summarizing tables for the (Root-mean-square deviation) RMSD data relative to alpha carbons and back bone, as well as the heavy structure. These tables show a significant difference of all the CAR.CD19 configurations with a linker spanning from 8 to 14aa, compared to CAR.CD19 with a longer 15aa linker. These differences are

suggesting that CAR.CD19 with a linker comprising from 8 to 14aa have a different masking potential respect to the CAR.CD19 with a longer linker. The root-mean-square deviation of atomic positions, or simply root-mean-square deviation (RMSD), is the measure of the average distance between the atoms (usually the backbone atoms) of superimposed proteins.

References

- 1 Frey, N. The what, when and how of CAR T cell therapy for ALL. *Best Pract Res Clin Haematol* **30**, 275-281, doi:10.1016/j.beha.2017.07.009 (2017).
- 10 2 Gokbuget, N. How should we treat a patient with relapsed Ph-negative B-ALL and what novel approaches are being investigated? *Best Pract Res Clin Haematol* **30**, 261-274, doi:10.1016/j.beha.2017.07.010 (2017).
- 15 3 Park, J. H. *et al.* Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. *N Engl J Med* **378**, 449-459, doi:10.1056/NEJMoa1709919 (2018).
- 4 Maude, S. L. *et al.* Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. *N Engl J Med* **378**, 439-448, doi:10.1056/NEJMoa1709866 (2018).
- 20 5 Ruella, M. *et al.* Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies. *J Clin Invest* **126**, 3814-3826, doi:10.1172/JCI87366 (2016).
- 6 Brudno, J. N. & Kochenderfer, J. N. Recent advances in CAR T-cell toxicity: Mechanisms, manifestations and management. *Blood Rev* **34**, 45-55, doi:10.1016/j.blre.2018.11.002 (2019).
- 25 7 Wang, Z., Wu, Z., Liu, Y. & Han, W. New development in CAR-T cell therapy. *J Hematol Oncol* **10**, 53, doi:10.1186/s13045-017-0423-1 (2017).
- 8 Philip, B. *et al.* A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy. *Blood* **124**, 1277-1287, doi:10.1182/blood-2014-01-545020 (2014).
- 30 9 Bonini, C. *et al.* HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* **276**, 1719-1724, doi:10.1126/science.276.5319.1719 (1997).
- 35 10 Di Stasi, A. *et al.* Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* **365**, 1673-1683, doi:10.1056/NEJMoa1106152 (2011).
- 11 Zhou, X. *et al.* Inducible caspase-9 suicide gene controls adverse effects from alloplete T cells after haploidentical stem cell transplantation. *Blood* **125**, 4103-4113, doi:10.1182/blood-2015-02-628354 (2015).
- 40 12 Ruella, M. *et al.* Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. *Nat Med* **24**, 1499-1503, doi:10.1038/s41591-018-0201-9 (2018).
- 45 13 Ruella, M. *et al.* A cellular antidote to specifically deplete anti-CD19 chimeric antigen receptor-positive cells. *Blood* **135**, 505-509, doi:10.1182/blood.2019001859 (2020).
- 14 Kemper, K., Rodermond, H., Colak, S., Grandela, C. & Medema, J. P. Targeting colorectal cancer stem cells with inducible caspase-9. *Apoptosis* **17**, 528-537, doi:10.1007/s10495-011-0692-z (2012).
- 50 15 Yagyu, S., Hoyos, V., Del Bufalo, F. & Brenner, M. K. An Inducible Caspase-9 Suicide Gene to Improve the Safety of Therapy Using

