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IMMUNOTHERAPY WITH MEMORY "STEM" TCELLS MODIFIED WITH CHIMERIC ANTIGENSPECIFIC RECEPTORS (CARs) FOR PATIENTS WITH LYMPHOID NEOPLASMS

Ana Carolina Caballero González

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Universitat Autònoma de Barcelona

PhD PROGRAM IN MEDICINE

DEPARTMENT OF MEDICINE

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Ana Carolina Caballero González

DIRECTORS: Javier Briones Meijide
Carmen Alvarez-Fernández

TUTOR: Jorge Sierra Gil

PhD PROGRAM IN MEDICINE - DEPARTMENT OF MEDICINE

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ACKNOWLEDGMENTS

Esta tesis doctoral fue conseguida mediante el esfuerzo y sacrificio de muchas personas y el apoyo incondicional de otras. Quisiera agradecer de todo corazón a todos los que de alguna manera contribuyeron al desarrollo de este trabajo que, a su vez, ayudó a mi desarrollo profesional y personal. A todos aquellos que me dieron "muestras" para hacer mis experimentos mi más sincero agradecimiento.

De manera especial, quisiera agradecer a las personas más importantes de mi vida: Mi familia. Jeanlu, mi esposo, por su amor, su apoyo incondicional y su paciencia en todo este trayecto que muchas veces me llevó a estar feliz, triste, frustrada, emocionada…por cosas que quizá él no entendía. Gracias por impulsarme a seguir mis sueños y por creer que yo "sería capaz". Gracias a mis padres, Fidel y Teresita, porque sin ustedes no sería la persona que soy hoy, gracias por enseñarme con amor y paciencia, por empujarme, por amarme, por ilusionarse con mis pequeños logros, por compartir con alegría mi trabajo. Gracias por haberme regalado la fe, que me mantiene a flote siempre. A mi hermano, Oscar, que me regala su cariño incondicional, me apoya, me alienta y que ayuda incansablemente a nuestros padres cada día. De mi familia aprendí a trabajar duro, que el esfuerzo tiene su recompensa y que hay que tener fe en Dios que nos acompaña siempre.

De corazón agradezco a mis directores de tesis: el Dr. Javier Briones y la Dra. Carmen Alvarez-Fernández. Primero, a ambos les agradezco muchísimo el haber aceptado trabajar conmigo a sabiendas de que tenía todo por aprender y casi nada para aportar: ¡Gracias! Ya durante la residencia, el Dr. Javier Briones permitió que pueda conocer el laboratorio de investigación y pueda tener contacto con los proyectos de su grupo en una corta rotación externa. Allí, me recibió la Dra. Carmen Alvarez-Fernández, quien con paciencia, alegría, risas y dedicación me enseñó poco a poco a trabajar en el laboratorio. Tras esa única experiencia, el Dr. Javier Briones me facilitó el integrarme al Grupo de Inmunoterapia Celular y Terapia Génica, me permitió el desarrollo tutelado de proyectos de investigación, se leyó, corrigió y mejoró todos los trabajaos que escribí (algunos más de 10 veces). Además, con su ayuda pude complementar mi formación clínica porque me permitió formar parte de la Unidad Multidisciplinar CAR para poder hacer "traslación" de mi aprendizaje de la terapia CAR a los pacientes porque tras el inicio del ensayo clínico me permitió formar parte del equipo clínico investigador y participar de la valoración de pacientes altamente complejos. De su criterio médico, aprendo cada día y lo agradezco mucho. La Dra. Carmen Alvarez-Fernández, por su parte, desde el primer día me enseñó a trabajar en el laboratorio de investigación y no hablo solo desde el punto de vista práctico

(que también, 100%), sino también del ejercicio mental, crítico y analítico. Tuvo paciencia para permitirme equivocarme y, que sea capaz de darme cuenta (lo cual era difícil). Estuvo allí cuando tuve que presentarme a "mil becas" y también cuando me las denegaron. Estuvo en mis altibajos, cuando creía que "nada iba a salir". Me asistió y ayudó a realizar mis experimentos incluso si implicaba trabajar noches y fines de semana. Pero también me permitió (con el tiempo) ser autónoma, me permitió ver que iba a aprendiendo y que tengo criterio…que "sé pensar". Mi equipo de directores de tesis es excelente, tiene puntos de vista complementarios que enriquecen el trabajo.

Muchas gracias a mi tutor, el Dr. Jorge Sierra, por acogerme en el Servicio de Hematología, por inculcar en el servicio el deseo de superación y alentar el desarrollo profesional científico. Muchas gracias por sus palabras de aliento y por su confianza. Le agradezco además que haya permitido mi continuidad en el servicio durante todos estos años.

No puedo dejar de agradecer a mis compañeras de laboratorio y amigas, Alba y Laurita. Su compañía, apoyo, comprensión y ayuda son invaluables. ¡Son tantas las risas, nervios (por presentaciones y deadlines) y anécdotas las que nos unen! Gracias por tanta paciencia para ayudarme en el laboratorio desde el principio, gracias por asistirme siempre que necesité, por esforzarse para hacer "hueco" entre sus actividades para leer o ayudarme en lo que estaba escribiendo o para escuchar por milésima vez una presentación que haya preparado. Alba, mi coR de laboratorio, ¡ahora yo también ya termino! Gracias por todos los días, amiga. Laurita, gracias por ser tan generosa con todo, incluyendo tu trabajo! Valoro todo lo que colaboraste para el desarrollo de mis experimentos. Paula, Ros, Eva y Cristina mil gracias por toda su ayuda. Muchas gracias por todas las veces que me ayudaron o asistieron en mis experimentos. También quiero agradecerles todas sus palabras de aliento, quiero decirles que siempre me sentí muy apoyada por todas ustedes (en todo).

Muchas gracias a todos mis compañeros del Servicio de Hematología del Hospital Sant Pau. Guadalupe e Irene son maravillosas, gracias por las palabras de aliento ("ya lo tienes", "ya no queda nada") y por el apoyo. Silvana, gracias por "mostrarme" los linfomas. A todos los compañeros: médicos, enfermeras, auxiliares, farmacéuticos, administrativos; muchas gracias por todo lo que me enseñan día a día.

Gracias a Dios por todo lo que me da y a la Virgencita por su amparo y protección,

Anita

ABBREVIATIONS

ACT: adoptive cell therapy

CAR: chimeric antigen receptor

CD19: cluster of differentiation 19

cHL: classical Hodgkin lymphoma

CD30: cluster of differentiation 30

CAR30: chimeric antigen receptor directed against CD30

CART30: chimeric antigen receptor therapy directed against CD30

$T_{SCM-LIKE}$: memory "stem" like T-cells

CART: chimeric antigen receptor therapy

HL: Hodgkin lymphoma

CAR19: chimeric antigen receptor directed against CD19

BCR: B-cell receptor

APC: antigen-presenting cells

TCR: T-cell receptor

CD3: cluster of differentiation 3

ITAMs: immunoreceptor tyrosine-based activation motifs

Ig: immunoglobulin

MHC: major histocompatibility complex

IFNγ: interferon gamma

IFNα: interferon alpha

IFNβ: interferon beta

CD4: cluster of differentiation 4

Th1: T helper 1

IL-2: interleukin 2

Th2: T helper 2

IL-4: interleukin 4

IL-5: interleukin 5

IL-6: interleukin 6

CD8: cluster of differentiation 8

IgSF: immunoglobulin superfamily

TNFRSF: tumor necrosis factor receptor superfamily

CD28: cluster of differentiation 28

ICOS: inducible T-cell co-stimulator

CTLA4: cytotoxic T-lymphocyte associated protein 4
PD1: programmed cell death protein 1
BTLA4: B- and T-lymphocyte attenuator 4
CD80: cluster of differentiation 80
PD-L1: programmed cell death ligand-1
CD137: cluster of differentiation 137 (4-1BB)
CD134: cluster of differentiation 134
CD27: cluster of differentiation 27
TNFRSF8: tumor necrosis factor receptor superfamily member 8
HVEM: herpes virus entry mediator
CD95: cluster of differentiation 95
BCMA: B-cell maturation antigen
PI3K: phosphatidylinositol 3-kinase
NF-kB: nuclear factor-kB
NFAT: nuclear factor of activated T-cells
IL-10: interleukin 10
IL-21: interleukin 21
IL-2: interleukin 2
LAT: linker for activation of T-cells
ZAP70: zeta-chain associated protein
PD-L2: programmed cell death ligand-2
PMBCL: primary mediastinal B-cell lymphoma
TIM-3: T-cell immunoglobulin and mucin-domain containing 3
TNF: tumor necrosis factor
CEACAM1: carcinoembryonic antigen cell adhesion molecule 1
LAG-3: lymphocyte activation gene 3
NKT: natural killer T-cells
NK: natural killer
pDC: plasmacytoid dendritic cells
DC: dendritic cells
Tregs: regulatory T-cells
sLAG-3: soluble form of lymphocyte activation gene 3
TRAF: TNF receptor associated factor
MAP: mitogen activated protein

4-1BBL: 4-1BB ligand
cIAP1/2: cellular inhibitors of apoptosis 1 or 2
ERK: extracellular signal regulated kinase
sCD30: soluble form of CD30 protein
TACE: TNFα converting enzyme
JNK: c-Jun N-terminal kinase
CD30L: CD30 ligand
CAR-T: chimeric antigen receptor T-cell
ICIs: immune checkpoint inhibitors
TILs: tumor-infiltrating lymphocytes
PBMCs: peripheral blood mononuclear cells
scFv: single chain peptide from heavy and light chain variable region of Ig
B-NHL: B-cell non-Hodgkin lymphoma
B-ALL: B-cell acute lymphoblastic leukemia
CART19: chimeric antigen receptor therapy directed against CD19
DLBCL: diffuse large B-cell lymphoma
OS: overall survival
R/R: relapse or refractory
ASCT: autologous stem cell transplantation
SOC: standard of care
ORR: overall response rates
CR: complete response
FL: follicular lymphoma
LD: lymphodepletion
Cy: cyclophosphamide
Flu: fludarabine
CRS: cytokine release syndrome
ICANS: immune effector cell-associated neurotoxicity syndrome
PFS: progression-free survival
FL3B: follicular lymphoma grade 3B
tFL: transformed follicular lymphoma
NR: not reported
DOR: duration of response
LDH: lactate dehydrogenase

PS: performance status
IPI: international prognostic index
AUC: area under the curve
IL-7: interleukin 7
IL-15: interleukin 15
MCP-1: monocyte chemoattractant protein 1
T_N: naïve T-cell
T_M: memory T-cell
T_{EF}: effector T-cell
T_{CM}: central memory T-cell
T_{EM}: effector memory T-cell
T_{SCM}: memory "stem" T-cell
CD45RO: cluster of differentiation 45 isoform RO
CD62L: L-selectin
CCR7: C-C chemokine receptor type 7
CD45RA: cluster of differentiation 45 isoform RA
CD95: cluster of differentiation 95
CXCR3: C-X-C Motif Chemokine Receptor 3
CD58: cluster of differentiation 58
CD22: cluster of differentiation 22
CD20: cluster of differentiation 20
BAFF-R: B-cell activating factor receptor
TSLPR: thymic stromal lymphopoietin receptor
T-ALL: T-acute lymphoblastic leukemia
AML: acute myeloid leukemia
NKG2DL: natural killer group 2 member D ligand
CLL1: C-type lectin like molecule 1
FLT3: FMS-like tyrosine kinase 3
WT1: Wilms tumor 1
CLL: chronic lymphocytic leukemia
ROR: receptor tyrosine kinase like orphan receptor
MM: multiple myeloma
SLAMF7: signaling lymphocytic activation molecule F7

GPRC5D: G protein-coupled receptor class-C group-5 member-D

T-NHL: T-cell non-Hodgkin's lymphoma

TRBC: T cell receptor β-chain constant domains

TME: tumor microenvironment

NSHL: nodular sclerosis Hodgkin lymphoma

MCHL: mixed cellularity Hodgkin lymphoma

LDHL: lymphocyte-depleted Hodgkin lymphoma

LRHL: lymphocyte-rich Hodgkin lymphoma

HRS: Hodgkin and Reed–Sternberg cell

APRIL: proliferation-inducing ligand

CD40L: CD40 ligand

BV: brentuximab-vedotin

CD95L: CD95 ligand

CD200: cluster of differentiation 200

HLA-G: human leukocyte antigen G

HLA-E: HLA class I histocompatibility antigen, alpha chain E

CCR4: C-C chemokine receptor type 4

ALCL: anaplastic large cell lymphoma

Benda: bendamustine

EGFRt: truncated human epidermal growth factor receptor

IV: intravenous

SC: subcutaneous

$T_{SCM\text{-}LIKE}^{H}$: high proportion of memory "stem" like T-cells

$T_{SCM\text{-}LIKE}^{H}$: low proportion of memory "stem" like T-cells

MFI: mean fluorescence intensity

CAR^{HI}: high intensity of CAR expression subgroup

CAR^{LO}: low intensity of CAR expression subgroup

HLA-DR: HLA class II histocompatibility antigen, DR alpha chain.

CD69: cluster of differentiation 69

CD25: cluster of differentiation 25

E:T: effector : target

ADC: antibody-drug conjugate

MMAE: monomethyl auristatin E

TARC: thymus and activation regulated chemokine

CXCR4: C-X-C chemokine receptor type 4

CXCR3: C-X-C chemokine receptor type 3

CCL5: C-C motif chemokine ligand 5

TAA: tumor-associated antigen

CAR30^{HI}: high intensity of CAR30 expression subgroup

CAR30^{LO}: low intensity of CAR30 expression subgroup

GMP: Good Manufacture Practices

AEMPS: Spanish Agency of Medicines and Medical devices

DL1: limiting dose 1

DL2: limiting dose 2

DL3: limiting dose 3

NOS: not otherwise specified

ESHAP: etoposide, cytarabine, cisplatin and methylprednisolone

ICE: ifosfamide, etoposide, carboplatin

DHAP: cytarabine, cisplatin, dexamethasone

GemOx: gemcitabine, oxaliplatin

ECOG: Eastern Cooperative Oncology Group Performance Status Scale

FEV1: forced expiratory volume during the first second

DLCO: diffusing capacity of the lungs for carbon monoxide

FVC: forced vital capacity

PD: progression disease

PR: partial response

M: male

F: female

LK: leukapheresis

SD: standard deviation

DLT: dose-limiting toxicities

CMV: cytomegalovirus

MN-pCT: myeloid neoplasm post-cytotoxic therapy

BS: body surface

TB: tuberculosis

RDT: radiotherapy

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ABSTRACT

Adoptive cell therapy (ACT) with T-cells modified with a chimeric antigen receptor (CAR) directed against CD19 has demonstrated outstanding results in B-cell lymphoma. However, its application in other hematologic malignancies that lack of CD19 expression, such as classical Hodgkin lymphoma (cHL) remains a clinical challenge. The CD30 protein is expressed in Hodgkin and Reed–Sternberg cells and is absent from most healthy tissues, representing an ideal target for ACT in this disease. Nevertheless, efficacy of CAR30 T-cells for cHL remains modest, with more than 50% of patients being unresponsive. CART30 therapy efficacy may be influenced by several factors related to the infused product, the blockade of CAR30 by the soluble CD30 protein and low persistence of $CAR30^+$ T-cells.

Here, we have developed a novel CAR30 targeting a membrane-proximal epitope within the non-cleavable part of CD30 protein to improve antitumor efficacy of CART30 therapy. Furthermore, we developed products enriched for memory "stem" T-cells ($T_{SCM\text{-}LIKE}$ cells) to improve engraftment, prolong persistence, and enhance antitumor activity. $CAR30^+$ $T_{SCM\text{-}LIKE}$ cells were successfully generated and expanded *ex vivo,* despite CD30 expression and fratricide killing of $CD30^+CAR30^+$T-cells. Our $CAR30^+$ $T_{SCM\text{-}LIKE}$ cells were not impaired by soluble CD30 and completely eradicated HL *in vivo*, with high persistence and long-lasting immunity. In addition, CAR30 T-cell products highly enriched for $T_{SCM\text{-}LIKE}$ cells have shown a survival advantage in contrast to more differentiated CAR30 T-cells.

Moreover, we have hypothesized that CAR expression level would have a significant impact on CART efficacy and tested this with $CAR30^+$ $T_{SCM\text{-}LIKE}$ enriched cells. By sorting T-cells according to CAR mean fluorescence intensity in two markedly different populations ($CAR30^{HI}$ and $CAR30^{LO}$), we showed that a high CAR expression enhances antitumor efficacy *ex vivo*, that is sustained after sequential re-exposures to tumor cells and is not associated to T-cell exhaustion or differentiation. Furthermore, we found a correlation between high surface CAR expression and antitumor effect with $CAR19^+$ T-cells, thus validating our findings with CAR30. Definitive proof of $CAR30^{HI}$ T-cells improved antitumor efficacy was demonstrated in a human HL xenograft mouse model, where CAR30-$T_{SCM\text{-}LIKE}$ enriched products with high intensity of CAR expression achieved superior tumor control *in vivo* and longer survival than those with a low intensity of CAR expression.

Our data highlights the superior efficacy of novel CAR30 directed against an epitope within the non-cleavable part of CD30 protein with highly enriched $T_{SCM\text{-}LIKE}$ products. Furthermore, our data shows that intensity of CAR expression on cell surface influences

antitumor efficacy, suggesting that modulation of CAR expression intensity should be considered as a critical factor to improve efficacy of anti-CD30 CAR T-cells.

RESUMEN

La terapia celular adoptiva con células T modificadas con un receptor quimérico antígeno—específico (CAR) dirigido contra CD19 ha demostrado resultados sobresalientes en las neoplasias de células B. Sin embargo, su aplicación en otras neoplasias hematológicas malignas que carecen de la expresión de CD19, como el linfoma de Hodgkin clásico (LHc), sigue siendo un desafío clínico. La proteína CD30 se expresa en las células de Hodgkin y Reed-Sternberg (HRS) y está ausente en la mayoría de los tejidos sanos, por lo que constituye una diana ideal para la terapia celular adoptiva en esta enfermedad. Sin embargo, la eficacia de las células T dirigidas contra CD30 (CART30) para el tratamiento del LHc sigue siendo modesta, ya que más del 50 % de los pacientes no responden. La eficacia de la terapia CART30 puede verse influenciada por varios factores relacionados con el producto infundido, el bloqueo del CAR30 por la presencia de proteína CD30 soluble y la baja persistencia de células CART30. Hemos desarrollado un nuevo CAR30 dirigido a un epítopo proximal a la membrana, dentro de la parte no escindible de la proteína CD30, con el objetivo de mejorar la eficacia antitumoral de la terapia CART30. Además, hemos desarrollado productos enriquecidos en células T de memoria "stem" (células $T_{SCM\text{-}LIKE}$) para prolongar la persistencia y aumentar la actividad antitumoral. Hemos generado las células $CAR30^{+}$ $T_{SCM\text{-}LIKE}$, que fueron expandidas *ex vivo*, a pesar de la expresión de CD30 en las células T activadas y la muerte fratricida de algunas estas células T $CD30^{+}CAR30^{+}$. Nuestras células $CAR30^{+}$ $T_{SCM\text{-}LIKE}$ no se vieron afectadas por la proteína CD30 soluble y, más aún, erradicaron por completo el HL *in vivo*, evidenciando su gran persistencia y su prolongada eficacia antitumoral. Además, los productos CART30 altamente enriquecidos en células $T_{SCM\text{-}LIKE}$ han logrado una ventaja de supervivencia *in vivo* comparados con los productos compuestos fundamentalmente por células CART30 más diferenciadas.

Por otra parte, hemos planteado la hipótesis de que la intensidad de expresión del CAR tendría un impacto significativo en la eficacia de la terapia CART. Esta hipótesis la hemos comprobado con nuestros productos CART30 enriquecidos con $T_{SCM\text{-}LIKE}$. Hemos separado las células CART30 según la intensidad de fluorescencia media del CAR, en dos poblaciones marcadamente diferentes ($CAR30^{HI}$ y $CAR30^{LO}$), demostramos que una alta expresión de CAR mejora la eficacia antitumoral *ex vivo*, que esa gran efectividad se mantiene después de reexposiciones secuenciales a células tumorales diana y que no está asociada al agotamiento o diferenciación celular. Además, encontramos una correlación entre la alta expresión de CAR en la superficie y el efecto antitumoral con las células CART19, lo cual valida nuestros hallazgos con las células CART30. Por último, la eficacia antitumoral

superior de las células CAR30^{HI} se demostró en un modelo *in vivo* de LH, en el que los productos CART30 enriquecidos con $T_{SCM\text{-}LIKE}$ con alta intensidad de expresión de CAR, lograron un mayor control tumoral y una supervivencia más prolongada que aquellos con una baja intensidad del CAR30.

Nuestros datos ponen en evidencia la óptima eficacia antitumoral obtenida con el nuevo CAR30 dirigido contra un epítopo incluido en la parte no escindible de la proteína CD30 y utilizado en productos altamente enriquecidos en células $T_{SCM\text{-}LIKE}$. Además, hemos mostrado que la intensidad de la expresión de CAR en la superficie celular influye en la eficacia antitumoral, lo que sugiere que la modulación de la intensidad de la expresión de CAR debe considerarse uno de los factores útiles para mejorar la eficacia de las células CART anti-CD30.

1 INTRODUCTION

1.1 Immune system overview

The immune system is a complicated and dynamic network of cells, molecules, and pathways that cooperate to protect us from infectious agents and provides us with a surveillance system to monitor the integrity of host tissues (1,2). It's function comprises two basic roles: recognition of foreign substances and organisms that have penetrated our outer defenses (i.e., the skin epithelium and the mucosal surfaces of the gut and reproductive and respiratory tracts) and elimination of such agents by a diverse repertoire of cells and molecules that act in concert to neutralize the potential threat (2). The immune response is mediated by sequential and coordinated responses that are called innate and adaptative.

Innate immunity (also called natural immunity or native immunity) is sometimes used to include physical, chemical, and microbiological barriers, but more usually encompasses the elements of the immune system (neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins) which provide immediate host defense.

The adaptive immune system (also called specific immunity or acquired immunity) develops precise antigen-specific reactions through T-cells and B-cells (3–5). The fundamental properties of the adaptive immune system reflect the properties of the lymphocytes that mediate these responses:

- *Specificity and diversity:* Immune responses are specific for different portions of a single complex protein, polysaccharide, or other macromolecules. The parts of complex antigens that are specifically recognized by lymphocytes are called determinants or epitopes. It is estimated that the immune system of an individual can discriminate 10^7 to 10^9 distinct antigenic epitopes. If an antigen binds to the antigen-specific cells, it activates them, and promotes its proliferation (clonal expansion).
- *Memory:* Immunologic memory occurs because each exposure to an antigen generates long-lived memory cells specific for the antigen. Memory cells react more rapidly and vigorously to antigen challenge than naïve lymphocytes.
- *Nonreactivity to self:* Self-tolerance is maintained by eliminating lymphocytes that express receptors specific for some self-antigens, inactivating self-reactive lymphocytes, or suppressing these cells by the actions of regulatory cells (6).

T-cells and B-cells are the central players in the adaptive immune system and can generate highly specific cell surface receptors to recognize practically any molecular structure. B-cell receptors and T-cell receptors are structurally similar molecules (Figure 1) (7).

Mature B-cells are distinguished by their expression of the B-cell receptor (BCR), a membrane bound immunoglobulin that binds to a specific antigen. A secreted form of immunoglobulin of the same antigen specificity is the antibody produced by terminally differentiated B-cells and plasma cells. The function of the BCR is to recognize and bind antigen, thus transmitting a signal that activates the B-cell, leading to clonal expansion and antibody production. Activated B-cells can act as antigen-presenting cells (APC) and also express costimulatory molecules required to activate T-cells (1).

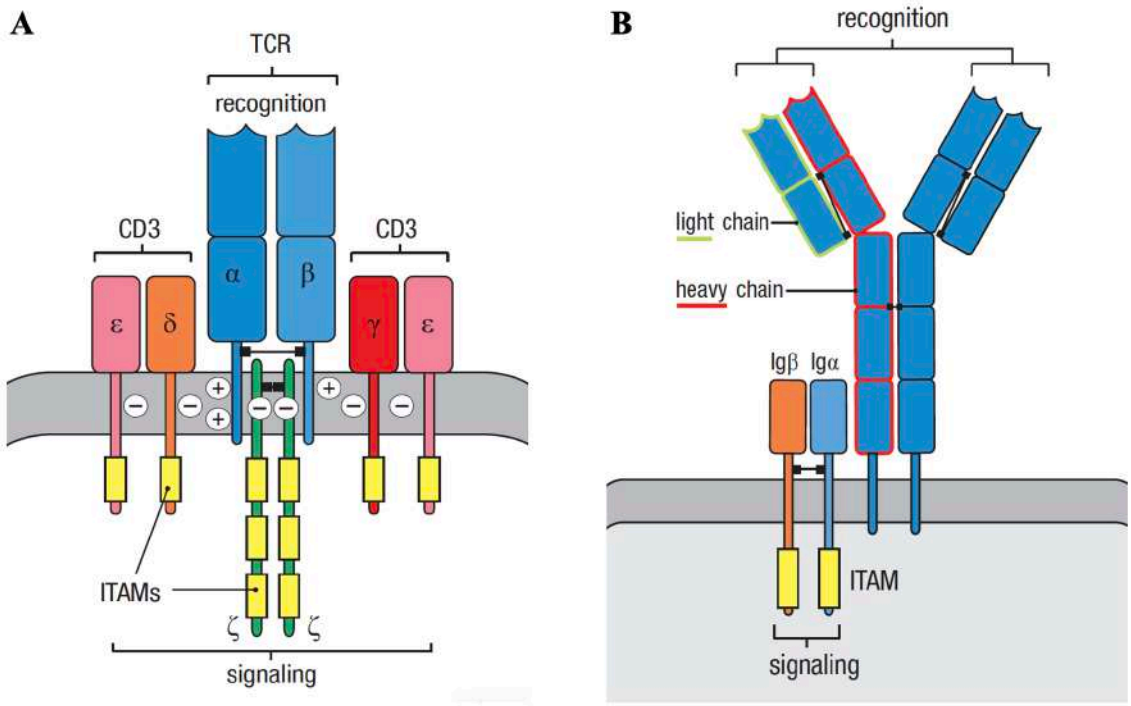

Figure 1: A. The T-cell receptor complex. The functional TCR complex is composed of the antigen-binding TCRα:β heterodimer associated with six signaling chains: two ε, one δ, one γ, collectively called CD3, and a homodimer of ζ chain. Signaling from the T-cell receptor is initiated by tyrosine phosphorylation within cytoplasmic regions called immunoreceptor tyrosine-based activation motifs (ITAMs). **B. The B-cell receptor complex** is made up of cell-surface immunoglobulin with one of each of the invariant signaling proteins Igα and Igβ. The immunoglobulin recognizes and binds antigen but cannot itself generate a signal. The antigen-nonspecific signaling molecules Igα and Igβ have ITAMs that enable them to signal when the BCR is ligated with antigen. *(Modified from Murphy K, 2022).*

In contrast to BCRs, T-cell receptors (TCRs) are made solely as membrane-bound proteins which are associated with an intracellular signaling complex. TCRs cannot recognize free antigen as immunoglobulin can, TCRs recognize only processed pieces of antigen (typically peptides) bound to cell membrane proteins called major histocompatibility complex (MHC) molecules (2,7).

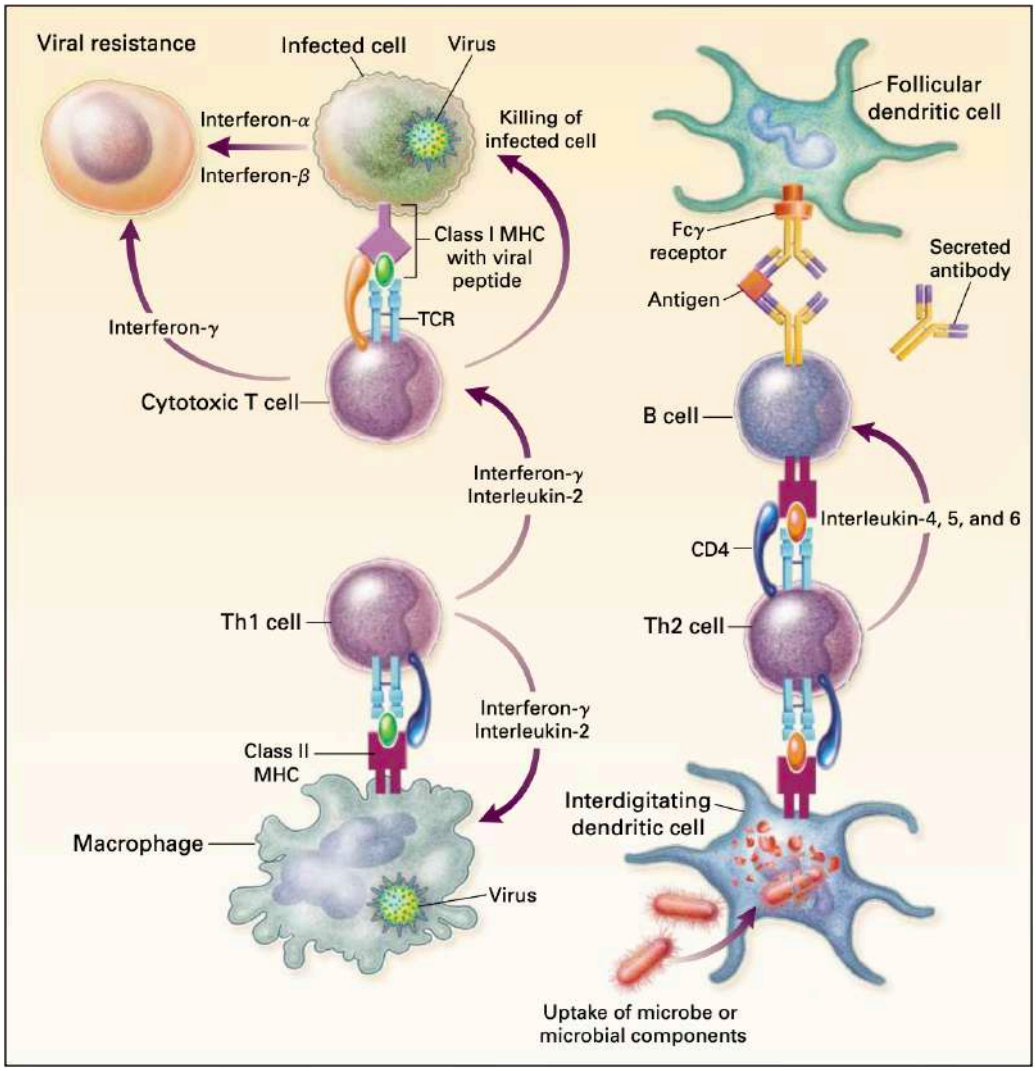

Figure 2: An Overview of Lymphocyte Responses. T-cells characteristically possess T-cell receptors (TCRs) that recognize processed antigen presented by major-histocompatibility- complex (MHC) molecules, as shown on the left-hand side of the figure. Most cytotoxic T-cells are positive for CD8, recognize processed antigen presented by MHC class I molecules, and kill infected cells. Activated cytotoxic T-cells secrete interferon γ (IFN γ) that, together with interferon α (IFN α) and interferon β (IFN β) produced by the infected cells themselves, sets up a state of cellular resistance to viral infection. As shown on the right-hand side of the figure, helper T-cells are generally positive for CD4, recognize processed antigen presented by MHC class II molecules, and can be divided into two major populations. Type 1 (Th1) helper T-cells secrete IFN γ and interleukin-2 (IL-2), which activate macrophages and cytotoxic T-cells to kill intracellular organisms; type 2 (Th2) helper T-cells secrete IL-4, IL-5, and IL-6, which help B-cells secrete protective antibodies. *(Delves P, 2000).*

The MHC molecules are highly polymorphic transmembrane glycoproteins with a cleft in the extracellular face of the molecule in which peptides can be bound. There are two types of MHC molecules: MHC class I and II. MHC class I that are expressed by nearly all nucleated cells and come largely from intracellular sources. MHC class II acquire peptides from extracellular sources and are expressed by antigen-presenting cells (4,5).

TCRs recognize features of both the peptide antigen and the MHC molecule to which it is bound. This is known as MHC restriction because any given TCR is specific for a particular peptide bound to a particular MHC molecule (2,7,8). The antigen recognition by the TCR is different for $CD4^+$ and $CD8^+$ T-cells. $CD4^+$ T-cells recognize antigens presented with MHC class II and $CD8^+$ T- cells with MHC class I (Figure 2). Endogenous antigens complexed with MHC class I molecules activate $CD8^+$ cytotoxic T-cells. Thus, any nucleated cells infected with a virus or producing abnormal tumor antigens can present these antigens with class I and be removed by cytotoxic attack. $CD4^+$ T-cells activation leads to production of cytokines which in turn activate a wide range of cells around them and only a small number of class II antigen-presenting cells can drive this activation (3).

The need for intracellular processing and expression with MHC ensures that only antigens derived from foreign molecules that have either invaded the interior of a host cell or have induced an inflammatory response to activate endocytosis by antigen-presenting cells, are recognized as foreign. Even more, binding of the TCR to the antigen-MHC complex alone, is not adequate to induce activation of the cell, a coreceptor stimulation is also required (3).

1.2 TCR structure and function

The TCR consists of a variable TCRαβ heterodimer that binds to ligands (Figure 1). The TCR forms a complex with the non-variable signal transduction CD3 complex, which contains CD3γ, CD3δ, CD3ε and TCRζ subunits. All CD3 complex subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains (9).

TCRαβ binds complexes of peptide and MHC molecules on the surface of APCs or target cells, which results in phosphorylation of the ITAMs in the cytoplasmic portions of the CD3 complex (9), and the transduction of the downstream signal to the nucleus to initiate transcriptional activation of various gene sequences, including those encoding cytokine that stimulate and regulate the proliferation of T-cells (5). This first intracellular signal is initiated with the help of the CD4 and CD8 coreceptors, which bind to MHC class II and class I

molecules, respectively, via their extracellular domains, and associate with nonreceptor kinases via their intracellular domains. The Src-family kinase, Lck, is constitutively associated with the cytoplasmic domains of CD4 and CD8 and is thought to be the kinase primarily responsible for phosphorylation of the ITAMs of the TCR (7).

Antigen receptor signaling alone is not sufficient to effectively activate T-cells. Instead, such isolated signal leads to anergy or apoptosis. Additional signals required to achieve T-cell activation come from the co-signaling receptors (5). Co-stimulatory receptors that promote the response, and co-inhibitory receptors that inhibit the response, are required to control the direction and magnitude of a given immune response (Figure 3) (10).

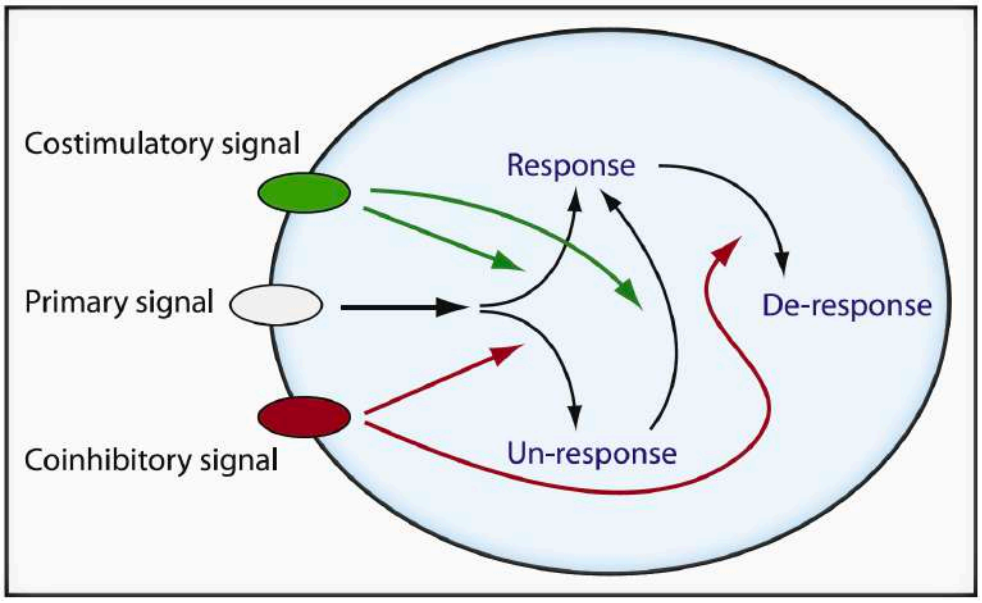

Figure 3: The Tide model for the control of immune response. The primary signal could either induce ‘‘response’’ when costimulatory signal is dominant, or trigger ‘‘un-response’’ in the presence of dominant coinhibitory signal. With a strong far-reaching coinhibitory signal, even an initial ‘‘response’’ could be terminated (‘‘de-response’’). *(Zhu Y, 2011)*

1.3 Co-signaling receptors

Most co-signaling molecules are members of the immunoglobulin superfamily (IgSF) and tumor necrosis factor receptor superfamily (TNFRSF) (Figure 4) (11).

The IgSF includes CD28 [i.e., CD28, ICOS, CTLA4, PD1, BTLA] and B7 [i.e., CD80, PD-L1] receptors subfamilies, which primarily interact with each other (11).

TNFRSF molecules includes: 4-1BB (CD137), OX40 (CD134), CD27 (TNFRSF7), GITR (CD357), CD30 (TNFRSF8) and HVEM (CD270), CD95, BCMA (11).

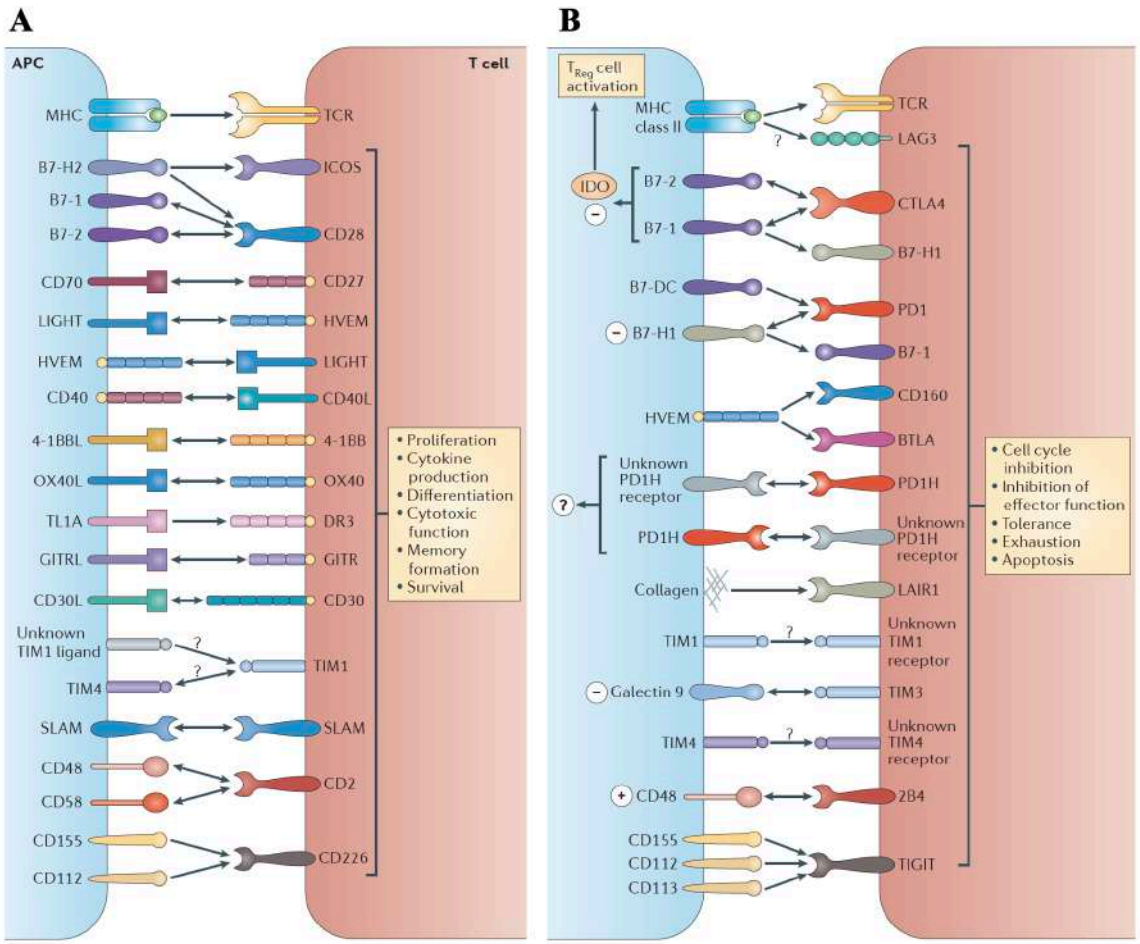

Figure 4: Co-signaling interactions in T-cells. A. Co-stimulatory molecules deliver positive signals to T-cells following their engagement by ligands and counter-receptors on antigen-presenting cells (APCs). Several co-stimulatory molecule interactions transduce a signal into their respective cell. **B.** Co-inhibitory molecules deliver negative signals into T-cells. *(Chen L, 2013)*

1.3.1 *Immunoglobulin superfamily receptors*

1.3.1.1 *Co-stimulatory receptors*

CD28 is expressed by naïve and activated T-cells and is required for optimal production of cytokines and proliferation. In general, primary T-cell responses are more dependent on CD28 than are secondary responses (11–13). ICOS and CTLA4 are restricted in their expression to activated and memory T-cells. CD28, ICOS, and CTLA4 have cytoplasmic domains that function as a binding site for the SH2 domains of p85 — an adaptor subunit of the lipid kinase phosphatidylinositol 3-kinase (PI3K) a common signaling intermediate, to initiate targeting of AKT that subsequently results in activation of several distal molecules. CD28 through this pathway promotes T-cell proliferation and survival through the activation

of the downstream targets nuclear factor-κB (NF-κB), nuclear factor of activated T-cells (NFAT), mammalian target of rapamycin (mTOR) and others (11–13).

ICOS displays an enhanced AKT signaling compared to CD28. ICOS signaling results in induction of interleukine-4 (IL-4), interleukine-10 (IL-10) and interleukine-21 (IL-21) but cannot induce interleukine-2 (IL-2) production. (11,12)

Despite being a co-inhibitory receptor, **CTLA4** cytoplasmatic domain also binds PI3K, and it has been proved that CTLA4 promotes $CD4^+$ T-cell migration through PI3K-dependent AKT activation. CTLA4 reduce proximal TCR signaling through dephosphorylation of the TCR–CD3ζ complex, of linker for activation of T-cells (LAT) and of 70 kDa zeta-chain associated protein (ZAP70), thereby inhibiting cell cycle progression and cytokine production (11,12,14).

1.3.1.2 *Co-inhibitory receptors*

Programmed death 1 (PD1) is a cell surface molecule that regulates the adaptive immune response and is expressed in B and T-cells as well as myeloid-derived cells. Engagement of PD1 by its ligands PD-L1 (B7-H1) or PD-L2 (B7-DC) transduces a signal that inhibits T-cell proliferation, cytokine production, and cytolytic function. Under normal conditions, PD1 expression establishes a threshold of activation that must be overcome before initiation of an immune response (15).

PD1 and its ligands are type I transmembrane glycoproteins sharing a basic structural pattern consisting of an immunoglobulin variable–type extracellular domain, a transmembrane region, and a cytoplasmic tail containing tyrosine-based signaling motifs. CD28, CTLA4, and ICOS form disulfide-based covalent homodimers, but PD1, PD-L1 and PD-L2 are known to exist and interact as monomers (15,16). PD1 engagement blocked the induction of PI3K activity (15).

PD-L1 is constitutively expressed in T-cells, B-cells, macrophages, and dendritic cells, and further upregulated following activation of these cells. The expression of PD-L1 is also detected on endothelial cells in the heart, β cells in the pancreas, glial cells in inflamed brain and muscle cells (17). PD-L1 genetic copy number gains and amplifications have been found in some types of cancers such as triple-negative breast cancer, classical Hodgkin lymphoma (cHL), primary mediastinal B-cell lymphoma (PMBCL), and squamous cell carcinomas of

the vulva and cervix(18). PD-L2 expression is observed only on activated macrophages and dendritic cells (17).

T-cell immunoglobulin and mucin-domain containing 3 (**TIM-3**) is a type I transmembrane protein which plays a key role in inhibiting Th1 responses and the expression of cytokines such as TNF and interferon γ (INF-γ) (19). Transient TIM-3 expression is detected on activated T-cells both *in vitro* and *in vivo*, but persistent expression is only observed following chronic stimulation (20). Dysregulation of TIM-3 expression has been associated with autoimmune diseases (19).

TIM-3 consists of 302 amino acid residues, with an extracellular domain consisting of a membrane distal N-terminal immunoglobulin domain, followed by a membrane-proximal mucin domain containing potential sites for glycosylation. The stalk domain is followed by a transmembrane domain and a cytoplasmic tail (19,21). The cytoplasmic tail functions as a binding site for the SH2 domain, where multiple SH2 domain-containing kinases, including Fyn, Lck, PI3K, P85, and Itk, have been shown to bind (22).

The interaction between Galectin-9, a soluble protein, and TIM-3 triggers cell death in effector Th1 cells. IFN-γ is critical for the induction of Galectin-9 expression, then Galactine-9 engages TIM-3 on T-cells and, ensures clonal contraction of responding Th1 cells. In addition, carcinoembryonic antigen cell adhesion molecule 1(CEACAM1) is required for TIM-3 glycosylation and protein stability. CEACAM1 and TIM-3 interaction is required for maintaining T-cell tolerance. Galectin-9 and CEACAM1 bind to different TIM-3 regions and might have cooperative effects in regulating TIM-3 signaling (19).

Expression of PD1 and TIM-3 on $CD4^+$ and $CD8^+$ T-cells has been reported, changes in their expression during T-cell activation have also been described. Interestingly, a recent study described that $CD4^+$ T-cells undergoing proliferation that express PD1 often exhibit lower expression of TIM-3, while TIM-3 expressing $CD8^+$ T cells have reduced PD1 expression (23).

Lymphocyte activation gene-3 (**LAG-3**, CD223) transmembrane molecule, consisting of four extracellular immunoglobulin superfamily-like domains, expressed on $CD4^+$ and $CD8^+$ T-cells, natural killer T-cells (NKT), NK cells, plasmacytoid dendritic cells (pDCs), and regulatory T-cells (Tregs). In most cell types, LAG-3 expression is regulated via activation except for pDCs and Tregs in which expression appears to be constitutive. Like CD4, LAG-

3 binds to MHC class II but with a much higher affinity. Once LAG-3 is bound to MHC class II, it transmits inhibitory signals (24). Very recent studies have revealed a new mechanism of action, independent of MHC binding: LAG-3 gets associated with the TCR:CD3 complex, inducing co-receptor and p56Lck dissociation, which impacts downstream signaling resulting in T-cell inhibition (25). LAG-3 also has a soluble form (sLAG-3) released by shedding at the cell surface that provides an additional layer of control and immune regulation. sLAG-3 is thought to impair monocyte differentiation into macrophages or DC, producing APC that ultimately have reduced immunostimulatory capacities (26).