- Human Induced Pluripotent Stem Cells. *Mol Ther* **23**, 1475-1485, doi:10.1038/mt.2015.100 (2015).
- 16 Zhou, X. *et al.* Serial Activation of the Inducible Caspase 9 Safety Switch After Human Stem Cell Transplantation. *Mol Ther* **24**, 823-831, doi:10.1038/mt.2015.234 (2016).
- 5 17 Quintarelli, C. *et al.* Efficacy of third-party chimeric antigen receptor modified peripheral blood natural killer cells for adoptive cell therapy of B-cell precursor acute lymphoblastic leukemia. *Leukemia* **34**, 1102-1115, doi:10.1038/s41375-019-0613-7 (2020).
- 10 18 Kalina, T. *et al.* EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* **26**, 1986-2010, doi:10.1038/leu.2012.122 (2012).
- 15 19 Maude SL, Teachey DT, Porter DL, Grupp SA. CD19-targeted chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Blood*. 2015;125(26):4017-4023. *Blood* **128**, 1441, doi:10.1182/blood-2016-07-730333 (2016).
- 20 20 Theunissen, P. *et al.* Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. *Blood* **129**, 347-357, doi:10.1182/blood-2016-07-726307 (2017).
- 20 21 Sedek, L. *et al.* Differential expression of CD73, CD86 and CD304 in normal vs. leukemic B-cell precursors and their utility as stable minimal residual disease markers in childhood B-cell precursor acute lymphoblastic leukemia. *J Immunol Methods* **475**, 112429, doi:10.1016/j.jim.2018.03.005 (2019).
- 25 22 Magdelaine-Beuzelin, C. *et al.* Structure-function relationships of the variable domains of monoclonal antibodies approved for cancer treatment. *Crit Rev Oncol Hematol* **64**, 210-225, doi:10.1016/j.critrevonc.2007.04.011 (2007).
- 30 23 DiJoseph, J. F. *et al.* Antibody-targeted chemotherapy of B-cell lymphoma using calicheamicin conjugated to murine or humanized antibody against CD22. *Cancer Immunol Immunother* **54**, 11-24, doi:10.1007/s00262-004-0572-2 (2005).
- 35 24 Kantarjian, H., Thomas, D., Wayne, A. S. & O'Brien, S. Monoclonal antibody-based therapies: a new dawn in the treatment of acute lymphoblastic leukemia. *J Clin Oncol* **30**, 3876-3883, doi:10.1200/JCO.2012.41.6768 (2012).

CLAIMS

1) Chimeric antigen receptor comprising or consisting of, from the N-terminus to the C-terminus:

a) a signal peptide,

5 b) a single chain antibody domain chosen from the group consisting of anti CD19 single chain antibody domain, anti CD20 single chain antibody domain or anti CD22 single chain antibody domain, said single chain antibody domain comprising or consisting of VL and VH sequences linked each other by a linker,

10 c) a hinge,

d) a trans membrane domain,

e) a co-stimulatory signaling domain, and

f) CD3Zeta chain sequence,

15 wherein said linker is a short flexible linker with a length from 7 to 14 amino acids, such as from 7 to 12, from 7 to 10 or 8 amino acids.

2) Chimeric antigen receptor according to claim 1, wherein

anti CD19 single chain antibody domain comprises anti CD19 FMC63 hybridoma VL and VH sequences, wherein anti CD19 FMC63 hybridoma VL sequence comprises CDR1 sequence QDISKY (SEQ ID NO:1), CDR2 sequence HTS and CDR3 sequence GNTLP (SEQ ID NO:2), whereas anti CD19 FMC63 hybridoma VH sequence comprises CDR1 sequence GVSLPDYG (SEQ ID NO:3), CDR2 sequence IWGSETT (SEQ ID NO:4) and CDR3 sequence AKHYYYGGSYAMDY (SEQ ID NO:5);

25 anti CD20 single chain antibody domain comprises anti CD20 VL and VH sequences, wherein anti CD20 VL sequence comprises CDR1 sequence SSVSY (SEQ ID NO:6), CDR2 sequence ATS and CDR3 sequence QQWTSNPPT (SEQ ID NO:7), whereas anti CD20 VH sequence comprises CDR1 sequence GYTFTSYN (SEQ ID NO:8), CDR2 sequence IYPGNGDT (SEQ ID NO:9) and CDR3 sequence ARSTYYGGDWYFNV (SEQ ID NO:10);

30 anti CD22 single chain antibody domain comprises anti CD22 VL and VH sequences, wherein anti CD22 VL sequence comprises CDR1

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sequence QSLANSYGNTF (SEQ ID NO:11), CDR2 sequence GIS and CDR3 sequence LQGTHQP (SEQ ID NO:12), whereas anti CD 22 VH sequence comprises CDR1 sequence GYRFTNYWIH (SEQ ID NO:13), CDR2 sequence INPGNNYA (SEQ ID NO:14) and CDR3 sequence TR.