1.3.2 *Tumor necrosis factor receptors superfamily*

1.3.2.1 *Co-stimulatory receptors*

TNFRSF molecules [i.e., 4-1BB (CD137), OX40 (CD134), CD27 (TNFRSF7), GITR (CD357), CD30 (TNFRSF8) and HVEM (CD270), CD95, BCMA] contain one or more extracellular cysteine-rich domains, whereas their ligands contain a conserved extracellular TNF homology domain (11). The ligands for the TNFRSF members are type II cell surface glycoproteins. The cytoplasmic domains of costimulatory TNFR family members contains TNF receptor-associated factor (TRAF) binding motifs. All of them can induce activation of NF-κB, mitogen activated protein (MAP) 3 kinases or MAP4 kinases. These co-signaling receptors are associated with cellular activation, differentiation, and survival (27).

4-1BB was originally identified as a molecule expressed in both $CD4^+$ and $CD8^+$ activated T-cells. $CD8^+$ T-cells can upregulate 4-1BB more rapidly and to higher levels than $CD4^+$ T-cells (27). Its ligand, 4-1BBL, is expressed on activated macrophages, dendritic cells, and B-cells (APCs). 4-1BB/4-1BBL interaction can promote T-cell expansion and cytokine production, inducing similar levels of IL-2 production as CD-28-mediated co-stimulation if signals through the TCR are strong. 4-1BB/4-1BBL interaction delivers a costimulatory signal mediated through the TRAF1 and TRAF2 trimers, which recruit the ubiquitin ligases, cellular inhibitors of apoptosis 1 or 2 (cIAPI1/2) that activate the downstream signaling pathways: NF-κB, extracellular signal regulated kinase (ERK), p38 MAP kinase (MAPK), and c-Jun N-terminal kinase (JNK) pathways (Figure 5) (11,27,28).

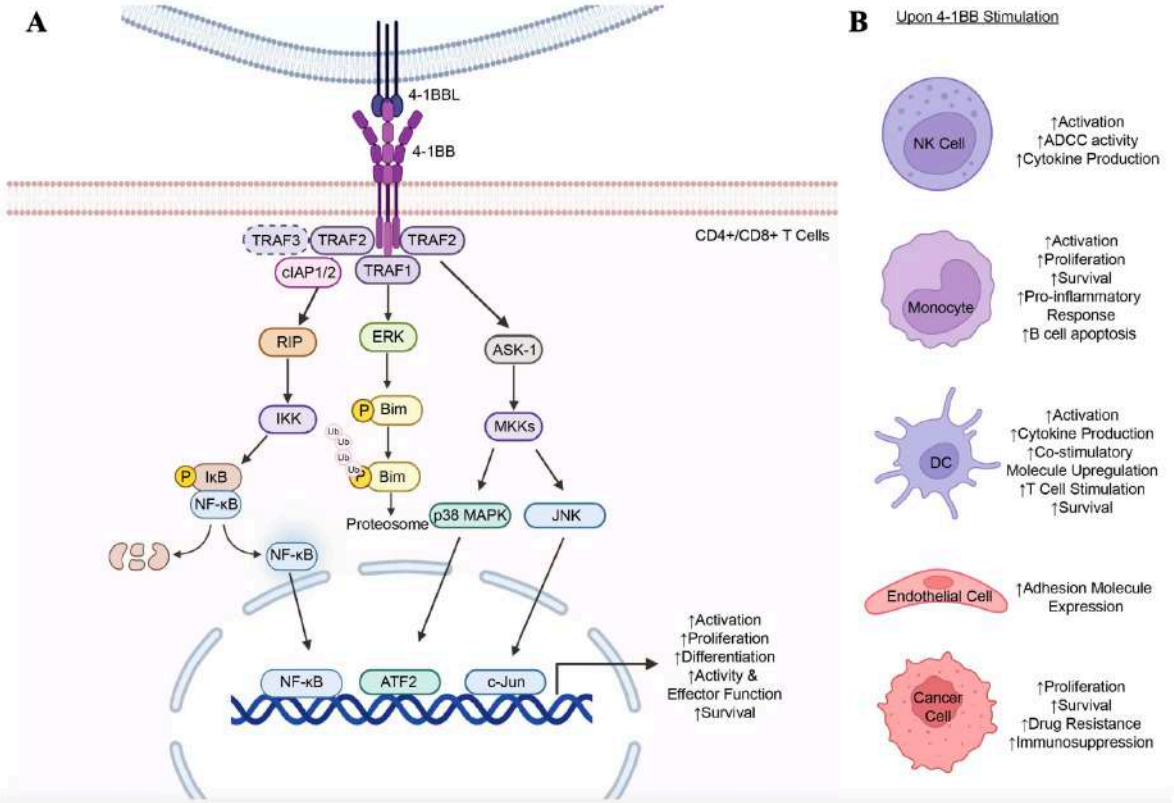

Figure 5: 4-1BB expression and signaling. A. Upon interaction with 4-1BBL, 4-1BB signaling is initially mediated by the recruitment of TRAF1, TRAF2, or, hypothetically, TRAF3 (in dashed lines). The TRAF proteins form a homo- or hetero-trimer and, in turn, recruits cIAP1/2 which further mediates the activation of downstream effectors that transduce signals down various signaling cascades to the nucleus including the NF-kB, ERK, p38 MAPK, and JNK pathways. Signaling down these pathways results in the increased expression of the anti-apoptotic proteins, Bfl-1 and Bcl-xL, decreased expression of the pro-apoptotic protein Bim, and increased proliferation, differentiation, effector functions, and survival of the T-cells. **B.** 4-1BB expression is observed in a wide range of cells including NK cells, monocytes, DCs, endothelial cells, and malignant cancer cells. Stimulation of 4-1BB on the respective cells result in varying cellular responses depending on the cell type. *(Kim A, 2022)*

CD30 (Ki-1 or TNFRSF8) was originally discovered on Hodgkin's lymphoma and Reed-Sternberg (HRS) cells (29), where overexpression of CD30 results in ligand-independent NF-κB activation and contributes to malignancy. CD30 is expressed on a small subset of activated T-cells, a minority population of stimulated B immunoblasts located at the edge of the germinal center and the extrafollicular region, as well as on virally infected T-cells, B-cells, and NK-cells, and on eosinophils (30).

The CD30 molecule is a 120 kD transmembrane glycoprotein receptor, with intracellular, transmembrane, and extracellular domains (30,31). Its 577 amino acid residues can be subdivided into an extracellular part with 361 residues and a cytoplasmatic part with 188

residues (31). The ectodomain is very cysteine-rich (34 cysteines), consists of two domains connected by a linker (31). This hinge provides additional flexibility at this part of the molecule (Figure 6). As many members of the TNFRSF, CD30 expresses a highly glycosylated stalk region that links the transmembrane domain and the membrane-proximal cysteine-rich domain. This stalk is often subject of a proteolytic cleavage resulting in the shedding of membrane-anchored proteins, leading to the release of the soluble ectodomain of CD30 (sCD30) (32). sCD30 is an 88 kD form which can be detected *in vivo* in inflammatory states and CD30-positive hematologic malignancies (33). Elevated levels of sCD30 have been observed in patients with anaplastic large T-cell lymphoma and Hodgkin lymphoma, which correlates with tumor load and confers a poor prognosis (34).

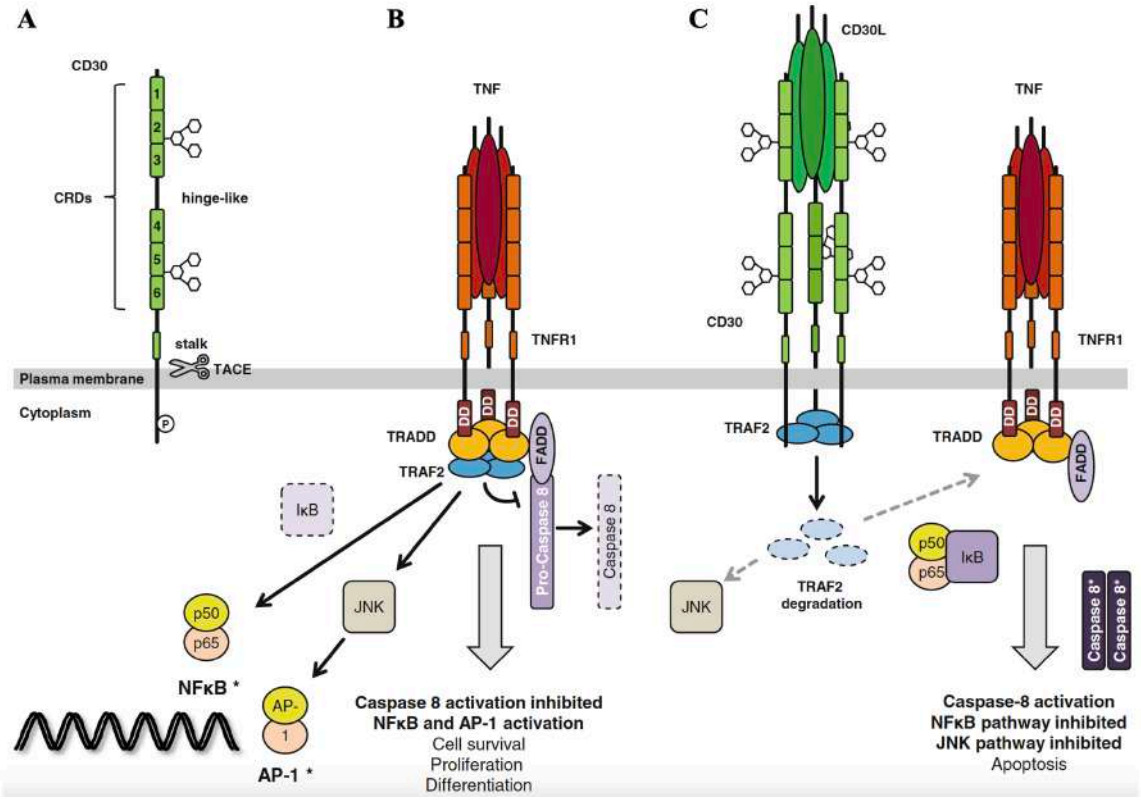

Figure 6: Schematic illustration of CD30 structure and signaling through TRAFs. A. The extracellular domain has six cysteine-rich regions in a duplicated structure. Regions 2 and 5 contain single N-glycosylation motifs and seem to be involved in activation of TNFα converting enzyme (TACE/ADAM17) cleaving CD30 within the membrane proximal extracellular stalk region. The cytoplasmic domain contains TRAF binding sequences and can also be phosphorylated. **B.** CD30 signals through TRAFs. **C.** Upon ligation with its natural ligand, CD30 forms homotrimers which recruit TRAF2 and induce its degradation diverting signaling through TNFR1 to apoptosis. *(Schirrmann T, 2014)*

In T-cells, cytoplasmatic tail of CD30 has been shown to bind TRAF1, TRAF2, TRAF3, and TRAF5 which activate p38-MAPK, c-Jun N-terminal kinase (JNK) and NF-kB transcription factor pathway (34,35). Depending on the cellular context, CD30 signaling can exert very different effects ranging from cell death to cellular proliferation (Figure 6) (34).

The natural ligand CD30L (CD153) is a membrane anchored protein belonging to the TNF family and is expressed on activated T, B, and NK cells, eosinophils, neutrophils, monocytes, and mast cells (34). It has been suggested that interactions between CD30 and its ligand result in bidirectional signaling with messages going to both the receptor and ligand bearing cells (35).

1.4 Immune system and cancer control

In most organs and tissues, as cells die, they are replaced by new cells formed from the proliferation and differentiation of local adult stem cells. Under normal circumstances, the production of new cells is regulated so that the number of any type of cell remains constant. Occasionally, a cell arises that no longer responds to normal growth control mechanisms; it proliferates in an unregulated manner and avoids apoptotic signals, eventually giving rise to cancer (1).

Cancer is characterized by a genomic instability in which numerous point mutations and structural alterations occur in the process of tumor progression. Such genomic variations could give rise to tumor antigens, which could be recognized by the immune system as non-self and elicit cellular immune responses (36). Surveillance for cancer cells is one of the regular maintenance roles of the immune system. Data collected in the past few decades from both animal models and clinical studies have clearly defined a role for the immune response in tumor cell identification and eradication. Both innate and adaptative immune system have been described to play a role in tumor control (1).

Effective immune responses could either eradicate malignant cells or impair their phenotypes and functions. However, cancer cells have evolved multiple mechanisms, such as defects in antigen presentation machinery, the upregulation of negative regulatory pathways, and the recruitment of immunosuppressive cell populations, to escape immune surveillance, resulting in the impeded effector function of immune cells and the abrogation of antitumor immune responses (36).

1.5 Immunotherapy

Immunotherapy is a monumental breakthrough for cancer treatment and has revolutionized the field of haemato-oncology (36). Immunotherapy for cancer refers to treatments designed to enhance the native ability of the immune system to eliminate cancer cells (37). The potential to use immune-based therapies for human malignancies is attractive because of the specificity of antibodies and T-cell recognition (38).

There are several types of immunotherapies: monoclonal antibodies, oncolytic virus therapies, cancer vaccines, cytokines therapies, adoptive cell transfer, and immune checkpoint inhibitors. Although the idea of unleashing the host immune system to eradicate cancer could trace back to a century ago, significant advances have been achieved in recent basic and clinical investigations (Figure 7) (36).

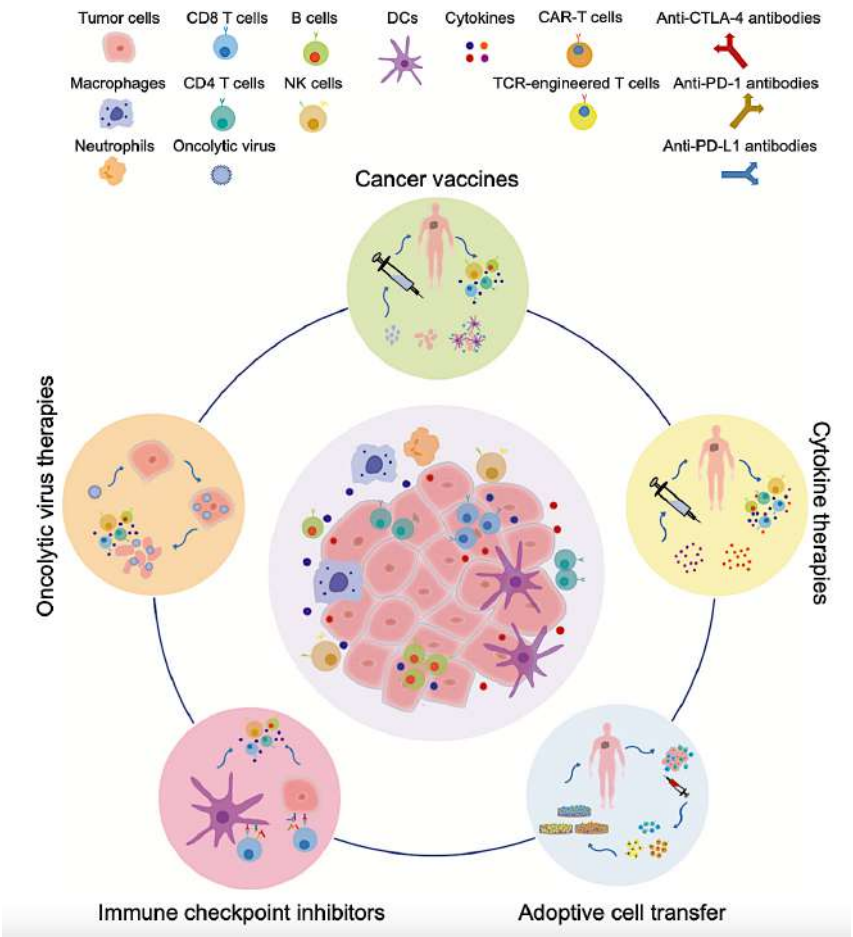

Figure 7: Different forms of cancer immunotherapy, including oncolytic virus therapies, cancer vaccines, cytokine therapies, adoptive cell transfer, and immune checkpoint inhibitors, have evolved and shown promise in clinical practice. The basic principles of each strategy and the corresponding cellular and molecular underpinnings involved in each step are depicted. DCs dendritic cells, NK natural killer, TCR T-cell receptor, CAR-T chimeric antigen receptor T-cell. *(Zhang Y, 2020)*

Adoptive cell transfer (ACT) and immune checkpoint inhibitors (ICIs) have obtained durable clinical responses, but their efficacies vary depending on the tumor, and ultimately, only some groups of cancer patients benefit from them (36).

1.6 Adoptive cell therapy

ACT is a highly personalized cancer therapy that involves the infusion to the cancer-bearing host of immunocompetent cells with direct anticancer activity (39). ACT is a robust form of immunotherapy and, may rely on T-cells harvested directly from the patient (autologous approach) or from healthy donors (allogeneic approach) (40,41).

ACT has several advantages and modalities compared to other approaches to cancer immunotherapy as large numbers of antitumor T-cells can be grown *ex vivo* and selected for high-avidity against the desired antigen, as well as for the effector functions required to mediated cancer regression (39,42).

Initially, ACT sought to sensitize and expand tumor-reactive T-cells *in vivo* and direct isolation of tumor-infiltrating lymphocytes (TILs) has been tested in multiple solid tumor studies with some durable responses, particularly in melanoma (43). In addition, the host can be manipulated prior to the administration of cells to provide a suitable microenvironment in the tumor (39,42). ACT has used either natural host cells that exhibit antitumor reactivity or host cells that have been genetically engineered with antitumor TCRs or chimeric antigen receptors (CARs) (39).

1.6.1 *Tumor infiltrating lymphocytes*

Tumor-infiltrating lymphocytes (TILs) represent a heterogeneous population of lymphocytes that naturally infiltrate solid tumors. In TIL-based ACT, naturally occurring TILs are isolated from a tumor biopsy and then expanded to large numbers *ex vivo* before being given back to the patient to fight the cancer cells (Figure 8). Treatment resistance represents a clinical challenge in TIL-based ACT. The most common cause of innate resistance is the absence of tumor-specific T-cells in the infusion product or the absence of well-presented immunogenic tumor antigens that prevent recognition by T-cells (44).

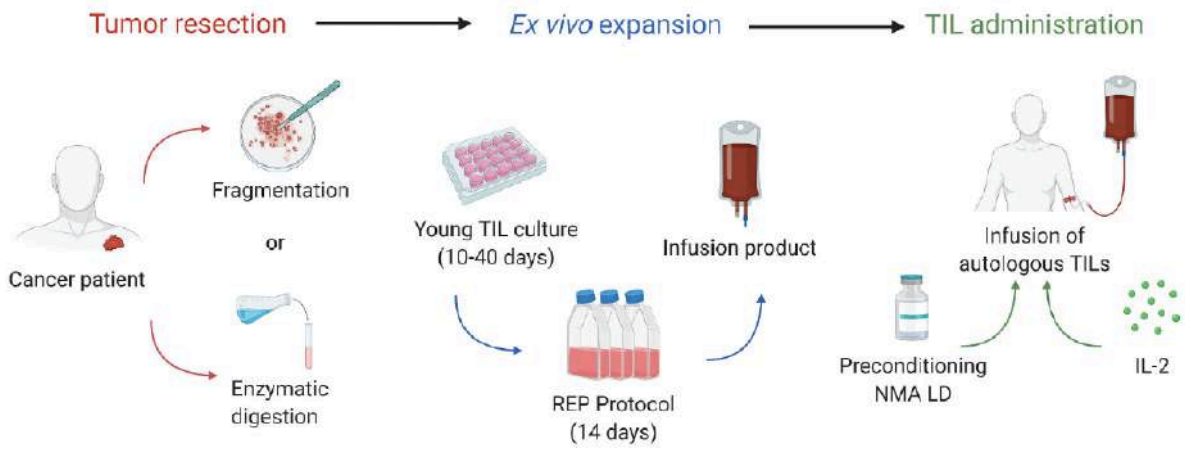

Figure 8. Adoptive cell transfer (ACT) with tumor-infiltrating lymphocytes (TILs). The tumor sample is surgically resected and either mechanically dissected into multiple fragments or enzymatically digested into a single-cell solution. *In vitro* expansion: the tumor sample is used to make the initial culture of young TILs. After 10–40 days, irradiated PBMCs, OKT3 (anti-CD3), and IL-2 are added, and the cells undergo rapid expansion (REP protocol). TIL administration: The patient receives lymphodepletion, TILs, and interleukin-2 (IL-2) infusion. *(J. S. Granhøj, 2022)*

1.6.2 *T-cells modified with antitumor TCR*

The specificity of T-cell recognition is provided by the TCR, and the transfer of an antitumor TCR α and β genes into recipient T-cells can endow the cell with the antigen-specificity of the introduced TCR (Figure 9) (38).

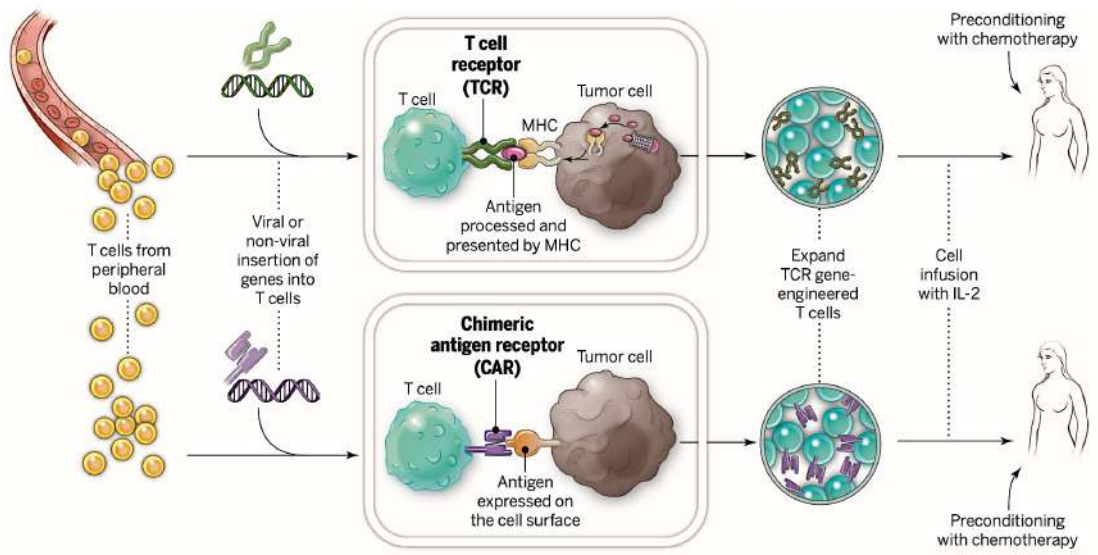

Figure 9. Gene-modification of peripheral blood lymphocytes. The top panel shows the insertion of a conventional TCR into T-cells, followed by the expansion and infusion into the patient. The bottom panel shows the insertion of a CAR into T-cells, the expansion, and their re-infusion. TCRs and CARs are different in their structures and the structures that they recognize. TCRs are composed of one α chain and one β chain, and they recognize antigens that have been processed and presented by one of the patient's own MHC molecules. *(Modified from Rosenberg S, 2015)*

To date, most of the clinical trials have adopted TCRs directed toward target tumor-associated antigens, such as gp100 and Melan-A/MART-1, or cancer germline antigens such as NYESO-1 and MAGE (41).

1.6.3 *T-cells modified with chimeric antigen receptors*

In late 1980s, first-generation chimeric antigen-specific receptors (CARs) were described. Initially, they were constructed by fusing TCR constant domain (segments α or β chains) to the variable region of an antibody, both the heavy chain and the light chain (45,46). Next, recognition domain was simplified into a single-chain peptide structure derived from heavy and light chain variable region of a specific immunoglobulin (scFv). The scFv was fused with ζ chain of the TCR CD3 complex, and thus a first-generation CAR was generated. (Figure 10) CAR T-cells containing CD3ζ demonstrated antitumor activity *ex vivo* but, exhibited limited *in vivo* antitumor effect, anergy, and lack of expansion (46–48).

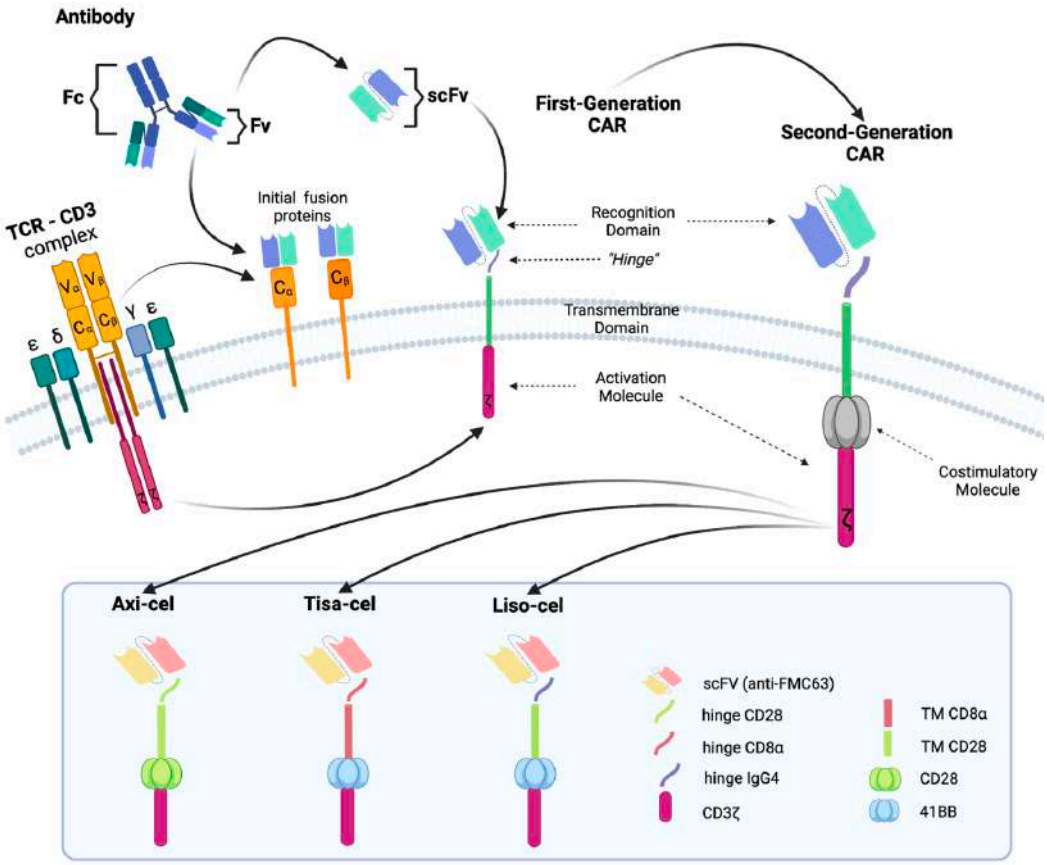

Figure 10. Illustration of the evolution of chimeric receptors design. Fragments from the native TCR-CD3 complex and antibodies were joined into fusion proteins that were improved to reach the basic structure of the second-generation CARs, which were successfully brought to the clinic. At the bottom, the figure shows the critical differences between the three FDA-approved second-generation CAR constructs. *(Caballero AC, 2022)*

In late 1990s, second-generation CARs directed to B-cell antigens were built by adding a co-stimulatory domain (CD28) to the initial first-generation CAR construct (Figure 10). These changes were based on the knowledge of TCR function to optimize *in vivo* expansion of activated T-cells (49). Second-generation CAR directed against CD19 (CAR19) was shown to be effective in B acute lymphoblastic leukemia (B-ALL) animal models (50). Early studies with CAR20 T-cells showed limited efficacy in patients with B-NHL, presumably because they were done with a first-generation CAR (51).

Finally, CAR T-cells moved into the clinical setting when treating a patient with refractory follicular lymphoma who reached partial response (PR) after CART19 therapy (52). Then, a few academic studies published their earliest clinical experiences with CAR19 T-cells paving the way for the development of CART19 clinical trials for patients with B-NHL (53–59).

1.7 Overview of CART19 therapy in refractory B-cell non-Hodgkin lymphomas

B-cell non-Hodgkin lymphomas (B-NHL) comprised some 544,000 new cases/year and caused 260,000 deaths worldwide in 2020 (60). DLBCL is the most common subtype, accounting for 30% to 40% of all newly diagnosed lymphomas in the world. DLBCL has an aggressive behavior and needs rapid treatment (61). First line of treatment is usually composed of repeated cycles of chemoimmunotherapy with rituximab combined with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) that allows to achieve long-term disease remissions in 60% of cases (61,62). Forty percent remains unresponsive, either due to primary refractoriness (10-15%) or relapse after having achieved an initial complete response (20-25%) (63).

Overall survival (OS) in relapsed or refractory (R/R) subgroup patients is poor. High-dose chemotherapy followed by autologous stem cell transplantation (ASCT) is the standard of care (SOC) in this situation for patients considered suitable for that treatment. About a half of R/R DLBCL patients are not eligible for intensive approach due to advanced age or comorbidities. There is no salvage regimen that has proven to be superior with overall response rates (ORR) 40-50% and only ~20% of complete responses (CR) (64). This means that only up to 50% of patients who are potentially candidates for intensive treatment would reach a required response to proceed to ASCT (61,63–65). Among patients who received

ASCT, less than 50% would be disease-free after 5 years (64). Refractory patients, defined as DLBCL that did not achieve objective response or relapsed ≤12 months after treatment, remain the greatest challenge. For these patients, chemotherapy salvage treatments yield ORR around 26% with less than 10% CRs, and their median OS was reported to be as short as 6.3 months in some studies (63). The poor outcomes shown by patients with R/R DLBCL constitutes an unmet medical need and have ACT to change the paradigm of lymphoma treatment.

Clinical trials with CART19 therapy have shown great efficacy in heavily pre-treated patients with DLBCL, high-grade B lymphoma and PMBCL. Outstanding results in academic studies led to the clinical development and subsequent approval by regulatory agencies of 3 different CAR19 T-cell products, which represent the first CART therapies approved for patients with cancer. These studies will be summarized next.

Axicabtagene ciloleucel (Axi-cel, Yescarta) is a CAR19 T-cell product originally designed by researchers at the National Cancer Institute (NCI) and later developed by Kite-Gilead (53,54,66). Autologous peripheral blood mononuclear cells (PBMCs) are genetically modified by γ retroviral vector to express a second-generation CAR with anti-CD19 scFv as recognition domain, CD3 ζ as activation, and CD28 as co-stimulation domain (Figure 10). Axi-cel has been approved to treat patients with R/R DLBCL, PMBCL or DLBCL transformed from follicular lymphoma (FL), R/R to at least 2 previous lines of treatment. The approval derived from the results of ZUMA-1, a phase 1/2 clinical trial that treated 101 patients (77 with DLBCL and 24 with PMBCL) with a single dose of 2×10^6 $CAR19^+$T-cells/kg. All patients received lymphodepletion (LD) regimen with cyclophosphamide (Cy) and fludarabine (Flu) for 3 days before infusion. The median age of treated patients was 58 years (range, 23 to 76). Bridging therapy before CART19 infusion was not allowed. Cytokine release syndrome (CRS) occurred in 93% of patients and 13% of them were grade 3 o higher. Neurologic events (ICANS) occurred in 64% of cases and 23% were grade ≥3. ORR was 82% with 54% CRs. The median duration of response was 11.1 months, and after a median follow-up of 27 months, the two-year progression-free survival (PFS) was 41% and OS was 50% (67).

Tisagenlecleucel (Tisa-cel, Kymriah) is a CAR19 T-cell product derived from studies at the University of Pennsylvania followed by clinical development by Novartis (55–57). T-cells are isolated from autologous cryopreserved leukapheresis, and then transduced with a

lentiviral vector encoding a second-generation CAR co-stimulated with 4-1BB (CD137), targeting CD19 and having CD3 ζ as activation domain (Figure 10). Tisa-cel has been approved for the treatment of R/R DLBCL after at least 2 previous lines. JULIET, a phase 2 clinical trial, was the pivotal study and included 111 patients (88 DLBCL and 21 DLBCL transformed from FL) who were treated with a single infusion of Tisa-cel. The median dose administered was $3x10^8$ $CAR19^+$T-cells (range $0.6 – 6×10^8$). Cy and Flu 25 for 3 days before infusion was also used as LD regimen. The median age of the included patients was 56 years (range, 22 to 76). Most of patients (92%) received bridging therapy before CART19 administration. CRS was observed in 56% of patients, but only 22% of grade >3 was detected. ICANS was less common (21%) and less severe (12% grade >3). ORR was 52% with 40% CR. PFS at 60 months was 31% for all patients (68,69).

Lisocabtagene maraleucel (Liso-cel), is a second-generation CAR19 T-cell product originally designed and formulated by investigators at the Fred Hutchinson Cancer Research Center and then continued by Juno and Bristol-Myers Squibb. To generate liso-cel, $CD4^+$ and $CD8^+$ T-cells are selected and separated into two different fractions to be cultured in parallel. T-cells are genetically modified by lentiviral vector encoding a second-generation CAR19 co-stimulated with 4-1BB (CD137) (Figure 10). The transduced $CD4^+$ and $CD8^+$ T-cell fractions are mixed in a 1:1 ratio to obtain the final formulation (58,59). Liso-cel is approved for the treatment of R/R DLBCL, PMBCL and follicular lymphoma grade 3B (FL3B) after at least two prior therapies. TRANSCEND NHL001 study led to FDA approval for Liso-cel. It was a phase 1/2 clinical trial and included 269 patients (173 DLBCL, 78 DLBCL transformed from indolent lymphoma, and 15 PMBCL). Median age was 63 years (range, 54 to 70). Bridging therapy was administered to 59% of patients. All patients received LD regimen with Cy and Flu for 3 days before infusion. CRS was observed in 42% of cases, but only 2% developed severe CRS (>3 grade). ICANS was detected in 30% of cases and 10% of them were higher than grade 3. Phase 1 sequentially evaluated 3 doses: $50×10^6$ $CAR19^+$ (dose 1), $100 ×10^6$ $CAR19^+$ (dose 2) and $150×10^6$ $CAR19^+$ (dose 3). Dose 2 ($100 ×10^6$ $CAR19^+$) was selected for phase 2 to test efficacy. The ORR was 73% with 53% of CRs. PFS at 12 months was 48% with an OS of 58%, for the overall patient population (70).

Table 1. Pivotal clinical trials with CART19 therapy for B-NHL: comparison of relevant features among studies

Clinical trial	ZUMA-1	JULIET	TRANSCEND
Product name	Axicabtagene ciloleucel	Tisagenlecleucel	Lisocabtagene maraleucel
Co-stimulatory domain	CD28	4-1BB	4-1BB
Leukapheresis	Fresh	Cryopreserved	Fresh
Starting material	PBMCs	Selected T-cells	Sorted $CD4^+$ and $CD8^+$ fractions
Transduction	Retroviral	Lentiviral	Lentiviral
Final composition	Bulk T-cells	Bulk T-cells	Defined ratio $CD4^+$:$CD8^+$ 1:1
Patients enrolled, n	111	165	344
Infused patients, n	101	111	269
Study population	DLBCL PMBCL tFL	DLBCL tFL	DLBCL DLBCL transformed from indolent lymphoma PMBCL FL grade 3B
Bridging therapy (% patients)	Not allowed	Allowed (92%)	Allowed (59%)
Lymphodepleting therapy	Cy 500 mg/m^2/day + Flu 30 mg/m^2/day 3 days	Cy 250 mg/m^2/day + Flu 25 mg/m^2/day 3 days	Cy 500 mg/m^2/day + Flu 30 mg/m^2/day 3 days
Stage III/IV	85%	76%	NR
>3 prior lines	69%	52%	51%
Refractory disease	77%	55%	67%
Previous ASCT	21%	49%	33%
Cell origin			
Germinal center B-cell-like subtype	49%	57%	NR
Activated B-cell-like subtype	17%	41%	NR
High-grade B-cell lymphoma	NR	27%	13%
Dose	2×10^6 CAR T-cells/kg	Median dose: 3×10^8 CAR T-cells/kg (0.1×10^8 - 6×10^8)	1×10^8 CAR T-cells (50×10^6 $CD8^+$ + 50×10^6 $CD4^+$ CAR T-cells)

DLBCL: diffuse large B-cell lymphoma; PMBCL: primary mediastinal B-cell lymphoma; tFL: transformed follicular lymphoma; NR: not reported.

Main differences in relevant features among these pivotal clinical trials are summarized in Table 1. Different primary endpoints, evaluations, and statistical analysis in these studies are added to distinct intrinsic features of the CAR19 T-cell product: starting material, dose, T-cell composition, culture conditions and constructs design. Despite this, surprisingly similar efficacy was seen among all three products with remissions around 50% in this previously incurable patient population. Although CART19 therapy has been successful in treating and probably curing some patients with R/R B-NHL, more than half of them remain refractory or relapse after CART19.

1.8 Factors influencing clinical outcome after CART19 therapy

Preclinical and clinical research has advanced rapidly to identify biological and clinical factors associated with response after CART19 therapy (Figure 11). Biological characteristics of the tumor, in addition to other factors such as T-cell subsets composition, cell features within the "starting material", *in vivo* expansion and persistence of CAR T-cells, have been described as critical determinants of response (46).

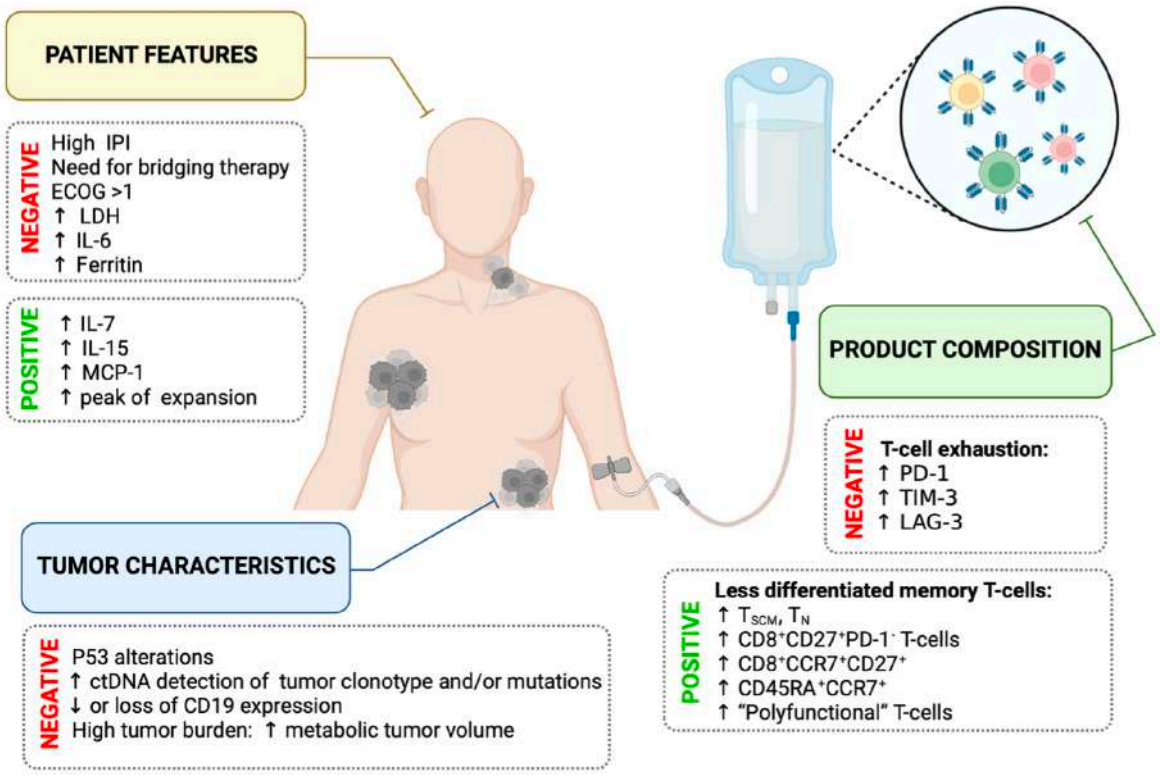

Figure 11. Summary of main factors influencing the efficacy of CART19 therapy. *(Caballero AC, 2022)*

1.8.1 *Patients features influencing CART19 therapy efficacy*

The pivotal studies of Tisa-cel and Axi-cel agreed that there were no clinical covariates predictive of efficacy (67,68). In contrast, Liso-cel pivotal trial, which was the largest and the most variable in terms of diagnoses, showed that duration of response (DOR) and PFS in patients with PMBCL and DLBCL transformed from FL were longer than for other subtypes, and that bridging therapy was associated with lower efficacy among included patients, likely reflecting a selection bias (70). Need for bridging therapy reflects higher

tumor burden or more rapidly progressive disease, and thus emerges as a negative prognostic feature.

Other patient-dependent features, such as age (>60years), ECOG 0/1, and normal lactate dehydrogenase (LDH) at the time of conditioning were also described to be associated to CRs after Axi-cel treatment (71). Performance status (PS) and normal LDH values before CAR19 T-cells infusion were also associated to longer PFS (71–73). Finally, a recent study evaluated international prognostic index (IPI) and age-adjusted IPI, two widely used indices to identify DLBCL patients with higher probability of survival following frontline therapy, found an association with them and PFS after CART19 treatment (74).

1.8.2 *Peak of expansion and persistence of CAR19 T-cells are associated with efficacy*

Higher peak and longer persistence of CAR19 T-cells were shown to determine a better PFS in R/R ALL patients (75). Persistence of CAR19 T-cells evaluated by flow cytometry was found to be longer in patients with DLBCL R/R who achieved response after treatment with Tisa-cel (76). Greater CAR19 T-cell peak and AUC in the first 28 days after infusion have been reported to be associated with objective and durable response in lymphoma patients treated with Axi-cel (77–79). Furthermore, the Liso-cel pivotal clinical trial evidenced a median maximum expansion (Cmax) and AUC significantly higher in responders compared to non-responders (70). Overall, data from different clinical studies demonstrate that a high peak expansion of CAR19 T-cells in peripheral blood correlates with a durable response in B-cell neoplasms (70,77,78,80).

It has been described that LD plays a role in both T-cell kinetics of expansion and clinical outcome in B-cell malignancies. Elimination of regulatory T-cells (Tregs) and improved function of transferred T-cells by increasing the availability of homeostatic cytokines are among the proposed mechanisms that explain the positive impact of LD on the efficacy of adoptive therapy. Interleukin-7 (IL-7) and interleukin-15 (IL-15) have supporting roles in the survival and proliferation of adoptively transferred T-cells and have been proved to be critical to enhance adoptive T-cells antitumor effect *in vivo* (81). Fludarabine combined with cyclophosphamide represents the most frequently LD regimen used and enhances $CD4^+$ and $CD8^+$ proliferation and persistence of 41BB-CAR19 T-cells in R/R ALL patients (75). Furthermore, the intensity of the LD regimen may positively impact on the outcome after

CART therapy, as it has been shown after adoptive therapy of TILs (82). Elevated levels of IL-7 and monocyte chemoattractant protein-1 (MCP-1) were associated with superior expansion and better PFS in patients with B-NHL treated with CART19 and, interestingly, this favorable cytokine profile was found more frequently in those patients receiving higher doses of that combo (73).

1.8.3 *T-cell subsets in the infusion product impact in CART19 efficacy*

As previously described, intrinsic patient characteristics, disease, and conditioning procedures may influence clinical outcome, but importantly, infusion product features may play a critical role in the efficacy of CART therapy. In fact, preclinical studies in xenogeneic animal models of B-cell lymphoma have shown increased antitumor effect of CAR19 T-cells with a defined composition of CD4:CD8 ratios (e.g., ratio 1:1). This concept has been moved to the clinic with the development of clinical trials with CART19 products with a defined 1 CD4:CD8 ratio in patients with DLBCL (70). Clinical efficacy of this strategy in patients with DLBCL seems to be comparable to other CART19 studies (67–69,83).

Furthermore, T-cells exist on a continuum of differentiation states characterized by the gradual acquisition or loss of phenotypic traits, functional properties, and patterns of gene expression (84,85). Following antigenic stimulation, "naïve" T-cells (T_N) proliferate and differentiate into memory T-cells (T_M) and effector T-cells (T_{EF}). T_M lymphocytes play a fundamental role in the immune response against cancer. In humans, the T_M cell population is heterogeneous with respect to phenotype and function but can be divided into 2 groups (86) central memory T-cells (T_{CM}), which express CD45R0, CD62L and CCR7; and effector memory T-cells (T_{EM}) lacking CD62L and CCR7 (Figure 12).

Several studies in animal models of cancer have shown that T_{CM} cells have a superior antitumor effect than differentiated T_{EM} and T_{EF} cells (87). A subtype of memory T-cells, the memory stem T lymphocytes (T_{SCM}), has been identified in humans with distinct gene expression and functional attributes to other T-cell subsets (88,89). These cells represent 2-3% of the entire T-cell population in peripheral blood from healthy individuals.