- 5 3) Chimeric antigen receptor according to claim 2, wherein anti-CD19 FMC63 hybridoma VL sequence comprises or consists of sequence

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVK
LLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLP
10 YTFGGGKLEIT (SEQ ID NO:15) and

anti-CD19 FMC63 hybridoma VH sequence comprises or consists of sequence

- EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKG
LEWLGVWGSSETTYNSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYC
15 AKHYYYGGSYAMDYWGQGTSVTVSS (SEQ ID NO:16);

anti-CD20 VL sequence comprises or consists of sequence

QIVLSQSPAILSASPGKVTMTCRASSSVSYIHWFQQKPGSSPKP
WIYATSNLASGVPVRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNP
PTFGGGKLEIK (SEQ ID NO:17) and

- 20 anti-CD20 VH sequence comprises or consists of sequence

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGR
GLEWIGAIYPGNGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSA
VYYCARSTYYGGDWYFNVWGAGTTVTVSA (SEQ ID NO:18);

anti-CD22 VL sequence comprises or consists of sequence

- 25 DVQVTQSPSSLSASVGDRVTITCRSSQSLANSYGNTFLSWYLHK
PGKAPQLLIYGISNRFSGVPDRFSGSGSGTDFTLTISLQPEDFATYYCL
QGTHQPYTFGQGTKVEIK (SEQ ID NO:19) and

anti-CD22 VH sequence comprises or consists of sequence

- EVQLVQSGAEVKKPGASVKVSCKASGYRFTNYWIHWVRQAPGQ
30 GLEWIGGINPGNNYATYRRKFQGRVTMTADTSTSTVYMELSSLRSEDTA
VYYCTREGYGNYGAWFAYWGQGLTVTVSS (SEQ ID NO:20).

- 4) Chimeric antigen receptor according to anyone of claims 1-3,

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wherein the linker which links VL and VH sequences is chosen from the group consisting of a flexible Flex linker glycines-rich, such as (G4S)₂ linker GGGGSGGGG (SEQ ID NO:35), G4SG2 linker GGGGSGG (SEQ ID NO:37) or G3SG4 linker GGGSGGGG (SEQ ID NO:38), SG4SG3 linker SGGGGSGGG (SEQ ID NO:186), (SG4)₂ S linker SGGGGSGGGGS (SEQ ID NO:187), (SG4)₂ SG linker SGGGGSGGGGSG (SEQ ID NO:188), (SG4)₂ SG3 linker SGGGGSGGGGSGGG linker (SEQ ID NO:189), (SG4)₂ SGGGGSGGGG (SEQ ID NO:190), (SG4)₂ SG2 SGGGGSGGGGSGG (SEQ ID NO:191), preferably, G3SG4 linker.

5 5)Chimeric antigen receptor according to anyone of claims 1-4, wherein said hinge comprises or consists of one or more of the following hinges:

CD8stalk

TTTTAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD

15 (SEQ ID NO:21);

Hinge

CD28

EVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFLPGPSKP (SEQ ID NO:22);

hinge CH2-CH3

ESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVV

20 VDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSR
LTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLGK (SEQ ID
NO:23); or

25 hinge CH3:

ESKYGPPCPSCPGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGF

YPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSR LTVDKSRWQEG
NVFSCSVMHEALHNHYTQKSLSLGLGK (SEQ ID NO:24), preferably
CD8stalk.

30 6) Chimeric antigen receptor according to anyone of claims 1-5, wherein said hinge is linked, at the N terminus, to a trackable marker, said trackable marker being linked, optionally by a second linker, to the single chain antibody domain.

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7) Chimeric antigen receptor according to claim 6, wherein the trackable marker is chosen from the group consisting of:

ΔCD34 ELPTQGTFSNVSTNVS (SEQ ID NO:25); or
NGFR

5 KEACPTGLYTHSGECCKACNLGEGVAQPCGANQTVCEPCLDSV
TFSDVVSATEPCKPCTECVGLQSMSAPCVEADDAVCRCAYGYQDETT
GRCEACRVCEAGSGLVFSCQDKQNTVCEECPDGTYSDEANHVDPCLP
CTVCEDTERQLRECTRWADAECEEIPGRWITRSTPPEGSDSTAPSTQEP
EAPPEQDLIASTVAGVVTTVMGSSQPVVTRGTTDN (SEQ ID NO:26),
10 preferably ΔCD34.

8) Chimeric antigen receptor according to anyone of claims 1-7, wherein the hinge CD8stalk is linked to the trackable marker ΔCD34.

9) Chimeric antigen receptor according to anyone of claims 1-8, wherein the trans membrane domain is chosen from the group consisting of
15 CD28TM FWVLVVVGGVLACYSLLVTVAFIIFWV (SEQ ID NO:27) or
CD8aTM IYIWAPLAGTCGVLLLSLVIT (SEQ ID NO:28), preferably CD8aTM.

10) Chimeric antigen receptor according to anyone of claims 1-9, wherein the co-stimulatory signaling domain is chosen from the group
20 consisting of

CD28 cytoplasmic sequence
RSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRS (SEQ ID NO:29),

CD137 (4-1BB) sequence
25 KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEEGGCEL (SEQ ID NO:30),

OX40 sequence
RDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI (SEQ ID NO:31), or
a sequence obtained by linking:

30 CD28 cytoplasmic sequence (SEQ ID NO:29) with CD137 (4-1BB) sequence (SEQ ID NO:30),

CD137 (4-1BB) sequence (SEQ ID NO:30) with CD28 cytoplasmic sequence (SEQ ID NO:29),

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CD28 cytoplasmic sequence (SEQ ID NO:29) with OX40 sequence (SEQ ID NO:31),

OX40 sequence (SEQ ID NO:31) with CD28 cytoplasmic sequence (SEQ ID NO:29),

5 OX40 sequence (SEQ ID NO:31) with CD137 (4-1BB) sequence (SEQ ID NO:30),

CD137 (4-1BB) sequence (SEQ ID NO:30) with OX40 sequence (SEQ ID NO:31)

10 11) Chimeric antigen receptor according to anyone of claims 1-10, wherein CD3Zeta chain sequence is

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG
KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRRGKGGHDGLYQGLS
TATKDTYDALHMQALPPR* (SEQ ID NO:32).

15 12) Chimeric antigen receptor according to anyone of claims 1-11, further comprising cytoplasmic moiety of CD8cyt: LYCNHRNRRRVCKCPR (SEQ ID NO:40) between the transmembrane domain and the co-stimulatory signaling domain.

20 13) Chimeric antigen receptor according to anyone of claims 1-12, wherein the signal peptide comprises or consists of MEFGLSWFLVAILKGVQC (SEQ ID NO:41).

14) Chimeric antigen receptor according to anyone of claims 1-13, wherein said anti-CD19 chimeric antigen receptor comprises or consists of the following sequence:

MEFGLSWFLVAILKGVQCSRDIMTQTSSLSASLGDRVTISCRASQDI
25 SKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL
EQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGGEVKLQESGPGL
VAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTY
NSALKSRLTIKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDY
WGQGTSTVTVSSGSELPTQGTFSTNVSTNVSPAPRPPTPAPTASQPLSLR
30 PEACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLVITLYCNHRN
RRRVCKCPRVDKRGKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEE
GGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRD
PEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRRGKGGHDGL

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YQGLSTATKDTYDALHMQALPPR (SEQ ID NO:72) or
 MEFLSWLFLVAILKGVQCSRDIQMTQTSSLSASLGDRVTISCRASQDI
 SKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL
 EQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGGEVKLQESGPGL
 5 VAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKLEWLGVWGETTYY
 NSALKSRLTIKDNSKSQVFLKMNSLQTDITAIYYCAKHYYYGGSYAMDY
 WGQGTSTVTVSSGSPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR
 GLDFACDIYWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPVDKRRGR
 KLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAP
 10 AYQQGQNQLYNELNLGRREEYDVLDRRGRDPEMGGKPRRKNPQEG
 YNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM
 QALPPR (SEQ ID NO:33) .

15 15) Nucleotide sequence comprising or consisting of a nucleotide
 sequence which encodes a chimeric antigen receptor according to anyone
 of claims 1-14.

16) Nucleotide sequence according to claim 15, wherein
 anti CD19 FMC63 hybridoma VL sequence is encoded by the
 nucleotide sequence

GACATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTC
 20 TGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGT
 AAATATTTAAATTGGTATCAGCAGAAACCAGATGGAAGTGTAACTC
 CTGATCTACCATACATCAAGATTACACTCAGGAGTCCCATCAAGGTTC
 AGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCT
 GGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAGGGTAATACGC
 25 TTCCGTACACGTTCCGAGGGGGGACTAAGTTGGAAATAACA (SEQ ID
 NO:52) and

anti CD19 FMC63 hybridoma VH sequence is encoded by the
 nucleotide sequence

GAGGTGAAACTGCAGGAGTCAGGACCTGGCCTGGTGGCGCC
 30 CTCACAGAGCCTGTCCGTACATGCACTGTCTCAGGGGTCTCATTAC
 CCGACTATGGTGTAAAGCTGGATTGCCAGCCTCCACGAAAGGGTCTG
 GAGTGGCTGGGAGTAATATGGGGTAGTGAAACCACATACTATAATTC
 AGCTCTCAAATCCAGACTGACCATCATCAAGGACAACTCCAAGAGCC