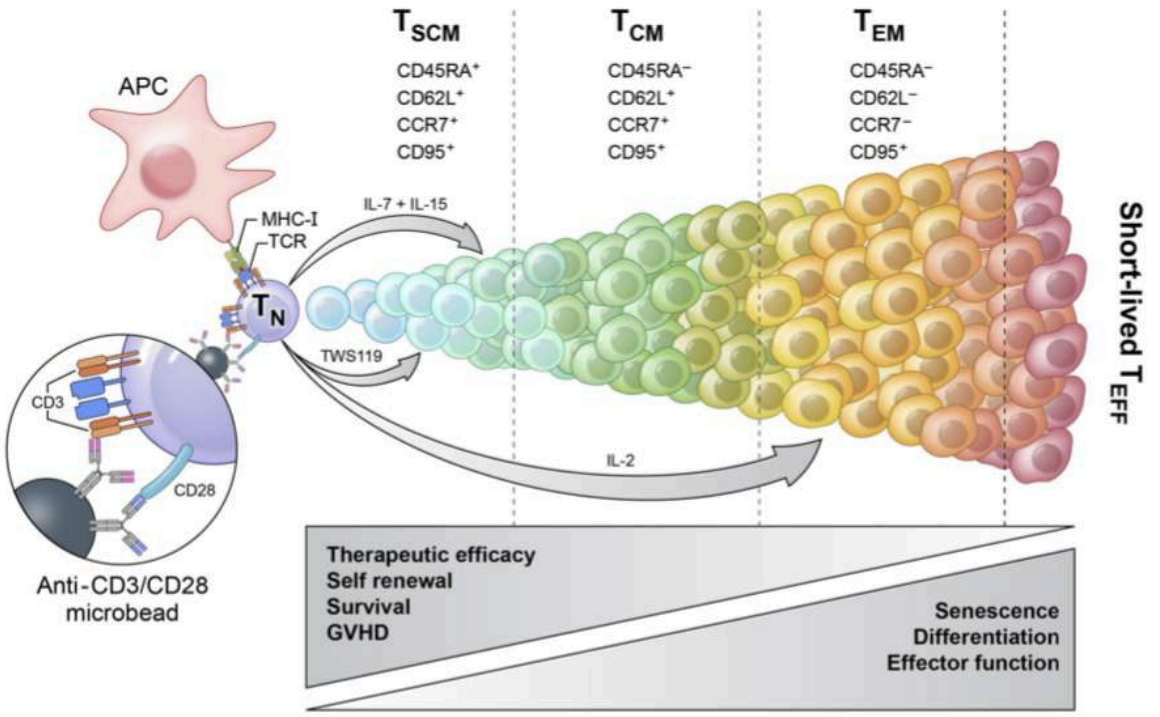

Figure 12. Programming T-cell fates for therapeutic use. After antigen encounter or stimulation with anti-CD3 and anti-CD28 antibody-conjugated microbeads, naive T-cells (T_N) enter a program of proliferation and differentiation that culminates in the generation of terminally differentiated short-lived effector T-cells (T_{EFF}). During this process of maturation, T-cells progressively acquire effector functions but simultaneously lose their capacities for self-renewal and survival, diminishing their therapeutic effectiveness. Cytokines and small molecules can be used to modulate this process and preferentially generate a desired T-cell subset. IL-2 induces effector memory T-cells (T_{EM}), while IL-7 and IL-15 can be used in combination to generate T memory stem cells (T_{SCM}). *(Modified from Gattinoni L, 2013)*

T_{SCM} cells are characterized by expressing naïve and memory markers such as CD45RA, CD62L, and CCR7 but, unlike T_N cells, they express high levels of CD95, CXCR3, CD58, and IL2Rβ (88,90). T_{SCM} cells have the ability to self-renew and to generate all the progeny of memory (T_{CM} and T_{EM}) and effector (T_{EF}) T-cells (89,91). Compared to central memory and effector T-cells, this unique population has a higher proliferative and persistence capacity *in vivo* and a superior antitumor effect in animal models of cancer (92), this attribute also holds true for those T_{SCM} gene-modified with an antigen-specific CAR; thus, adoptive transfers of sorted memory T-cell subsets (e.g., T_{SCM}, T_{CM}, T_{EM}) transduced with a mesothelin-redirected CAR into a xenogeneic model of human mesothelioma demonstrated a superior antitumor effect of the T_{SCM} subset, which was also associated to enhanced proliferative capacity and persistence of CAR T-cells (88,93).

Cellular CART products infused into patients are quite heterogeneous, related to T-cell subset composition as well as exhaustion, these differences may contribute significantly to

the efficacy of the CART19 therapy. Furthermore, patients with DLBCL have substantial differences in their frequencies of peripheral blood T-cell subsets (naïve, T_{CM} or T_{EM}) (58,59), due to factors such as age and chemotherapy regimens received (94), and these variabilities have an impact on the cell quality of the apheresis products used for CAR T-cell manufacturing. Hence, use of appropriate *ex vivo* culture methods that promote enrichment of T_{SCM} and T_{CM} subsets within the CART product would potentially enhance their clinical efficacy.

Indeed, preclinical studies in xenogeneic animal models of B-cell lymphoma have shown that CART19 cells manufactured from $CD4^+$ and $CD8^+$ T_N and T_{CM} cells have improved antitumor effect compared with those CART derived from more differentiated T-cell subsets (58,95). Even more, recent technical advances using culture conditions with IL-7/IL-15 and the addition of IL-21 may enhance the enrichment for T_{SCM} cells in the final CART product (96–98), which can be further increased with the addition of drugs blocking T-cell differentiation, such as glycogen synthase-3 inhibitors (85). Overall, preclinical studies convincingly demonstrated that *in vivo* administration of less-differentiated memory T-cells results in enhanced engraftment, expansion, and persistence which are features required for clinical efficacy of CART therapy for B-cell malignancies (54,68,70,79).

In addition, preliminary clinical data in 14 patients with B-cell lymphoma treated with CAR19 T-cells demonstrate an association between the presence of T_{SCM} (> 5% of total T-cells) in the infusion product and the *in vivo* expansion (99). Moreover, a recent study analyzed for gene expression using a transcriptome profiling approach, 24 infusion products of DLBCL patients treated with Axi-cel and revealed that patients with CRs and durable responses received CART19 products enriched in $CCR7^+$ $CD27^+$ memory $CD8^+$ T-cells compared with those patients with progressive disease (100).

1.8.4 *Antigen expression in tumor cells influences CAR19 T-cell antitumor effect*

Antigen downregulation and antigen escape are among the main causes of relapse in patients with B-cell malignancies after CART19 therapy (101). Initial studies on this mechanism of resistance come from clinical trials of CART19 in patients with B-ALL, in which, up to 30% of the relapsing patients had CD19-negative tumors. Differences in numbers of patients suffering CD19-negative relapses also depend on the co-stimulation of the CAR used (4-

1BB vs. CD28), with preliminary data suggesting a higher frequency in patients receiving 4-1BB-costimulated CARs (102–105).

The impact of the occurrence of CD19-negative relapses in patients with DLBCL after CART19 therapy has been less studied, in part because lymph node biopsies at the time of relapse are difficult to obtain, and the techniques regularly used to analyze for CD19 expression on tumors (e.g., immunohistochemistry) are unreliable. CD19 expression at the time of relapse has not been systematically studied in pharmaceutical-sponsored clinical trials of CART19 for DLBCL patients. Estimations of 20-30% CART19-negative relapses have been documented (101,106) but these numbers may be underestimated since studies on CD19 expression were not done in most relapsed patients.

A recent retrospective multicentric study from the US Lymphoma CAR T-cell Consortium reported a 30% of CD19-negative progression after Axi-cel treatment. Measurement of CD19 expression was assessed by flow cytometry and/or immunohistochemistry, although cutoff for CD19 positivity varied widely between centers (106). Another study revealed that up to 60% of DLBCL patients treated with Axi-cel had tumors at the time of relapse with diminished or CD19-negative expression (107). Interestingly, in this study a correlation was also found between pre-CART therapy density of expression of CD19 antigen on tumor cells, as detected by sensitive flow cytometry, and outcome. Investigators set up a threshold limit for defining low versus high expression of CD19 molecules on tumor cells and showed an increased risk of progression (50%) after Axi-cel therapy in patients with low-expression CD19 DLBCL.

CD19 antigen escape also happened in patients receiving a bi-specific CART. In a small study with an in-house developed tandem CD19/CD22 4-1BB-z CART for DLBCL (n=21), 4 out of 14 patients had CD19-negative tumors at relapsing, while preserving CD22 antigen expression (107).

Mechanisms explaining CD19 escape include antigen loss due to splice variants that specifically lacks the exon containing the CD19 extracellular epitope recognized by all three FDA-approved CART19 used in the clinic (108). Alternatively, CD19 variants that lack the transmembrane domain may also occur and therefore loss of surface expression. Expression of the CAR on the tumor B-cell and interaction with CD19 antigen may result in masking it from recognition by the CAR T-cells and therefore conferring resistance to CART19 (109).

However, this mechanism, described in a single patient with ALL, has not been detected in DLBCL patients so far.

In summary, the efficacy of CART19 therapy and subsequent clinical outcome in DLBCL are influenced by patient's features, tumor characteristics and the composition of the T-cell product.

1.9 Alternative CAR T-cell targets for B-cell malignancies

Most of the clinical trials using CAR-T cells are studies in B-cell malignancies and the most common target is CD19. For a variety of reasons, CD19 expressing blood cancers appear most conducive to CAR-T cell therapy. High levels of tumor expression of the target antigen, ease of physical access to tumor cells through the blood and lymphatics, and the tolerability of the on-target off-tumor effect of B-cell aplasia make CD19 a unique target. However, <5% of all new cancer diagnoses are CD19 expressing malignancies and the situation for the other hematological malignancies and solid tumors remains a long way behind, although an increasing range of potential antigen targets have led to many trials opening to address this gap (110).

The target ought to be a protein, carbohydrate, or glycolipid molecule particularly common in cancer cells. The specificity of the target antigen is essential to prevent toxic effects; the ideal target should be minimally expressed in normal tissue. For the security and efficacy of CAR-T cells, ideal targets should include high levels of malignant cell coverage, specificity, and stability. Indeed, antigens that play a crucial role in disease pathophysiology are more suitable as targets (111).

Alternative surface molecules, including CD20, CD22 and the immunoglobulin light chain are also frequently expressed in B-cell tumors and CARs targeting these alternative lymphoma-associated antigens are currently under development. Another example is B-cell maturation antigen (BCMA), a member of the TNF receptor family is at present the most promising target for multiple myeloma (Figure 13) (112).

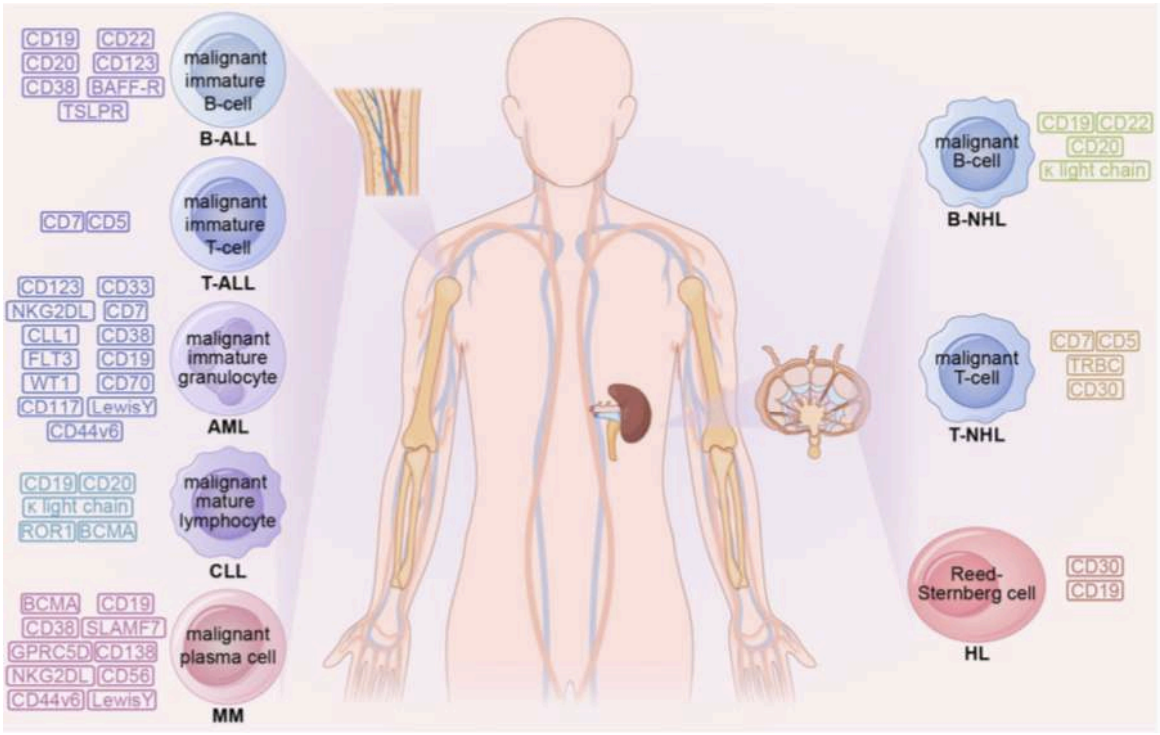

Figure 13. Common CAR T-cell therapy targets in hematological malignancies. B-ALL: B-acute lymphoblastic leukemia; BAFF-R: B-cell activating factor receptor; TSLPR: thymic stromal lymphopoietin receptor; T-ALL: T-acute lymphoblastic leukemia; AML: acute myeloid leukemia; NKG2DL: natural killer group 2 member D ligand; CLL1: C-type lectin like molecule 1; FLT3: FMS-like tyrosine kinase 3; WT1: Wilms tumor 1; CLL: chronic lymphocytic leukemia; ROR: receptor tyrosine kinase like orphan receptor; BCMA: B-cell maturation antigen; MM: multiple myeloma; SLAMF7: signaling lymphocytic activation molecule F7; GPRC5D: G protein-coupled receptor class-C group-5 member-D; B-NHL: B-cell non-Hodgkin's lymphoma; T-NHL: T-cell non-Hodgkin's lymphoma; TRBC: T-cell receptor β-chain constant domains; HL: Hodgkin's lymphoma. *(Qu C, 2022)*

1.10 Classical Hodgkin lymphoma outline

Classical Hodgkin lymphoma (cHL) is one of the most frequent lymphomas in the western world, with an incidence of about 3 new cases per 100,000 individuals per year (113). Depending on clinical stage, risk factors and the treatment given, 60–90% of all patients can be cured from this lymphoid malignancy after multi-agent chemotherapy treatment and improved radiation techniques (114). Yet about 10%–30% of patients develop relapse or refractory cHL and have a much poorer prognosis (especially those 60 years or older and those with early-less than 12 months- relapse), with a reported 3-year PFS rate of 50% and an OS rate of 68% (115). However, severe, life-threatening treatment-related side effects occur, which include organ toxicity and secondary malignancies. Thus, the treatment

approaches must be carefully balanced between optimal disease control and the risk of long-term sequelae (114,115).

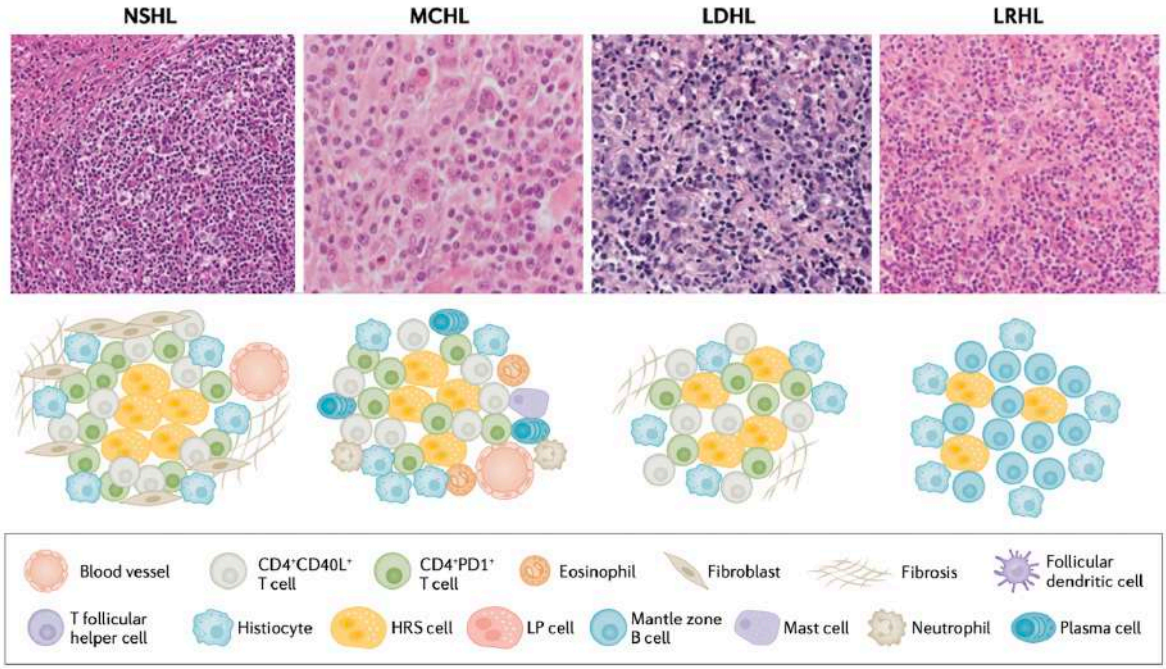

Figure 14. Morphological and cellular characteristics of classical Hodgkin lymphoma. Histology images (top row) and corresponding drawings (bottom row) show the cell types of the tumor microenvironment (TME) of the four subtypes of classic Hodgkin lymphoma (cHL). The TME in cHL demonstrates a variable cellularity that is different in each subtype. In nodular sclerosis Hodgkin lymphoma (NSHL), the TME is specifically characterized by fibroblast-like cells and fibrosis. In mixed cellularity Hodgkin lymphoma (MCHL), the TME consists of a polymorphous reactive infiltrate with B-cells and T-cells, neutrophils, histiocytes, plasma cells and mast cells. In lymphocyte-depleted Hodgkin lymphoma (LDHL), the TME is usually composed of histiocytes and irregular fibrosis. In lymphocyte-rich Hodgkin lymphoma (LRHL), the TME is variable but usually consists of histiocytes and lymphocytes. HRS cell, Hodgkin and Reed–Sternberg cell. *(Modified from Connors J, 2020)*

cHL is typically composed of a low number of malignant Hodgkin and Reed–Sternberg (HRS) cells accompanied by a massive infiltration with nonmalignant reactive cells (Figure 14) (116,117). Through genetic analysis of isolated HRS cells it was clarified that they represent transformed B-cells, because these cells carry immunoglobulin (Ig) heavy and light chain V gene rearrangements, which are specific for B-cells, although most B-cell specific lineage markers are completely lost (113). A high density of CD30 protein is selectively expressed on the surface of HRS cells. In contrast, normal tissues exhibit no or very low expression of CD30, making this antigen a preferred target antigen for cHL immunotherapy (116,118).

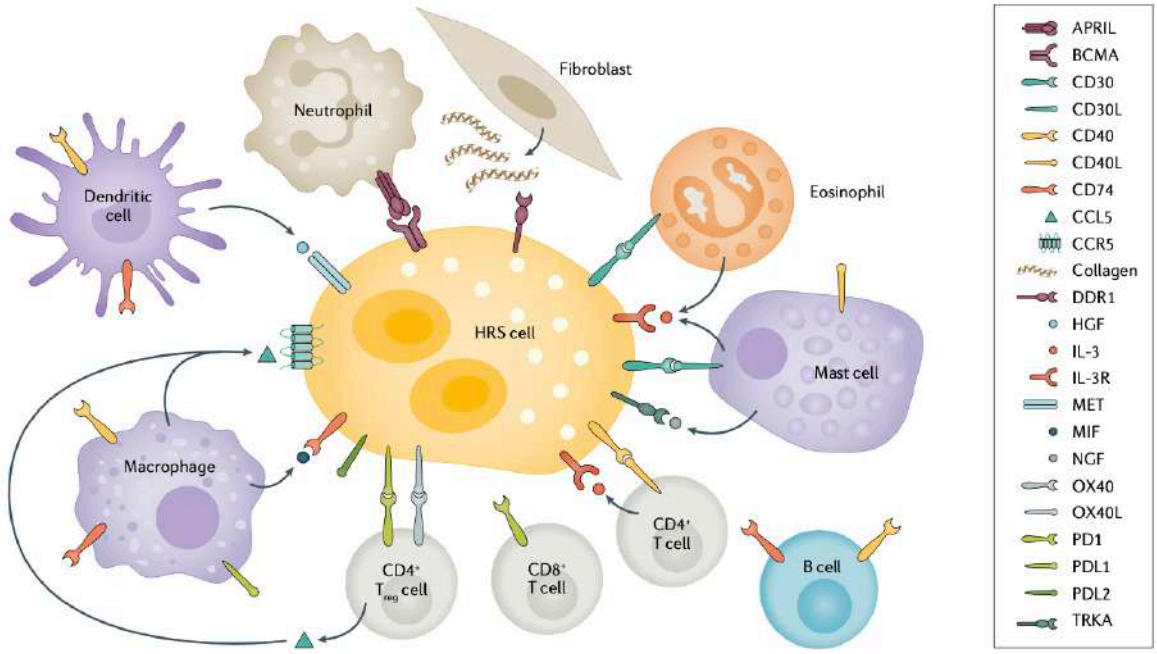

Figure 15. Tumor microenvironment (TME). Reed–Sternberg (HRS) cell surrounded by the inflammatory cellularity of the TME. The inflammatory cell infiltrate produces molecules that bind to proteins expressed on the cell membrane of the HRS cell. The reactive cells of the TME express and release molecules that have a crucial function in the growth and survival of tumor cells. Eosinophils and mast cells, which express CD30 ligand (CD30L), as well as neutrophils, which express a proliferation-inducing ligand (APRIL), are commonly mixed with HRS cells. In addition, $CD4^+$ T-cells, which express CD40 ligand (CD40L), surround HRS cells. These TME cells have a major role in immune evasion as they express PD1. Importantly, a subset of $CD4^+$ regulatory T (Treg) cells interact with HRS cells. Overall, the non-neoplastic cells of the cHL TME interact with HRS cells in a complex ligand–receptor crosstalk. *(Modified from Connors J, 2020)*

CD30 has been explored extensively as a target for antibody-based therapy, including both naked and immuno-conjugated antibodies. The most remarkable results have been achieved with brentuximab vedotin (BV), an antibody drug conjugate directed against CD30, which has indeed shown good tolerance as well as promising activity in $CD30^+$ lymphomas, with an ORR of 75% and CR rate of 34% in patients with relapsed or refractory cHL (119,120). Although BV appears to have excellent responses, these are not usually durable with only 22% of patients with relapsed or refractory cHL being disease-free after 5 years (119,121).

HRS cells express surface molecules that inhibit cytotoxic T-cells and/or NK-cells, including CD95 ligand (CD95L), PD-L1, PD-L2, CD200, HLA-G, and HLA-E. The latter two factors inhibit NK- cells, whereas CD95L can induce apoptosis of $CD95^+$ cytotoxic T-cells. Notably, the immunosuppressive PD-L1 is also expressed by macrophages in the tumor microenvironment, which likely contributes to the suppression of $PD1^+$ immune cells. A

fraction of PD-L1 detected on the surface of macrophages may derive from HRS cells themselves, transferred to macrophages though trogocytosis (113).

Tumor cells expressing PD-L1 and PD-L2 can escape the immune surveillance of mature cytotoxic T-cells via the PD1 pathway, which is one of the most critical mechanisms that leads to cHL (115). Nivolumab and pembrolizumab are monoclonal antibodies, that block signaling through the PD1 pathway, thus releasing the inhibition of T-cells and augmenting antitumor immune responses (122). Among heavily pretreated HL patients, nivolumab treatment showed a high ORR of 87% and a CR rate of 17% (123). Pembrolizumab has shown a median PFS of 13.2 months, an ORR of 65.6% and a CR rate of 25% in R/R cHL patients. However, most of these responses are not durable over time (124).

1.11 Overview of CART therapy in Hodgkin lymphoma

The limited expression of CD30 by normal tissues and its consistent overexpression by cHL have encouraged the development of anti-CD30 CAR T-cell therapy (Table 2). In contrast to the CART studies developed for B-cell lymphoma and myeloma, with dozens of clinical trials conducted, a few studies have been initiated for cHL.