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AAGTTTTCTTAAAAATGAACAGTCTGCAAAGTATGACACAGCCATTT
ACTACTGTGCCAAACATTATTACTACGGTGGTAGCTATGCTATGGACT
ACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA (SEQ ID NO:53);

anti CD20 VL sequence is encoded by the nucleotide sequence

5 CAGATCGTGCTGAGCCAGAGCCCCGCCATCCTGAGCGCCAGC
CCCGGCCGAGAAGGTGACCATGACCTGCAGGGCCAGCAGCAGCGTG
AGCTACATCCACTGGTTCCAGCAGAAGCCCGGCAGCAGCCCCAAGC
CCTGGATCTACGCCACCAGCAACCTGGCCAGCGGCGTGCCCGTGAG
GTTGAGCGGCAGCGGCAGCGGCACCAGCTACAGCCTGACCATCAGC
10 AGGGTGGAGGCCGAGGACGCCGCCACCTACTACTGCCAGCAGTGGA
CCAGCAACCCCCCACCTTCGGCGGCGGCACCAAGCTGGAGATCAA
G (SEQ ID NO:54) and

anti CD20 VH sequence is encoded by the nucleotide sequence

CAGGTGCAGCTGCAGCAGCCCGGCGCCGAGCTGGTGAAGCC
15 CGGCGCCAGCGTGAAGATGAGCTGCAAGGCCAGCGGCTACACCTTC
ACCAGCTACAACATGCACTGGGTGAAGCAGACCCCGGCAGGGGCC
TGGAGTGGATCGGCGCCATCTACCCGGCAACGGCGACACCAGCTA
CAACCAGAAGTTCAAGGGCAAGGCCACCCTGACCGCCGACAAGAGC
AGCAGCACCGCCTACATGCAGCTGAGCAGCCTGACCAGCGAGGACA
20 GCGCCGTGTACTACTGCGCCAGGAGCACCTACTACGGCGGCGACTG
GTACTTCAACGTGTGGGGCGCCGGCACCACCGTGACCGTGAGC
(SEQ ID NO:55);

anti CD22 VL sequence is encoded by the nucleotide sequence

GACGTGCAGGTGACCCAGAGCCCCAGCAGCCTGAGCGCCAG
25 CGTGGGCGACAGGGTGACCATCACCTGCAGGAGCAGCCAGAGCCTG
GCCAACAGCTACGGCAACACCTTCCTGAGCTGGTACCTGCACAAGCC
CGGCAAGGCCCCCAGCTGCTGATCTACGGCATCAGCAACAGGTTC
AGCGGCGTGCCCGACAGGTTGAGCGGCAGCGGCAGCGGCACCGAC
TTCACCCTGACCATCAGCAGCCTGCAGCCCGAGGACTTCGCCACCTA
30 CTACTGCCTGCAGGGCACCCACCAGCCCTACACCTTCGGCCAGGGC
ACCAAGGTGGAGATCAAG (SEQ ID NO:56) and

anti CD22 VH sequence is encoded by the nucleotide sequence

GAGGTGCAGCTGGTGCAGAGCGGCGCCGAGGTGAAGAAGCC

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CGGCGCCAGCGTGAAGGTGAGCTGCAAGGCCAGCGGCTACAGGTTC
ACCAACTACTGGATCCACTGGGTGAGGCAGGCCCCCGGCCAGGGCC
TGGAGTGGATCGGCGGCATCAACCCCGGCAACAACTACGCCACCTA
CAGGAGGAAGTTCCAGGGCAGGGTGACCATGACCGCCGACACCAGC
5 ACCAGCACCGTGTACATGGAGCTGAGCAGCCTGAGGAGCGAGGACA
CCGCCGTGTACTACTGCACCAGGGAGGGCTACGGCAACTACGGCGC
CTGGTTCGCCTACTGGGGCCAGGGCACCCCTGGTGACCGTGAGCAGC
(SEQ ID NO:57).

17) Nucleotide sequence according to any one of claims 15-16,
10 wherein the nucleotide sequence encoding anti-CD19 chimeric antigen
receptor is:

ATGGAGTTTGGACTTTCTTGGTTGTTTTTGGTGGCAATTCTGAAGGGT
GTCCAGTGTAGCAGGGACATCCAGATGACACAGACTACATCCTCCCT
GTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTC
15 AGGACATTAGTAAATATTTAAATTGGTATCAGCAGAAACCAGATGGAA
CTGTAAACTCCTGATCTACCATACATCAAGATTACACTCAGGAGTCC
CATCAAGGTTCAAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACC
ATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAG
GGTAATACGCTTCCGTACACGTTTCGGAGGGGGGACTAAGTTGGAAAT
20 AACAGGCGGAGGAAGCGGAGGTGGGGGCGAGGTGAAACTGCAGGA
GTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCGTCACA
TGCACTGTCTCAGGGGTCTCATTACCCGACTATGGTGTAAGCTGGAT
TCGCCAGCCTCCACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGG
GGTAGTGAAACCACATACTATAATTGAGCTCTCAAATCCAGACTGACC
25 ATCATCAAGGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGT
CTGCAAACTGATGACACAGCCATTTACTACTGTGCCAAACATTATTAC
TACGGTGGTAGCTATGCTATGGACTACTGGGGTCAAGGAACCTCAGT
CACCGTCTCCTCAGGATCCGAACCTCCTACTCAGGGGACTTTCTCAA
ACGTTAGCACAAACGTAAGTCCCGCCCCAAGACCCCCCACACCTGC
30 GCCGACCATTGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCCTGCC
GGCCAGCTGCCGGCGGGGGCCGTGCATACAAGAGGACTCGATTTTCGC
TTGCGACATCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCC
TTCTGCTCAGCCTGGTTATTACTCTGTACTGTAATCACCGGAATCGCC

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GCCGCGTTTGTAAGTGTCCCAGGGTCGACAAACGGGGCAGAAAGAA
ACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACT
CAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAG
GAGGATGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCC
5 CGCGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAG
GACGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGA
CCCTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGC
CTGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCGCTACAGTGA
GATTGGGATGAAAGGCGAGCGCCGGAGGGGGCAAGGGGCACGATGG
10 CCTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCC
TTCACATGCAGGCCCTGCCCCCTCGCTAA (SEQ ID NO:58) or
ATGGAGTTTGGACTTTCTTGGTTGTTTTTGGTGGCAATTCTGAAGGGT
GTCCAGTGTAGCAGGGACATCCAGATGACACAGACTACATCCTCCCT
GTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTC
15 AGGACATTAGTAAATATTTAAATTGGTATCAGCAGAAACCAGATGGAA
CTGTTAACTCCTGATCTACCATACATCAAGATTACACTCAGGAGTCC
CATCAAGGTTCAAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACC
ATTAGCAACCTGGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAG
GGTAATACGCTTCCGTACACGTTTCGGAGGGGGGACTAAGTTGGAAAT
20 AACAGGCGGAGGAAGCGGAGGTGGGGGCGAGGTGAACTGCAGGA
GTCAGGACCTGGCCTGGTGGCGCCCTCACAGAGCCTGTCCGTCACA
TGCACTGTCTCAGGGGTCTCATTACCCGACTATGGTGTAAGCTGGAT
TCGCCAGCCTCCACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGG
GGTAGTGAAACCACATACTATAATTCAAGCTCTCAAATCCAGACTGACC
25 ATCATCAAGGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGT
CTGCAAACTGATGACACAGCCATTTACTACTGTGCCAAACATTATTAC
TACGGTGGTAGCTATGCTATGGACTACTGGGGTCAAGGAACCTCAGT
CACCGTCTCCTCAGGATCCCCCGCCCCAAGACCCCCACACCTGCG
CCGACCATTGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCCTGCCG
30 GCCAGCTGCCGGCGGGGCGGTGCATACAAGAGGACTCGATTTGCT
TGCGACATCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCCT
TCTGCTCAGCCTGGTTATTACTCTGTACTGTAATCACCGGAATCGCCG
CCGCGTTTGTAAGTGTCCCAGGGTCGACAAACGGGGCAGAAAGAAA

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CTCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACTC
AAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAGGA
GGATGTGAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCG
CGTACCAGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGA
5 CGAAGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACC
CTGAGATGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCT
GTACAATGAACTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGA
TTGGGATGAAAGGCGAGCGCCGGAGGGGGCAAGGGGCACGATGGCC
TTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTT
10 CACATGCAGGCCCTGCCCCCTCGCTAAA (SEQ ID NO:34).

18) Nucleotide sequence according to any one of claims 15-17, said nucleotide sequence further comprising a nucleotide sequence encoding a suicide gene inducible amino acid sequence linked to the nucleotide sequence encoding said chimeric antigen receptor by a nucleotide
15 sequence encoding a 2A self-cleaving peptide.

19) Nucleotide sequence according to claim 18, wherein the suicide gene inducible amino acid sequence is a chimeric Caspase-9 polypeptide or comprises a herpes simplex virus thymidine kinase.