Initial preclinical studies to develop CART therapy directed against CD30 to treat cHL were developed in late 1990s. This first construction included a binding domain, constituted by an anti-CD30 scFv derived from the HRS3 monoclonal antibody, and the signaling domain comprised by the high affinity receptor for immunoglobulin E (FcεRI) γ-chain (116). As previously described, first generation CARs showed some antitumor effect, but failed to expand or persist *in vivo*. (47,48,116)

Then, another first-generation CAR was tested using the same recognition domain but a different activation site (CD3ζ chain). This time, to improve CAR T-cell function, it was proposed to engineer antigen-specific T-cells (Epstein Barr virus – specific T-cells), in order to add co-stimulation from APCs when their native TCR is engaged, and some *in vivo* tumor growth control was observed (125).

Finally, the same research group designed a second generation CAR30. The CAR30 structure contained CD30-binding scFv (HRS3), CD3ζ and CD28 co-stimulation. They evaluated migration and antitumor activity of T-cells genetically modified to express the CAR30 compared to those engineered to express CAR30 and CCR4. Although both showed

temporal tumor growth control *in vivo*, it was higher in the $CAR30^{+}CCR4^{+}$ products, secondary to a potential higher intratumor localization of engineered T-cells (126).

All these preclinical studies resulted in a pioneer phase 1 dose escalation study including 9 patients [6 patients with cHL, 1 patient with DLBCL evolved to cHL, 2 anaplastic large T-cell lymphoma (ALCL) patients] that were infused with autologous T-cells that were gene-modified with a retroviral vector to express a second generation CAR30. The CAR30 construction contained a CD28 costimulatory domain. Three dose levels, from 0.2×10^{8} to 2×10^{8} CAR30-Ts/m^2, were infused without a LD regimen. Two patients with HL and 1 ALCL patient entered CR and 3 had transient stable disease (127).

After this first experience, the same research group conducted 2 parallel phase I/II studies at 2 independent centers involving patients with relapsed or refractory cHL. In this study, they administered CART30 therapy after LD with either bendamustine alone, bendamustine and fludarabine, or cyclophosphamide and fludarabine. Forty-one patients were treated. There were no dose-limiting toxicities associated with CART30 infusion. CRS grade 1 was observed in 24% of patients, no neurotoxicity event was seen. Transient nonpruritic, nontender skin rash was observed in 48% of cases. The ORR in the 32 patients with active disease who received fludarabine-based lymphodepletion was 72%, including 19 patients (59%) with CR. These preliminary data are encouraging and suggest that CART30 therapy may be a promising tool for the treatment of R/R cHL. Although this study shows remarkable responses in heavily treated patients, most of them relapse within the first 2 years with only 20% being disease-free (128).

Another research group from China developed a phase 1 clinical trial that has treated 18 patients with CART30 therapy. This study included 1 patient with primary cutaneous ALCL and 17 with cHL. Autologous PBMCs were modified using a lentiviral vector encoding a second-generation CAR30, co-stimulated with 4-1BB. The recognition domain was composed of a single-stranded fragment (scFv) derived from AJ8786061. All patients received LD therapy but different regimens were used among them. In this study efficacy was low with ORR of 39% and 0% CR rate (118). Taken together, this highlights the need for refined, improved CART30 for patients with refractory cHL.

Table 2. Clinical trials with CART30 therapy for the treatment of CD30$^+$ lymphomas

CLINICAL TRIAL NCT	CAR DESIGN	LD REGIMEN	DOSE	INSTITUTION
NCT04653649	CAR30-4-1BBz	Benda/Flu; Cy /Flu:	2×10^6 CAR$^+$T-cells/kg 1×10^7 CAR$^+$T-cells/kg	H. Sant Pau (Spain)
NCT02690545	CAR30-CD28z	Benda/Flu	2×10^7 CAR$^+$T-cells /m^2 1 - 2×10^8 CAR$^+$T-cells/m^2	U. North Carolina. (US)
NCT02690545	CAR30-CD28z	Cy / Flu:	2×10^7 CAR$^+$T-cells/m^2 1-2×10^8 CAR$^+$T-cells/m^2	Baylor College. Houston. (US)
NCT03049449	CAR30-CD28z	Cy / Flu:	$0{,}3\times10^6$ - 2×10^7 CAR$^+$T-cells/kg	National Cancer Institute. (US)
NCT02259556	CAR30-4-1BBz	Cy / Flu Gemcitabine Paclitaxel	1 - 3×10^7 CAR$^+$T-cells/kg	Pla Chinese Hospital. (China)
NCT03602157	CAR30-CCR4-CD28z	Benda/Flu	2×10^7 - $1\times10^{8\cdot}$ CAR$^+$T-cells/m^2	U. North Carolina. (US)

LD: lymphodepletion, Benda/Flu: Bendamustine/Fludarabine; Cy / Flu: Cyclophosphamide/Fludarabine.

2 HYPOTHESIS

We have hypothesized that the CAR T-cell products characteristics would have a critical impact on the efficacy of CART30 therapy, and that the association of favorable attributes such as proper CAR design, optimal T-cell composition, and CAR expression in T-cells would contribute to enhance antitumor efficacy.

3 OBJECTIVES

3.1 Main objective

To develop a novel adoptive T-cell product highly enriched for memory "stem" T-cells genetically modified to express a second-generation chimeric antigen receptor directed against an epitope of the membrane-proximal CD30 protein and co-stimulated with 4-1BB for the treatment of classical Hodgkin lymphoma.

3.2 Secondary objectives

- To generate products enriched for memory "stem" T-cells engineered with our anti-CD30 chimeric antigen receptor (CAR30) and then demonstrate their *ex vivo* cytotoxic activity against human Hodgkin lymphoma tumor cell lines, proving that their antitumor effect is not impaired by the presence of soluble CD30 protein.

- To assess the *in vivo* antitumor efficacy of our CAR30 T-cell products enriched for memory "stem" T-cells in a Hodgkin lymphoma model and to compare their antitumor effect with CAR30 T-cell products composed mostly of effector T-cells.

- To evaluate whether CAR expression level has an impact on CAR30 T-cell composition, T-cells activation, and inhibition profile and, most importantly, *ex vivo* antitumor effect a against human Hodgkin lymphoma tumor cell lines.

- To determine if the intensity of CAR expression influences the antitumor efficacy *in vivo* by comparing two CAR30 products with different intensity of CAR expression in a Hodgkin lymphoma model.

4 COMPENDIUM OF PUBLICATIONS

4.1 First research article

Memory stem T-cells modified with a redesigned CD30-chimeric antigen receptor show an enhanced antitumor effect in Hodgkin lymphoma

*Carmen Alvarez-Fernández[1,2,3†], Laura Escribà-Garcia[1,2,3†], **AC Caballero[1,2,3,4]**, Eva Escudero-López[1,2,3,4], Cristina Ujaldón-Miró[1,2,3,4], Rosanna Montserrat-Torres[1,2,3], Paula Pujol-Fernández[1,2,3], Jorge Sierra[1,3,4] & Javier Briones[1,2,3,4]*

[†]Equal first-author contributors

[1]Hematology Service, Hospital de la Santa Creu y Sant Pau, Barcelona, Spain

[2]Laboratory of Experimental Hematology-IIB, Institut Recerca Hospital de la Santa Creu y Sant Pau, Barcelona, Spain

[3]Jose Carreras Leukemia Research Institute, Barcelona, Spain

[4]Department of Medicine, Autonomous University of Barcelona, Barcelona, Spain

Clin Transl Immunology. 2021 Apr 29;10(4):e1268.

DOI: 10.1002/cti2.1268

Clinical & Translational Immunology

Clinical & Translational Immunology 2021; e1268. doi: 10.1002/cti2.1268
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ORIGINAL ARTICLE

Memory stem T cells modified with a redesigned CD30-chimeric antigen receptor show an enhanced antitumor effect in Hodgkin lymphoma

Carmen Alvarez-Fernández[1,2,3†], Laura Escribà-Garcia[1,2,3†], AC Caballero[1,2,3,4], Eva Escudero-López[1,2,3,4], Cristina Ujaldón-Miró[1,2,3,4], Rosanna Montserrat-Torres[1,2,3], Paula Pujol-Fernández[1,2,3], Jorge Sierra[1,3,4] & Javier Briones[1,2,3,4]

[1]Hematology Service, Hospital de la Santa Creu y Sant Pau, Barcelona, Spain
[2]Laboratory of Experimental Hematology-IIB, Institut Recerca Hospital de la Santa Creu y Sant Pau, Barcelona, Spain
[3]José Carreras Leukemia Research Institute, Barcelona, Spain
[4]Autonomous University of Barcelona, Barcelona, Spain

Correspondence
J Briones or C Alvarez-Fernández, Hematology Service, Hospital Santa Creu y Sant Pau, Mas Casanovas 90, 08041 Barcelona, Spain.
E-mails: jbriones@santpau.cat (JB) or calvarezf@santpau.cat (CAF)

†Equal contributors.

Received 4 June 2020;
Revised 18 December and 4 March 2020;
Accepted 4 March 2021

doi: 10.1002/cti2.1268

Clinical & Translational Immunology 2021; 10: e1268

Abstract

Objectives. Adoptive cell therapy (ACT) with mature T cells modified with a chimeric antigen receptor has demonstrated improved outcome for B-cell malignancies. However, its application for others such as Hodgkin lymphoma remains a clinical challenge. CD30 antigen, expressed in Hodgkin lymphoma cells, is absent in most healthy tissues, representing an ideal target of ACT for this disease. Despite that, efficacy of CD30-chimeric antigen receptor (CAR) T cells for Hodgkin lymphoma remains modest. Here, we have developed and tested a novel CD30-CAR T to improve efficacy of CD30-CAR therapy, using a targeting epitope within the non-cleavable part of CD30 receptor, and memory stem T cells (T_{SCM}) to improve engraftment, persistence and antitumor activity. **Methods.** $T_{SCM\text{-}like}$ cultures were generated and expanded *ex vivo* and transduced at day 1 or 2 with a lentiviral vector encoding the CD30-CAR. Therapeutic *in vivo* experiments were performed using NSG mice injected with L540 (sc) or L428 (iv) and treated with CD30-CAR T cells when the tumor was established. **Results.** CD30-CAR $T_{SCM\text{-}like}$ cells generated and expanded *ex vivo*, despite CD30 expression and fratricide killing of $CD30^+$ CAR T cells, were not impaired by soluble CD30 and completely eradicated Hodgkin lymphoma *in vivo*, showing high persistence and long-lasting immunity. In addition, highly enriched CD30-CAR $T_{SCM\text{-}like}$ products confer a survival advantage *in vivo*, in contrast to more differentiated CAR T cells, with higher tumor infiltration and enhanced antitumor effect. **Conclusion.** This study supports the use of a refined CD30-CAR T cells with highly enriched $T_{SCM\text{-}like}$ products to improve clinical efficacy of CAR T for Hodgkin lymphoma.

Keywords: chimeric antigen receptor, immunotherapy, memory stem T cells

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INTRODUCTION

T cells expressing chimeric antigen receptors (CARs) have revolutionised the field of ACT for cancer in the last few years.[1] CAR technology allows engineered T cells to recognise tumor-associated antigens (TAA) with high specificity in a MHC-independent manner, improving T-cell proliferation and antitumor efficacy.[2,3] A number of clinical trials with CD19-CAR-modified T cells (CAR19) have developed in the last years for B-cell malignancies with high rates of complete remission and a significant proportion of patients being long-term progression-free.[4–7] However, the application of CAR T-cell therapy to other haematological tumors such as Hodgkin lymphoma (HL) resulted in lower remission rates, and thus, it remains a clinical challenge.

CD30 protein, a member of the tumor necrosis factor receptor family, is highly expressed by HL malignant cells,[8] while its expression is highly restricted in normal cells (i.e. expressed in eosinophils and some subtypes of activated T and B cells), making this receptor a very convenient target for Hodgkin and other $CD30^+$ lymphomas.

Two reported clinical trials using CD30-CAR T cells against HL have demonstrated that CAR T-cell infusion is well tolerated, with no relevant toxicities.[9,10] However, although clinical results in those heavily treated patients are promising, responses are modest, and in contrast to what is seen in patients treated with a CAR19, very few patients achieve long-term remission.[10]

Although the mechanisms explaining this modest clinical efficacy are not well understood, previous studies suggest that factors such as membrane location of the targeted epitope and persistence of CAR T cells in treated patients are critical points for a successful CAR T-cell therapy.[9,10] In addition, the CD30 single-chain variable fragment (scFv) used in those studies could be blocked by soluble CD30 protein (found at high concentrations in most of HL patients), which may significantly limit CD30-CAR efficacy.

In addition, clinical trials with CAR T cells have used mostly T-cell products with a differentiated phenotype [effector memory T cells (T_{EM}) and terminally differentiated effector T cells (T_{EMRA})], which leads to a poor persistence of T cells *in vivo*.[6,9] Previous studies of ACT in mice[11] and non-human primates[12] showed that effector T cells (T_{EFF}) have a robust cytotoxic capacity, but only less differentiated T-cell subsets, such as naïve, central memory (T_{CM}) and the recently described memory stem T cells (T_{SCM}),[13] are capable to generate the complete T-cell repertoire, displaying *in vivo* long persistence, greater proliferative capacity and enhanced antitumor efficacy than T_{EFF}. T_{SCM}, a particular subpopulation characterised by the high expression of CD45RA, CD62L, CCR7 and CD95, had brought attention through its association with clinical efficacy of CAR therapy.[13–15]

The clinical significance of this T-cell subset has been shown recently in patients receiving CAR19 cells in whom *in vivo* expansion of CAR T cells and clinical responses have been correlated with the frequency, within the infused product, of a T-cell subset with a phenotype closely related to T_{SCM}.[16,17]

Here, we have developed a novel CAR targeting CD30 with the aim of improving the antitumor efficacy of CD30-CAR therapy. Design novelties include the use of a scFv targeting a membrane-proximal epitope to maximise the CAR therapy antitumor effect and the interaction of the CD30-CAR with a protein domain localised in the stalk, non-cleavable region of the CD30 molecule, to avoid the potential blockade by the soluble CD30 protein.[18,19] In addition, we generated highly enriched modified CD30-CAR $T_{SCM\text{-}like}$ products to take advantage of their intrinsic potential for durable engraftment, *in vivo* proliferation and tumor homing in order to improve the CAR therapeutic efficacy.[10,13,14] Our work demonstrates that CD30-CAR-modified $T_{SCM\text{-}like}$ cells can be efficiently transduced and expanded *ex vivo* despite CD30 protein expression. Importantly, we show that, despite the presence of soluble CD30 protein, our CD30-CAR $T_{SCM\text{-}like}$ cells show enhanced antitumor activity against HL, tumor homing and persistence *in vivo*, compared with cell products with more differentiated CD30-CAR T cells. These data may significantly contribute to the design of improved CD30-CAR cell therapies for patients with HL and other $CD30^+$ lymphoid malignancies, which may translate into better clinical outcomes.

RESULTS

Generation of $T_{SCM\text{-}like}$ cells modified with a novel CD30-CAR

To generate CD30-CAR $T_{SCM\text{-}like}$ enriched cells, isolated $CD8^+$ and $CD4^+$ naïve T cells from healthy donors were cultured with low doses of IL-7, IL-15

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and IL-21, and activated with a short CD3/CD28 costimulation. $T_{SCM\text{-}like}$ cells were the most prevalent T-cell subset at day 10 of culture (62.4 ± 6.49% $CD4^+$ T cells, 67.29 ± 8.62% of $CD8^+$ T cells; Figure 1a) and were efficiently transduced with a third-generation CD30-CAR lentiviral vector, showing a CD30-CAR expression of 87.03 ± 1.69% in bulk T cells (Figure 1b) and 94.3 ± 1.34% in $CD4^+$ and 75.3 ± 2.14% in $CD8^+$ $T_{SCM\text{-}like}$ cells, respectively (Supplementary figure 1).

A concern with a CAR targeting CD30 protein is the potential elimination of activated $CD30^+$ T cells by CD30-CAR T cells (i.e. 'fratricide killing'). In this regard, we detected a significant increase in the proportion of $CD30^+$ T cells during the culture peaking at day 4 compared with untransduced (UN) T cells (38.86 ± 1.92% vs 6.8 ± 3.24 in $CD4^+$ T cells; and 40.92 ± 3.69% vs 11.92 ± 4.87 in $CD8^+$ T cells, CD30-CAR T cells and UN cells, respectively) that further decrease at the end of the culture (3.9 ± 0.89% vs 0.18 ± 0.27 in $CD4^+$ T cells; and 6.65 ± 2.26% vs 0.20 ± 0.26% in $CD8^+$ T cells, CD30-CAR T cells and UN cells, respectively; Figure 1c). Interestingly, the decrease in the proportion of $CD30^+$ T cells in transduced T-cell culture was correlated with an increase in T-cell viability from 78.95 ± 0.83% at day 4 to 88.55 ± 0.38% at the end of the culture (Figure 1d), while UN T-cell viability was not affected during cell culture (Figure 1d), suggesting a possible elimination of $CD30^+$ T cells by CD30-CAR T cells.

Moreover, the fold expansion of transduced T cells is significantly lower than that of non-transduced T cells, which could be also related to fratricide killing.

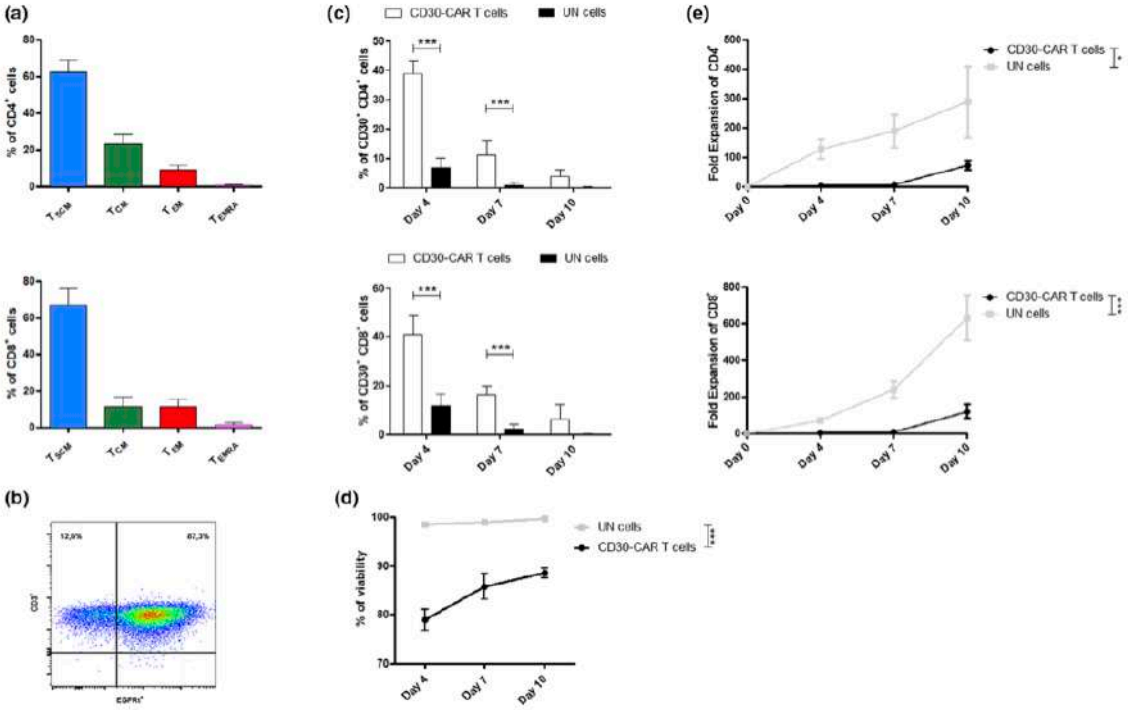

Figure 1. $T_{SCM\text{-}like}$ are highly enriched, CD30-CAR-transduced and expanded *in vitro* despite CD30 expression. Naïve T cells from healthy donors (n = 7) were cultured with CD3/CD28 costimulation in the presence of IL-7, IL-15 and IL-21 during 10 days. **(a)** Frequencies of $CD4^+$ and $CD8^+$ T-cell subpopulations at the end of culture. $T_{SCM\text{-}like}$ cells were the most prevalent T-cell population (mean ± SD). **(b)** Representative plot of T cells transduced at day 2 with CD30-CAR-encoding lentivirus and CD30-CAR expression and analysed by flow cytometry using an anti-CD3 and anti-EGFRt antibodies. **(c)** Expression of CD30 receptor on $CD4^+$ and $CD8^+$ T-cell subpopulations in untransduced cells (white bars) and transduced cells (black bars) during the culture (days 4, 7 and 10; mean ± SD). **(d)** Percentage of T-cell viability in untransduced cells (grey line) and transduced cells (black line) at days 4, 7 and 10 of culture (mean ± SD). **(e)** Fold expansion of $CD4^+$ and $CD8^+$ T cells during 10-day culture in untransduced cells (grey line) and transduced cells (black line; mean ± SD). Data are representative of seven independently repeated experiments.

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Despite the transient CD30 protein expression and a significant decrease in fold expansion compared with UN cells, CD30-CAR $T_{SCM\text{-}like}$ cells could be efficiently expanded *ex vivo* (day 10: 63.16 ± 25.66 vs 289.5 ± 120.81 fold expansion of $CD4^+$ T cells; and 101.98 ± 50.69 vs 632.52 ± 125.85 fold expansion of $CD8^+$ T cells, CD30-CAR T cells and UN cells, respectively; Figure 1e).

Enriched CD30-CAR $T_{SCM\text{-}like}$ cells show *in vitro* antitumor efficacy and persistence against HL that is not blocked by soluble CD30

Next, we evaluated the antitumor capacity of CD30-CAR $T_{SCM\text{-}like}$ enriched T cells using two different HL tumor cell lines, L428 and L540. CD30-CAR $T_{SCM\text{-}like}$ enriched cells showed a potent and specific cytolytic activity against both $CD30^+$ HL lines (tumor cell death at 5:1 E:T ratio; 89.92 ± 5.95% vs 0% with control $T_{SCM\text{-}like}$ cells in L540, and 71.11 ± 8.91% vs 0% with control $T_{SCM\text{-}like}$ cells in L428), while $CD30^-$ target cells were not killed (Figure 2a).

Hodgkin lymphoma patients present high amounts of soluble CD30 protein (sCD30) in serum, which can potentially abrogate the antitumor activity of CAR T cells because of CAR blockade.[10] We have observed that both HL lines secrete high amounts of CD30 protein to extracellular environment (1790 ± 50.2 pg mL^{-1} for L428 and 2388.75 ± 137.53 pg mL^{-1} for L540; Supplementary figure 2).

To simulate a scenario with comparable amounts of sCD30 protein found in advanced HL patients,[10] a cytotoxicity assay was performed in the presence of saturating concentrations of recombinant sCD30 protein (20 μg). In spite of that, we observed that the efficacy of our CD30-CAR T cells was not compromised and killed $CD30^+$ tumor cells effectively (Figure 2b).

Moreover, we measured a number of cytokines released by CD30-CAR $T_{SCM\text{-}like}$ enriched cells after 24-h co-culture with $CD30^+$ HL lines and the $CD30^-$ Raji cell line. After exposure to CD30 antigen, CD30-CAR $T_{SCM\text{-}like}$ enriched cells secreted high amounts of INF-γ (48 437.5 ± 1654.63 pg mL^{-1} for L540 and 9052.39 ± 1889.16 pg mL^{-1} for L428 vs 1193.75 ± 8.84 pg mL^{-1} for Raji), TNF-α (1709.16 ± 34.17 pg mL^{-1} for L540 and 1353.33 ± 580.31 pg mL^{-1} for L428) and IL-2 (2833.67 ± 367.98 pg mL^{-1} for L540 and 2232.78 ± 977.33 pg mL^{-1} for L428 vs 315 ± 162.63 pg mL^{-1} for Raji). Interestingly, low amounts of secreted IL-6 (178.45 ± 82.1 pg mL^{-1} for L540 and 565.17 ± 156.58 pg mL^{-1} for L428 vs 130.38 ± 13.6 pg mL^{-1} for Raji) and IL-10 (134.71 ± 34.95 pg mL^{-1} for L540 and 122.18 ± 20.47 pg mL^{-1} for L428 vs 5.99 ± 2.11 pg mL^{-1} for Raji) were detected in these same conditions (Figure 2c).