20) Nucleotide sequence according to anyone of claims 15-19,
20 which is

ATGCTCGAGATGCTGGAGGGAGTGCAGGTGGAGACTATTAGC
CCCGGAGATGGCAGAACATTCCCCAAAAGAGGACAGACTTGCGTCG
TGCATTATACTGGAATGCTGGAAGACGGCAAGAAGGTGGACAGCAG
CCGGGACCGAAACAAGCCCTTCAAGTTCATGCTGGGGAAGCAGGAA
25 GTGATCCGGGGCTGGGAGGAAGGAGTCGCACAGATGTCAGTGGGAC
AGAGGGCCAACTGACTATTAGCCCAGACTACGCTTATGGAGCAACC
GGCCACCCCGGGATCATTCCCCCTCATGCTACACTGGTCTTCGATGT
GGAGCTGCTGAAGCTGGAAAGCGGAGGAGGATCCGGAGTGGACGG
GTTTGGAGATGTGGGAGCCCTGGAATCCCTGCGGGGCAATGCCGAT
30 CTGGCTTACATCCTGTCTATGGAGCCTTGCGGCCACTGTCTGATCAT
TAACAATGTGAACTTCTGCAGAGAGAGCGGGCTGCGGACCAGAACA
GGATCCAATATTGACTGTGAAAAGCTGCGGAGAAGGTTCTCTAGTCT
GCACTTTATGGTCGAGGTGAAAGGCGATCTGACCGCTAAGAAAATGG

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TGCTGGCCCTGCTGGAAGTGGCTCGGCAGGACCATGGGGCACTGGA
TTGCTGCGTGGTCGTGATCCTGAGTCACGGCTGCCAGGCTTCACATC
TGCAGTTCCTGGGGCAGTCTATGGAAGTACGGCTGTCCAGTCAG
CGTGGAGAAGATCGTGAACATCTTCAACGGCACCTCTTGCCCAAGTC
5 TGGGCGGGAAGCCCAAAGTGTCTTTATTCAGGCCTGTGGAGGCGA
GCAGAAAGATCACGGCTTCGAAGTGGCTAGCACCTCCCCCGAGGAC
GAATCACCTGGAAGCAACCCTGAGCCAGATGCAACCCCTTCCAGGA
AGGCCTGAGGACATTTGACCAGCTGGATGCCATCTCAAGCCTGCCCA
CACCTTCTGACATTTTCGTCTCTTACAGTACTTTCCCTGGATTTGTGA
10 GCTGGCGCGATCCAAAGTCAGGCAGCTGGTACGTGGAGACACTGGA
CGATATCTTTGAGCAGTGGGCCCATTCTGAAGACCTGCAGAGTCTGC
TGCTGCGAGTGGCCAATGCTGTCTCTGTGAAGGGGATCTACAAACAG
ATGCCAGGATGCTTCAACTTTCTGAGAAAGAAAGTGTCTTTAAGACC
TCCGCATCTAGGGCCCCGCGGGAAGGCCGAGGGAGCCTGCTGACAT
15 GTGGCGATGTGGAGGAAAACCCAGGACCACCATGGATGGAGTTTGG
ACTTTCTTGTTGTTTTTGGTGGCAATTCTGAAGGGTGTCCAGTGTAG
CAGGGACATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTC
TGGGAGACAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGT
AAATATTTAAATTGGTATCAGCAGAAACCAGATGGAAGTGTAAACTC
20 CTGATCTACCATACATCAAGATTACACTCAGGAGTCCCATCAAGGTTC
AGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCT
GGAGCAAGAAGATATTGCCACTTACTTTTGCCAACAGGGTAATACGC
TTCCGTACACGTTCCGAGGGGGGACTAAGTTGGAAATAACAGGCGG
AGGAAGCGGAGGTGGGGGCGAGGTGAAACTGCAGGAGTCAGGACC
25 TGGCCTGGTGGCGCCCTCACAGAGCCTGTCCGTACATGCACTGTC
TCAGGGGTCTCATTACCCGACTATGGTGTAAAGCTGGATTGCCAGCC
TCCACGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGGGTAGTGAA
ACCACATACTATAATTCAGCTCTCAAATCCAGACTGACCATCATCAAG
GACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTGCAAACT
30 GATGACACAGCCATTTACTACTGTGCCAAACATTATTACTACGGTGGT
AGCTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTC
CTCAGGATCCGAACTTCCTACTCAGGGGACTTTCTCAAACGTTAGCA
CAAACGTAAGTCCCGCCCCAAGACCCCCCACACCTGCGCCGACCAT