It has been reported that the ability of CAR T cells to maintain functionality in the context of high tumor burden and repeated stimulation is critical for their therapeutic efficacy. To evaluate functional persistence of CD30-CAR, $T_{SCM\text{-}like}$ cells were repeatedly stimulated with $CD30^+$ tumor cells and different functional aspects were analysed.

Upon re-exposure to $CD30^+$ target cells, CD30-CAR $T_{SCM\text{-}like}$ enriched T cells maintained their functionality with a potent and specific cytolytic activity against L540 (tumor cell death at 5:1 E:T ratio; 88.75% after three antigen exposition vs 0% with UN cells; Figure 3a). Interestingly, a significant increase in CAR expression was observed within 24 h of co-incubation with $CD30^+$ target cells (2959.5 ± 702.1 vs 5982 ± 871.6 MFI, prior exposure and after 24 h, respectively). However, this increase declined 72 h after antigen exposition, reaching similar MFI levels to pre-exposition, which were stable after subsequent antigen encounter (Figure 3b). Although an increase in $PD1^+TIM3^+$ cells was observed after the first co-culture with $CD30^+$ target cells (Figure 3c; 15.35 ± 1.48% vs 32.75 ± 5.58% of $PD1^+$-$TIM3^+$ cells, pre-exposition and 24 h post-exposition, respectively), a significant decrease was observed after 72 h. The low frequency of $PD1^+TIM3^+$ cells and the maintenance of CAR functionality after successive re-expositions suggest that the increase in $PD1^+$-$TIM3^+$ cells observed after the first antigen encounter was related to activation rather than exhaustion (Figure 3c; 32.75 ± 5.58% vs 3.01 ± 2.11% of $PD1^+$-$TIM3^+$ cells, $P < 0.006$, 24 and 72 h post-exposure, respectively).

Enriched CD30-CAR $T_{SCM\text{-}like}$ cells exhibited a potent antitumor effect *in vivo* against HL, enhanced persistence and long-term immunity

After showing that CD30-CAR $T_{SCM\text{-}like}$ enriched cells have significant *in vitro* cytotoxicity against HL, we evaluated their therapeutic potential *in vivo* using two different HL models (iv L428 and sc L540). Mice ($n = 4$ per group) were treated with

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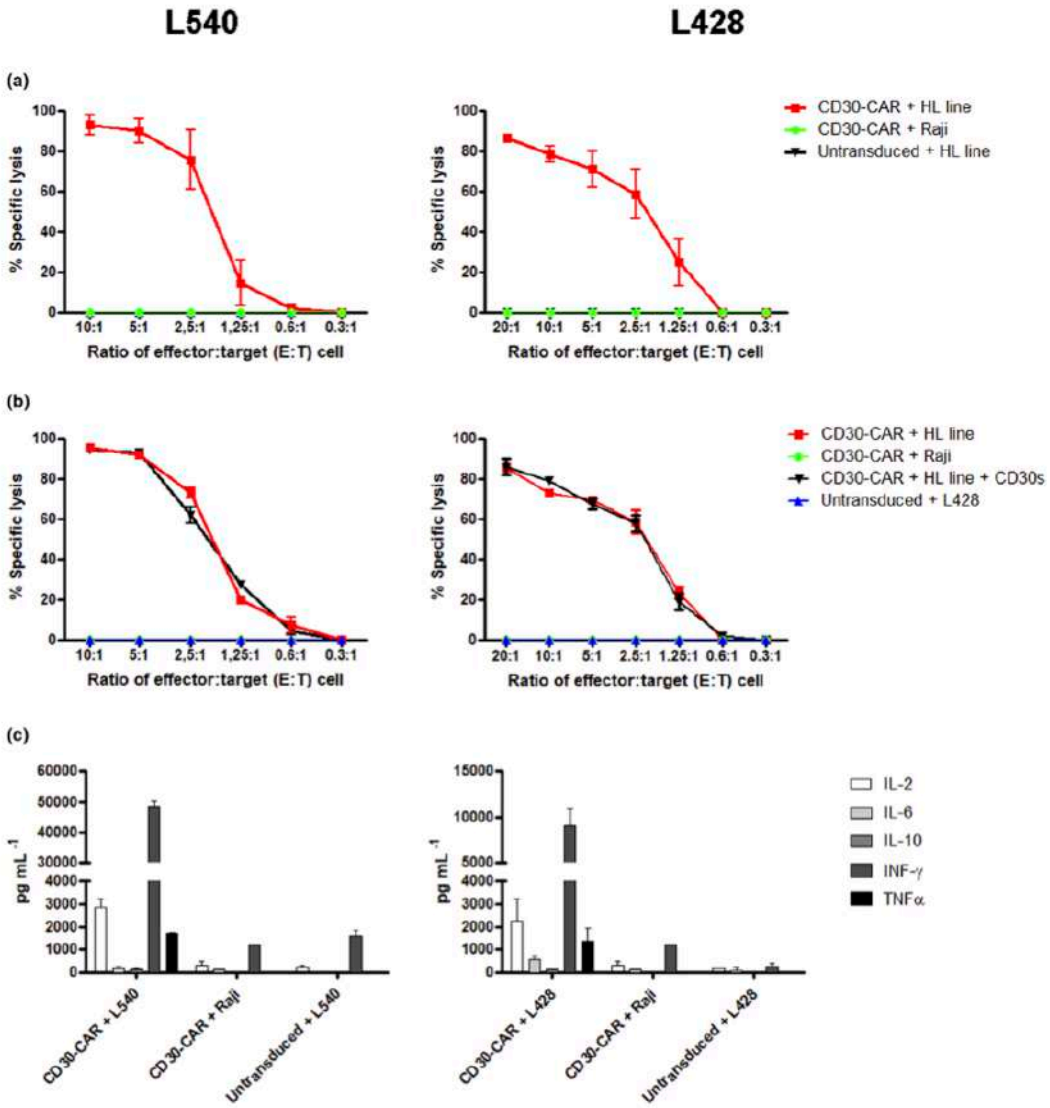

Figure 2. CD30-CAR $T_{SCM\text{-}like}$ enriched culture efficiently eradicates HL *in vitro*. **(a)** $T_{SCM\text{-}like}$ enriched cells expressing CD30-CAR ($n = 7$) were exposed to $CD30^+$ target cells (L540 or L428 tumor cells) and control $CD30^-$ cell line (Raji) at different effector:target (E:T) ratios. Specific cytolytic activity was measured at 24 h by bioluminescence assay. Untransduced $T_{SCM\text{-}like}$ cells were used as negative control (mean ± SD). **(b)** Cytolytic capacity of untransduced and CD30-CAR $T_{SCM\text{-}like}$ cells ($n = 3$) was measured against $CD30^+$ target cells (L540 and L428) at 24 h, in the presence or absence of saturated concentration of soluble CD30 protein (sCD30; 20 μg), at different E:T ratios. Raji cell line ($CD30^-$) was used as negative target control (mean ± SD). **(c)** Cytokine secretion of CD30-CAR $T_{SCM\text{-}like}$ enriched culture ($n = 3$) 24 h after L540, L428 and Raji co-culture. Untransduced $T_{SCM\text{-}like}$ cells were used as negative control. Cytokine levels of IL-2, IL-6, IL-10, IFN-γ and TNF-α were measured by cytometry-based multiplex analysis (mean ± SD). Data are representative of seven **(a)** or three **(b, c)** independently repeated experiments. For each donor, technical duplicate was used **(c)**.

two different iv doses of CD30-CAR $T_{SCM\text{-}like}$ enriched cells (low dose: 5×10^6 and high dose: 10×10^6). The treatment with the higher dose induced a complete response in all mice bearing either L540 or L428 tumors (Figure 4a and b, respectively), being 25 and 37 days after treatment administration the time needed to achieve complete tumor clearance, respectively. However, mice receiving the lower dose of CD30-CAR $T_{SCM\text{-}like}$ enriched cells showed a decreased

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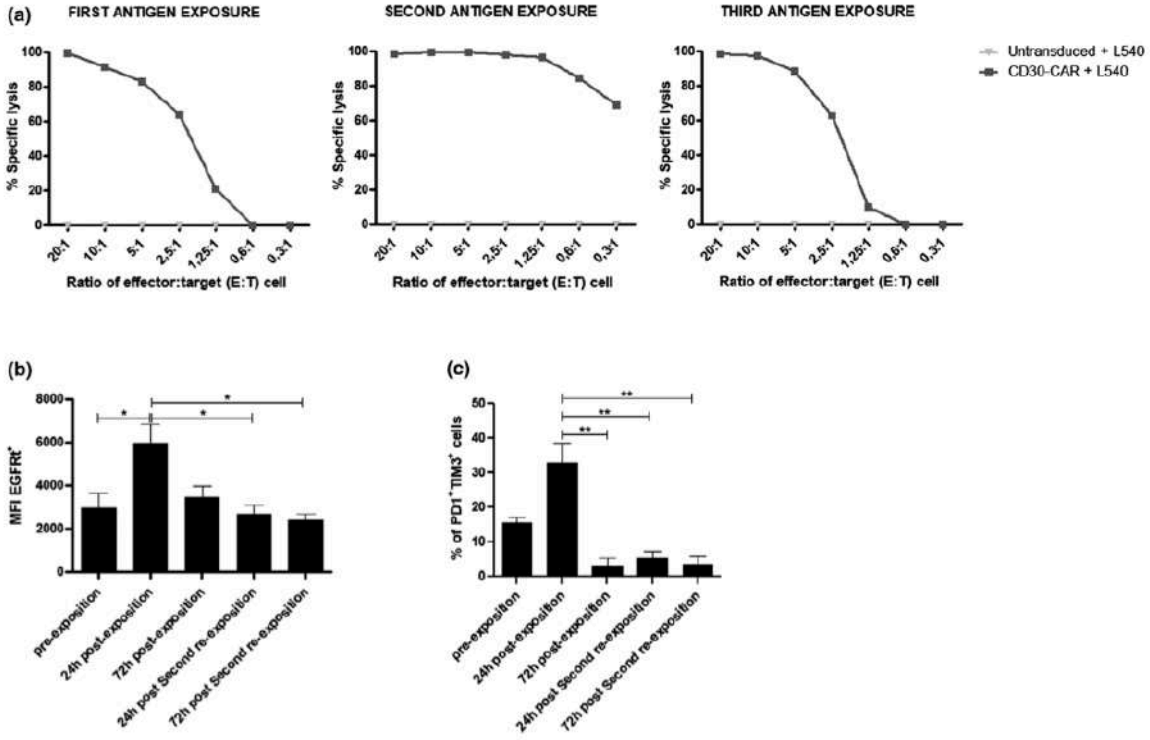

Figure 3. CD30-CAR $T_{SCM\text{-}like}$ function persists after $CD30^+$ antigen re-exposition. **(a)** Representative graphic of untransduced and CD30-CAR $T_{SCM\text{-}like}$ enriched cells exposed to $CD30^+$ target cells (L540) at different effector:target (E:T) ratios every 72 h after each antigen re-exposition (total stimulations: 3). Specific cytolytic activity was measured after 24 h of each antigen stimulation by bioluminescence. **(b)** Mean fluorescence intensity (MFI) of CD30-CAR expression and **(c)** percentage of PD-1-TIM-3^+ T cells during antigen re-exposures (mean ± SD). $*P < 0.05$; $**P < 0.01$. Data are representative of three independently repeated experiments.

antitumor efficacy in both tumor models (50% and 75% of tumor clearance in L540 and L428, respectively; Figure 4a and b). Tumor-bearing mice that received the highest dose (i.e. 10×10^6) of non-transduced T cells were not able to eliminate the tumor.

Furthermore, we took advantage of the subcutaneously implanted tumor (L540) to analyse tumor-infiltrating T lymphocytes (TILs) in those mice in which the lower dose of CD30-CAR T cells was not effective. Remarkably, a significant increase in the proportion of $PD1^+TIM3^+$ $CD4^+$ and $CD8^+$ T cells was detected in the tumor at day 48 compared with the infused CD30-CAR T cells ($CD4^+$: 4.41 ± 5.12% vs 33.6 ± 10.6%, $P < 0.036$; $CD8^+$: 0.43 ± 0.35% vs 13.05 ± 1.62%, $P < 0.0086$; Figure 4c), suggesting a long-term exhaustion of these T cells.

To elucidate whether treatment with CD30-CAR $T_{SCM\text{-}like}$ enriched cells could induce long-term immunity against HL, animals that survived after systemic L428 tumor injection ($n = 4/4$) were challenged again with the same tumor dose at day 79 with no further CD30-CAR T cells. All mice remained tumor-free 81 days after tumor rechallenge, whereas all age-matched control mice died of tumor progression (Figure 5a).

Next, since CAR T persistence is an important attribute correlated with antitumor efficacy, we look for T cells expressing $CD30\text{-}CAR^+$ in lymphoid organs of mice that survived after L428 tumor rechallenge. We detected the presence of $CD30\text{-}CAR^+$ T cells in bone marrow and lymph nodes (72.55 ± 1.34% and 75.45 ± 3.6% of CAR^+ T cells from the entire T-cell population, respectively; Figure 5b). Remarkably, $T_{SCM\text{-}like}$ CD30-CAR cells represent the predominant T-cell population detected in both bone marrow and lymph nodes (43.75 ± 0.07% and 41.8 ± 7.21%, respectively; Figure 5c).

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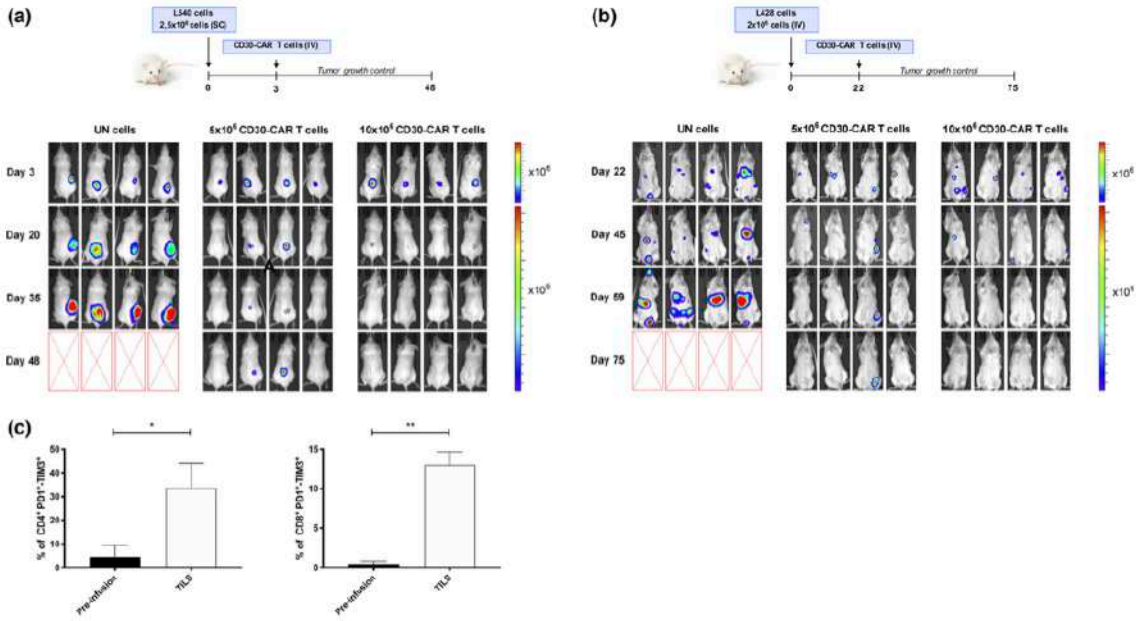

Figure 4. Therapeutic treatment with CD30-CAR $T_{SCM\text{-}like}$ enriched cell products induces a potent antitumor response against HL *in vivo*. NSG mice ($n = 4$ for both experimental and control groups) were injected with **(a)** 2.5×10^6 L540 tumor cells (sc) on day 0 and treated 3 days later with 5 or 10×10^6 CD30-CAR $T_{SCM\text{-}like}$ enriched cells (iv), or **(b)** 2×10^6 L428 tumor cells (iv) on day 0 and treated with 5 or 10×10^6 CD30-CAR $T_{SCM\text{-}like}$ enriched cells (iv) 22 days after tumor challenge. In both models, control mice received 10×10^6 untransduced (UN) $T_{SCM\text{-}like}$ enriched cells. Mice were monitored every other day for survival, and tumor growth was measured by *in vivo* bioluminescence. **(c)** Expression of exhaustion markers (PD1 and TIM3) in $CD4^+$ and $CD8^+$ tumor-infiltrating T cells found in L540-bearing mice treated with 5×10^6 CD30-CAR $T_{SCM\text{-}like}$ enriched cells, 48 days after tumor challenge, compared with $CD4^+$ and $CD8^+$ pre-infused T cells (mean ± SD). $^*P < 0.05$; $^{**}P < 0.01$. Data are representative of two independent experiments.

Collectively, these data show that CD30-CAR $T_{SCM\text{-}like}$ enriched cells have long-term persistence after a single infusion and provide the establishment of long-lasting antitumor immunity.

In addition, the proportion of T cells expressing $PD1^+$, $TIM3^+$ and $LAG\text{-}3^+$ were analysed in T cells from bone marrow and lymph nodes. We observed a low proportion of T cells expressing either $PD1^+$, $TIM3^+$ or $LAG\text{-}3^+$ in lymph nodes ($5.84 \pm 2.22\%$ of $PD1^+$; $4.72 \pm 4.1\%$ of $TIM3^+$; $0.3 \pm 0.28\%$ of $LAG\text{-}3^+$) and bone marrow ($18.45 \pm 3.32\%$ of $PD1^+$; $3.06 \pm 1.91\%$ of $TIM3^+$; $3.62 \pm 3.05\%$ of $LAG\text{-}3^+$; Figure 5d).

Highly enriched CD30-CAR $T_{SCM\text{-}like}$ ($T_{SCM\text{-}like}^H$) cells confer superior antitumor capacity compared with lowly enriched CD30-CAR $T_{SCM\text{-}like}$ ($T_{SCM\text{-}like}^L$) cells

To analyse whether a high proportion of less differentiated CD30-CAR T cells within the infused product could enhance the antitumor efficacy compared with more differentiated T cells, we studied the *in vivo* therapeutic antitumor activity of two different cell products, highly enriched $T_{SCM\text{-}like}$ ($T_{SCM\text{-}like}^H$; 70.95 ± 0.06 in $CD4^+$ and $82.25 \pm 10.1\%$ in $CD8^+$) and lowly enriched $T_{SCM\text{-}like}$ ($T_{SCM\text{-}like}^L$; 28.45 ± 12.1 in $CD4^+$ and $18.11 \pm 11.14\%$ in $CD8^+$) T cells (Figure 6a).

To prove potential clinically meaningful differences in antitumor efficacy between both cell products, mice were treated with a single infusion of a suboptimal dose of CD30-CAR T cells (5×10^6 iv) in both cases, and a delay in the treatment administration time regarding the tumor challenge. Under this stringent therapeutic condition, mice treated with $T_{SCM\text{-}like}^H$ CD30-CAR cells showed a significant reduced tumor growth throughout the entire study, compared with those mice treated with $T_{SCM\text{-}like}^L$ CD30-CAR cells (Figure 6b; $P < 0.001$). That lower tumor growth translates into a significant superior survival of mice treated with $T_{SCM\text{-}like}^H$ CD30-CAR compared with those treated with $T_{SCM\text{-}like}^L$ CD30-CAR cells

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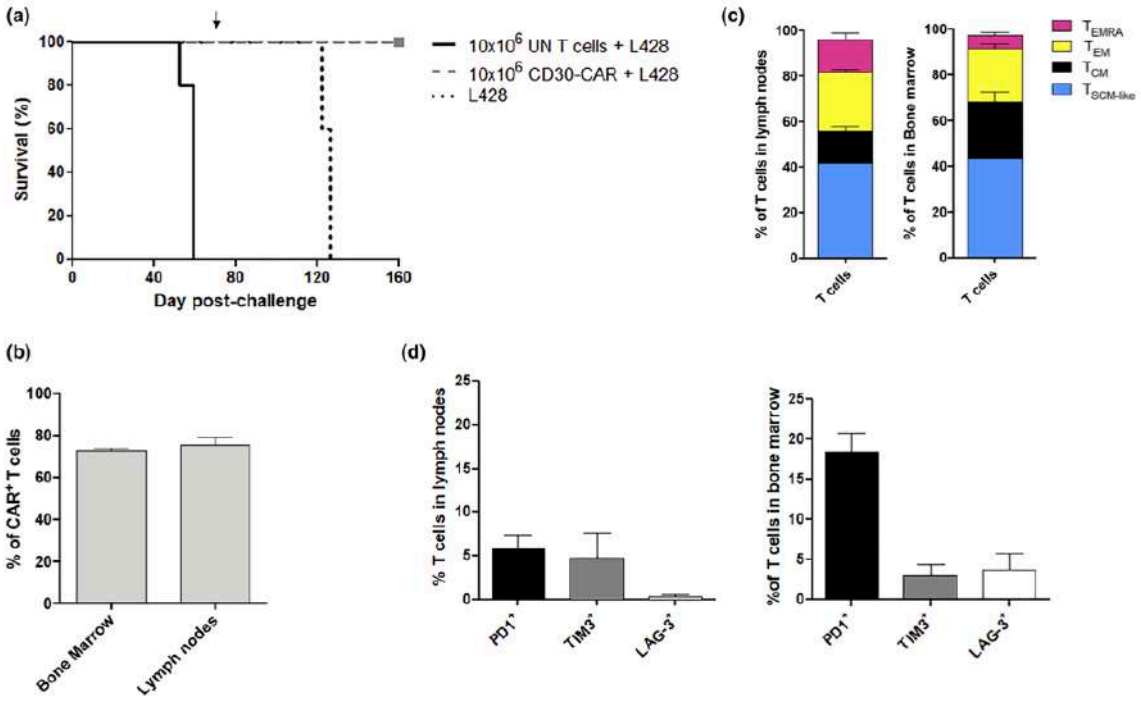

Figure 5. $T_{SCM\text{-}like}$ enriched cells expressing CD30-CAR have high persistence *in vivo* and confer long-lasting immunity against HL. **(a)** NSG mice surviving from L428 tumor administration ($n = 4$) were rechallenged again with the same tumor dose (2×10^6 cells per mouse, iv), 79 days (arrow) after the first tumor challenge (grey discontinued line). An age-matched mouse group ($n = 4$) was injected with 2×10^6 L428 tumor cells (iv) as tumor control (pointed line). Black line represents control mice receiving 10×10^6 untransduced (UN) $T_{SCM\text{-}like}$ enriched cells. Mice were followed every other day for survival, and tumor growth control was done by *in vivo* bioluminescence. **(b)** Bone marrow and lymph nodes from surviving CD30-CAR-treated mice were analysed for CAR T cell by flow cytometry (mean ± SD). **(c)** T-cell subpopulations were analysed in bone marrow and lymph nodes by flow cytometry (mean ± SD). **(d)** Expression of exhaustion markers (PD1, TIM3 and LAG-3) was analysed by flow cytometry in T cells found in lymph nodes and bone marrow (mean ± SD). Data are representative of two independent experiments.