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TGCTTCTCAACCCCTGAGTTTGAGACCCGAGGCCTGCCGGCCAGCT
 GCCGGCGGGGCGGTGCATACAAGAGGACTCGATTTTCGCTTGCGACA
 TCTATATCTGGGCACCTCTCGCTGGCACCTGTGGAGTCCTTCTGCTC
 AGCCTGGTTATTACTCTGTACTGTAATCACCGGAATCGCCGCCGCGT
 5 TTGTAAGTGTCCCAGGGTCGACAAACGGGGCAGAAAGAACTCCTGT
 ATATATTCAAACAACCATTTATGAGACCAGTACAACTACTCAAGAGG
 AAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAGGAGGATGT
 GAACTGAGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCGCGTACC
 AGCAGGGCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGAAGA
 10 GAGGAGTACGATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGA
 TGGGGGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGCCTGTACAA
 TGAAGTGCAGAAAGATAAGATGGCGGAGGCCTACAGTGAGATTGGG
 ATGAAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGCCTTTAC
 CAGGGTCTCAGTACAGCCACCAAGGACACCTACGACGCCCTTCACAT
 15 GCAGGCCCTGCCCCCTCGCTAA (SEQ ID NO:180).

21) Vector comprising the nucleotide sequence according to anyone of claims 15-20, wherein said vector is a DNA vector, a RNA vector, a plasmid, a lentivirus vector, adenoviral vector, retrovirus vector, such as γ -retroviral vector, or non viral vector.

20 22) Cell, such as T cell, such as alfa/beta and gamma/delta T cell, NK cells, NK-T cells, comprising the chimeric antigen receptor according to anyone of claims 1-14 and/or the vector or plasmid according to claim 21.

23) Cell according to claim 22, further comprising a suicide gene
 25 inducible amino acid sequence such as a chimeric Caspase-9 polypeptide or comprises a herpes simplex virus thymidine kinase.

24) Cell according to claim 23, wherein the chimeric Caspase-9 polypeptide comprises or consists of:

FKBP12 binding region comprising or consisting of a short 5' leader
 30 peptide MLEMLE (SEQ ID NO:43) and the mutant of human FKBP12(V36F) of sequence

GVQVETISPGDGRTFPKRQTCVVHYTGMLDGKKVDSSRDNRN
 KPFFKMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIP

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PHATLVFDVELLKLE (SEQ ID NO:44), which is linked by a linker, such as SGGGSG (SEQ ID NO:45) linker, to Caspase-9 polypeptide

VDGFGDVGALESLRGNADLAYILSMEPCGHCLIINNPNFCRESGL
RTRTGSNIDCEKLRRRFSSLHFMVEVKGDLTAKKMVLALLELARQDHGA
5 LDCCVVVILSHGCQASHLQFPGAVYGTGCPVSVEKIVNIFNGTSCPSLG
GKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSNPEPDATPFQEGLRT
FDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDIFEQWA
HSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (SEQ ID
NO:46), which is linked by a linker, such as ASRAPR (SEQ ID NO:47)
10 linker, to

a Polynucleotide 2A self-cleaving peptide chosen from the group consisting of T2A AEGRGSLTCDVEENPGP (SEQ ID NO:48), P2A ATNFSLLKQAGDVEENPGP (SEQ ID NO:49), E2A QCTNYALLKLAGDVESNPGP (SEQ ID NO:50) or F2A: VKQTLNFDLLKLAGDVESNPGP (SEQ ID NO:51), preferably T2A.
15

25) Cell according to any one of claims 22-24, which is obtained in culture conditions wherein both IL-7 and IL-15 are present, for example in the culture conditions of the activation step, transduction step and/or expansion step of the process for the preparation of said cell.

20 26) Pharmaceutical composition comprising the nucleotide sequence according to claims 16-20, or the vector according to claim 21, or the cell according to claims 22-25 together with one or more excipients and/or adjuvants.

27) Chimeric antigen receptor according to anyone of claims 1-15, 25 nucleotide sequence according to anyone of claims 16-20, vector according to claim 21, cell according to claims 22-25, pharmaceutical composition according to claim 26, for medical use.

28) Chimeric antigen receptor according to anyone of claims 1-15, nucleotide sequence according to anyone of claims 16-20, vector according to claim 21, cell according to claims 22-25, pharmaceutical composition according to claim 26, for use in the treatment of CD19+, CD20+ or CD22+ cancers, for example B cell lymphomas (Non-Hodgkin's Lymphoma (NHL)), acute lymphoblastic leukemia (ALL), myeloid leukemia
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and chronic lymphocytic leukemia (⁷⁷CLL), B-cell derived autoimmune diseases.