(median survival: 51 vs 37 days post-challenge, respectively, $P = 0.006$; Figure 6c).

Importantly, although the frequency of tumor-infiltrating T cells founded in tumors of $T_{SCM\text{-}like}^{H}$- and $T_{SCM\text{-}like}^{L}$-treated mice was similar (29 ± 4% and 23.5 ± 2.5% from total tumor mass, respectively; data not shown), the proportion of CD30-CAR^+ T cells within the tumor-infiltrating T cells was significantly higher in mice treated with $T_{SCM\text{-}like}^{H}$ than in $T_{SCM\text{-}like}^{L}$ mice (78.78 ± 10.64% vs 53.75 ± 3.43%, respectively, $P = 0.002$; Figure 6d), suggesting a higher trafficking of CD30-CAR $T_{SCM\text{-}like}^{H}$ cells to the tumor site.

Moreover, we detect a higher amount of CD30-CAR T cells in blood in those mice treated with $T_{SCM\text{-}like}^{H}$ cells than in mice treated with $T_{SCM\text{-}like}^{L}$ cells (740 ± 106.22 CAR^+ T cell mL^{-1} vs 137.66 ± 18.45 CAR^+ T cell mL^{-1}, respectively, $P = 0.0006$; Figure 6e and Supplementary figure 3).

DISCUSSION

Most HL patients may be cured with current chemotherapy treatments; however, about 20% have a relapsing/refractory (R/R) disease, with very poor outcome.[20] Immunotherapy targeting CD30 (i.e. brentuximab vedotin) or immune checkpoint molecules for these R/R patients may yield complete responses, but long-term control of the disease is infrequent.[21,22] Despite that, responses observed with these agents emphasise the sensitivity of HL to immunotherapy strategies.

Chimeric antigen receptor T-cell therapy redirected to CD30 has been tested in R/R HL patients.[9,10] Although results are promising,

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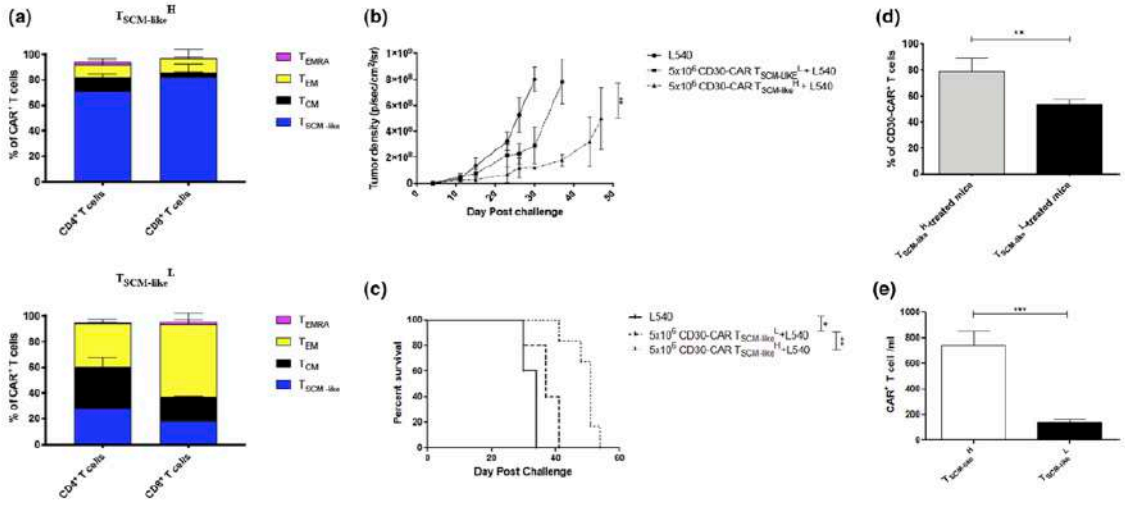

Figure 6. CD30-CAR $T_{SCM\text{-}like}^{H}$ products have higher antitumor efficacy and T-cell persistence than CD30-CAR $T_{SCM\text{-}like}^{L}$ products. **(a)** Composition of $T_{SCM\text{-}like}^{H}$ and $T_{SCM\text{-}like}^{L}$ cultures at the day of treatment (mean ± SEM). **(b, c)** NSG mice ($N = 4$/group) were challenged with 2.5×10^6 L540 tumor cells (sc) on day 0 and treated when the tumor was well established (day 5) with 5×10^6 CD30-CAR $T_{SCM\text{-}like}^{H}$ (pointed line and ▲) or $T_{SCM\text{-}like}^{L}$ (discontinued line and ■) cells (iv). A group of mice were injected with 2.5×10^6 L540 tumor cells (sc) as tumor control (black line and ●). Mice were monitored every other day for (B) tumor growth and **(c)** survival, measured by *in vivo* bioluminescence (mean ± SD). **(d)** Percentage of CD30-CAR expression in tumor-infiltrating T cells was analysed in $T_{SCM\text{-}like}^{H}$ and $T_{SCM\text{-}like}^{L}$ treated mice by flow cytometry **(e)** CD30-CAR T-cell detection in blood from mice treated with $T_{SCM\text{-}like}^{H}$ or $T_{SCM\text{-}like}^{L}$ cultures. (mean ± SD). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data are representative of two independent experiments.

clinical efficacy is limited, in contrast to data from B-cell tumors treated with CAR19.[23] Although CD30 remains an excellent target for CAR T therapy of HL, a number of obstacles should be solved to clinically improve their efficacy, such as persistence of CAR T cells, trafficking to tumor and increase of tumor cytotoxicity.

Here, we have evaluated the efficacy of a CD30-CAR targeting a membrane-proximal epitope on the CD30 molecule, to maximise its antitumor effect and to avoid the inhibition with soluble CD30. Furthermore, we have explored the use of CAR T-cell products highly enriched in $T_{SCM\text{-}like}$ cells to foster engraftment, persistence and trafficking into HL tumors.

Although the effectiveness of CD30-CAR targeting a distal epitope on the CD30 molecule in previous studies was apparently not affected by soluble CD30 molecule,[24,25] recent clinical trials have shown a correlation between sCD30 levels and the limited antitumor effect with CD30-CAR T cells.[10] The affinity of the CD30-specific scFv used to construct the CAR that recognises a distal epitope within the cleavable part of CD30 molecule[9,10,19] may be a consequence of the limited activity of CD30-CAR. This recognition could lead to CAR blockade by CD30 present in a soluble form in the plasma of HL patients with advanced/aggressive disease, significantly diminishing its antitumor effect.[26,27] Here, we show that the effectiveness of our CD30-CAR was not blocked by the presence of high amounts of soluble CD30 protein (20 μg, equivalent to 12 000 units mL^{-1}). In addition, epitope location appears to be a major determinant of CAR T efficacy, with membrane-proximal epitopes being better than those located on a distal region.[28] In line with this, our CD30-CAR could be advantageous over others since not only it is not affected by sCD30, but also it may have an enhanced cytolytic activity because of the proximal location of the targeted epitope.

It has become evident that long-term persistence, high engraftment, expansion and survival of adoptively transferred T cells are critical parameters for a good antitumor efficacy.[6,9,10,13,16] Thus, recent studies have been focused on the study of the most appropriate T-cell subsets for ACT and CAR T-cell therapy, and evidences indicate that the use of less differentiated T cells, and particularly T_{SCM}, results in greater persistence, cell expansion and

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antitumor efficacy[11,13,29-32] in contrast to unselected bulk T cells.[6,9,32]

According to previous studies, the potential elimination of activated CD30$^+$ T cells responding to other antigens and CD30-CAR T cells themselves is not a limitation for $T_{SCM\text{-}Like}$ cell expansion *ex vivo* with an optimal cell viability and CAR expression.[3,25] Moreover, we observed a correlation between the decrease in CD30$^+$ T cells along with the culture and an increase in cell viability, which is not observed in UN T cells, suggesting fratricide elimination of CD30$^+$ T cells. Other mechanisms could explain the reduction in CD30$^+$ T cells including the internalisation of the protein or the sequestration of CD30 protein in the cytoplasm of T cells, similar to what has been shown with other CARs targeting molecules expressed in activated T cells.[33]

Long-lasting responses have been associated with the persistence of CAR T cell and potent T-cell expansion.[5,34,35] In addition, the trafficking of CAR T cells to tumors is of critical importance for obtaining an antitumor effect. In this study, we have generated CD30-CAR $T_{SCM\text{-}like}$ enriched products to increase persistence and trafficking into HL tumors. We observed that an optimal dose of CD30-CAR $T_{SCM\text{-}like}$ enriched products had a potent and specific antitumor efficacy against HL *in vitro* and *in vivo*, clearing completely both a systemic and a localised (simulating an extra-nodal lesion) HL tumor. Interestingly, we found tumor-infiltrating T cells coexpressing high levels of PD1 and TIM3 in those animals treated with a suboptimal dose that were not able to eradicate the tumor, which is correlated with a profound T-cell dysfunction.[36-38] T cells expressing PD1 in combination with inhibitory markers such as TIM3 and LAG-3 are associated with relapses or refractory disease,[38,39] and may be a mechanism for decreasing the effectiveness of CAR T-cell therapy. Targeting of TIM3 and PD1 pathways, in combination with CAR T-cell therapy, could increase the tumor control and restore T-cell function, a strategy that already has been proved in B-cell lymphoma patients treated with CAR19.[40] Our approach described here may represent a way to reduce the known immunosuppressive effect of HL tumor microenvironment (TME) on T cells.[41] T cells generated under IL-7 and IL-15 show increased expression of CXCR4,[42] a chemokine that promote T-cell migration to peripheral tissues and tumors. In addition, under those conditions, T cells also express CXCR3 that interacts with CCL5, a chemokine secreted by HL cells, facilitating tumor infiltration of T cells,[41,43] which is in line with our finding that CD30-CAR $T_{SCM\text{-}like}$ enriched products have increased infiltration into HL tumors. Importantly, the use of IL-21 within the culture would contribute further to the enhanced antitumor efficacy of our CD30-CAR T cells, promoting the generation of less differentiated memory T cells and increasing antitumor activity.[44] Besides, CD30-CAR T cells may kill Hodgkin's TME regulatory T cells, which express high levels of CD30, thus contributing to ameliorate immunosuppression.

Other strategies described to improve resistance of adoptive T cells to HL-derived immunosuppression included the use of CD30-CAR cells gene-modified to express CCR4,[45] to increase tumor homing, and a dual targeting of HL tumor and myeloid suppressor cells by using a CD123-redirected CART,[46] all of them are being translated to the clinic.

According to previous studies with $T_{SCM\text{-}like}$ cells,[11,13,29-32] we observed a long-term persistence of CD30-CAR $T_{SCM\text{-}like}$ cells that were able to fully eradicate the tumor after a second tumor rechallenge. Moreover, CD30-CAR expression persisted and $T_{SCM\text{-}like}$ remained the most frequent cellular subpopulation in lymph nodes and bone marrow of those mice. Importantly, CD30-CAR T cells found after the second tumor challenge display a low expression of PD1, TIM3 and LAG-3, demonstrating that these cells were not exhausted and remained functional.[36-39]

Differentiated CAR T cells redirected to CD30 or CD19 have limited antitumor efficacy, mostly because of poor proliferation and migration to tumor sites.[6,9,10,25] Importantly, in patients receiving bulk CD19-CAR T cells, *in vivo* expansion of these cells has been correlated with the frequency, within the infusion product, of a T-cell subset closely related to T_{SCM}[16,17,47]; however, the frequency of these cells is low in the majority of cell products currently used in clinical trials.[16,34] To gain insight into the antitumor activity of CD30-CAR with different proportions of $T_{SCM\text{-}like}$ cells, we established a therapeutic model with two different proportions of $T_{SCM\text{-}like}$ cells, namely 'highly enriched' (>50% of CD30-CAR $T_{SCM\text{-}like}$ cells) and 'lowly enriched' (<30% of CD30-CAR $T_{SCM\text{-}like}$ cells). Remarkably, we found higher persistence, trafficking to tumor and frequency of circulating CD30-CAR T cells in mice receiving CD30-CAR $T_{SCM\text{-}like}^{H}$ products than in those

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treated with $T_{SCM\text{-}like}^{L}$, which, importantly, translated into a significant survival advantage.[9,10,17] Collectively, although we did not attempt to quantify the minimum threshold of $T_{SCM\text{-}like}$ proportion in infused CAR T-cell products that confer maximum antitumor activity, our data suggest that an elevated frequency of this cell subset expressing the CAR could significantly improve the outcome of CAR T therapy.

Although xenograft mouse models are extensively used to study antitumor efficacy of T-cell adoptive therapy, they have some limitations, particularly related to the absence of relevant TME and lack of interaction with the host immune system.[48] For T-cell therapy, some models could also fail to correctly evaluate off-tumor toxicity, which is an important issue when T-cell therapies are translated to the clinical scenario. With all these pros and cons, NSG models allow studying persistence and efficacy of human CAR T cells and thus representing a good option to test the effect of novel CARs.

In summary, we demonstrate that $T_{SCM\text{-}like}$ can be efficiently transduced and *ex vivo*-expanded with a novel CD30-CAR that surpasses fratricide killing of CD30-expressing T cells and confers potent *in vivo* antitumor efficacy against HL. We have shown that highly enriched CD30-CAR $T_{SCM\text{-}like}$ products have enhanced tumor trafficking and antitumor activity compared with those composed predominantly of more differentiated cell subsets.

These proof-of-concept studies support the use of a refined CD30-CAR with highly enriched $T_{SCM\text{-}like}$ cell products for improving clinical efficacy of CAR T therapy of patients with HL.

METHODS

Blood samples and peripheral blood mononuclear cell isolation

Peripheral blood samples were obtained from healthy donors ($n = 7$) after informed consent following the protocol approved by the Ethics Committee of Hospital de la Santa Creu i Sant Pau (Barcelona). Peripheral blood mononuclear cells were isolated by Ficoll–Hypaque density gradient centrifugation (Lymphoprep; Axis-shield, Dundee, UK) and frozen until use.

Mice

Female NOD.Cg-Prkdc scid IL2rg^{tm1Wjl}/SzJ (NSG) mice (6–7 weeks of age; Charles River, France) were used for *in vivo* experiments. Animals were housed under specific pathogen-free conditions at the Laboratory Animal Facility at Hospital Sant Pau. All experiments and care of animals were conducted according to the European Animal Care guidelines and approved by the Ethical Committee of Animal Experimentation at Hospital Sant Pau.

Cell lines

HEK-293T cells (LentiX; Clontech, Mountain View, California, USA) were cultured in DMEM supplemented with 10% heat-inactivated foetal bovine serum (FBS), penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}; Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The CD30^{+} HL-derived cell lines, L428 and L540, were obtained from the German Collection of Cell Cultures (DSMZ, Braunschweig, Germany). Raji cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Tumor cell lines were cultured in RPMI-1640 medium supplemented with 10–20 % FBS, penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}; Thermo Fisher Scientific). All cell cultures were performed at 37 °C in a fully humidified atmosphere with 5% CO_2 in air. Tumor cell lines were modified with a lentiviral vector to express firefly luciferase (ffluc) and enhanced green fluorescence protein (eGFP; Addgene, Watertown, Massachusetts, USA).

For *in vivo* experiments, ffluc-transduced tumor cells were thawed from a common frozen stock and grown *in vitro* for 3 days before use. On the day of tumor injection, cells were washed with complete medium and diluted to the appropriate concentration in 0.1 mL of phosphate-buffered saline (PBS) per mouse.

Construction of CD30-CAR-encoding lentiviral vector

A codon-optimised scFv comprising the variable heavy and light chains of the anti-CD30 monoclonal antibody (mAb; clone T105),[19] separated by a Whitlow peptide linker,[49] was synthesised (GeneArt; Thermo Fisher, Regensburg, Germany), and cloned into the third-generation lentiviral expression vector pHIV7,[50,51] where it was fused to a CD8 hinge, a 4.1BB-CD3ζ signalling module in *cis* with a T2A element, and the truncated human epidermal growth factor receptor (EGFRt), as previously described.[52]

Lentivirus production

Lentivirus was produced in HEK-293T cells co-transfected with the pHIV7 lentiviral vector and the packaging vectors pCHGP-2, pCMV-rev2 and pCMV-VSV-G, (Addgene) using calcium phosphate (Clontech). Medium was changed 16 h after transfection, and the cells were incubated for another 36–48 h. Lentiviral supernatants were collected, centrifuged to remove cellular debris for 15 min at 2217 *g* and concentrated for 2 h at 55126 *g*. The lentiviral pellet was resuspended in TBS-5 buffer, snap-frozen on dry ice and stored at −80°C.

T-cell isolation and culture

Naïve T cells were isolated using a Human Naïve Pan T Cell Isolation Kit (StemCell Technologies, Vancouver, British

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Columbia, Canada) according to the manufacturer's instructions. To obtain $T_{SCM\text{-}like}$ highly enriched cultures, purified naïve T cells (higher than 95 %) were cultured and expanded as previously described.[15] Briefly, cells were activated during 48 h with anti-CD3/CD28 magnetic beads (Life Technologies, Waltham, Massachusetts, USA) in 1:2 bead/T-cell ratio and then cultured with IL-7, IL-15 and IL-21 at 25 ng mL^{-1} each (Stem Cell Technologies, Vancouver, British Columbia, Canada). 2.5×10^5 cells mL^{-1} per well were seeded in a 24-well plate.

To generate products with low proportion of $T_{SCM\text{-}like}$ cells ($T_{SCM\text{-}like}^{L}$), T cells were isolated using a Human Pan T Cell Isolation Kit (StemCell Technologies) according to the manufacturer's instructions. Purified T cells were activated with anti-CD3/CD28 magnetic beads (Life Technologies) in 1:1 bead/T-cell ratio, and cultured with IL-2 at 50 UI mL^{-1} (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany). 5×10^5 cells mL^{-1} per well were seeded in a 48-well plate.

T cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}; Thermo Fisher Scientific). All cell cultures were performed at 37°C in a fully humidified atmosphere with 5% CO_2 in air. Cytokines and medium were replaced every 3–4 days, and cells were counted every 3–4 days by trypan blue dye exclusion.

T-cell transduction

Transduction was performed 24 h (for $T_{SCM\text{-}like}^{L}$ cultures) or 48 h (for $T_{SCM\text{-}like}^{H}$ cultures) after initial stimulation by 800 *g* spinoculation for 45 min at 32°C in the presence of polybrene (5 μg mL^{-1}; Sigma-Aldrich, St. Louis, Missouri, USA). CAR expression was detected by flow cytometry at days 6–7 of culture.

Cytometry and CAR expression analysis

Cells were stained for 30 min at 4°C in PBS containing 1% FBS and 0.01% NaN_3 (Sigma-Aldrich; staining buffer). All antibodies were titrated before use, and corresponding isotypes for each antibody were used as controls. For T-cell population analysis, cells were labelled with fluorescent antibodies against human CD3-FITC (REA613), CD4-APC-Vio-770 (clone VIT4), CD8-VioGreen (clone BW135/80), CD45RA-PerCPVio700 (clone T6D11), CD45RO-PE (clone UCHL1), CD27-VioBlue (clone M-T271), CD95-FITC (clone DX2) and CCR7-PE vio770 (clone REA108; all from Miltenyi Biotech). Different T-cell subpopulations were determined following a previously published gating strategy.[15,53] Alexa Fluor 647-labelled anti-EGFRt antibody (cetuximab; Roche, Basel, Switzerland) was used to detect CAR expression. CD30 expression was detected using anti-CD30-APC (clone Ki-2; Miltenyi Biotec). In addition, anti-PD1 (clone PD1.3.1.3), anti-TIM3 (clone F38-2E2) and anti-LAG-3 (clone REA351) antibodies (all from Miltenyi Biotec) were used to analyse T-cell exhaustion. All data were acquired on a MACSQuant Analyzer 10 (Miltenyi Biotec). Data from flow cytometry were analysed using the FlowJo version 10 software (TreeStar, Ashland, Oregon, USA).

In some cases, bone marrow, lymph nodes, tumor and blood of treated and control mice were harvested at the end of the experiment, and CAR T cells were analysed by flow cytometry. Briefly, tumor and axillar lymph nodes were disaggregated by mechanical procedures and collected in 5 and 3 mL of complete medium, respectively, and bone marrow was flushed from the long bones (femur and tibia). Cellular suspensions were filtered through a 70-μm cell strainer (BC Falcon; Cultek S.L.U., Foster City, California, USA) and centrifuged at 385 *g* during 5 min. In the case of bone marrow and blood, erythrocytes were lysed using an ammonium chloride solution (Pharm Lyse Buffer; BD Biosciences, Franklin Lakes, New Jersey, USA) during 3 min in agitation at room temperature. Finally, cells were counted and maintained in 5 mL of complete medium until use. Blood samples were incubated with Fc Block and corresponding antibodies prior to 10-min erythrocyte lysis (Pharmlyse Buffer; BD Bioscience), centrifuged at 385 *g* during 5 min and acquired on MACSQuant Analyzer 10 (Miltenyi Biotec).

Functional *in vitro* analysis of CD30-CAR T cells

In vitro cytolytic activity of CD30-CAR $T_{SCM\text{-}like}$ cells was analysed by a bioluminescence-based assay using ffluc-transduced $CD30^+$ tumor cells (L540 and L428, at different effector:target (E:T) ratios)), after 24-h co-culture.

Persistence of CD30-CAR T cells was evaluated by continued re-exposure assays. $T_{SCM\text{-}like}$ CD30 CAR T cells were co-cultured again with $CD30^+$ tumor cells 72 h after exposition, and cytolytic activity was analysed by bioluminescence-based assay using ffluc-transduced $CD30^+$ tumor cells. Levels of IFN-γ, IL-2, TNF-α, IL-6 and IL-10 were analysed in supernatants obtained after a 24-h co-culture of CAR T cells with L540, L428 and Raji cell lines (E:T ratio 5:1). Cytokines were detected using Multi-Analyte Flow Assay (LEGENDplex; BioLegend, San Diego, California, USA) according to the manufacturer's instructions. Acquisition was performed on a MACSQuant Analyzer 10 (Miltenyi Biotec), and data analysis was done using LEGENDplex Analysis software (BioLegend).

In vivo efficacy of CD30-CAR T cells

Mice (four per group) were injected intravenously (iv) with L428 tumor cells (2×10^6 cells per mouse) or subcutaneously (sc) with L540 (2.5×10^6 cells per mouse). When tumors were well established (22 days for L428 tumor model and 3 days for L540), mice were treated with a $T_{SCM\text{-}like}$ enriched cell suspension ($5\text{–}10 \times 10^6$ CAR^+ T cells per mice, iv). Control mice received the same dose of untransduced T cells. In some experiments, mice surviving to L428 lymphoma were rechallenged with a second tumor dose (2×10^6 cells per mouse, iv) 79 days after the first tumor injection. An untreated age-matched mice group receiving the same tumor dose was used as control.

To analyse differences between products with a high ($T_{SCM\text{-}like}^{H}$ cultures) or low ($T_{SCM\text{-}like}^{L}$ cultures) proportion of CD30-CAR $T_{SCM\text{-}like}$ cells, L540 tumor-bearing mice ($n = 4$)

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received either 5×10^6 CAR^+ highly enriched or lowly enriched $T_{SCM\text{-}like}$ cells (iv) 5 days after tumor injection.

Tumor growth was measured by *in vivo* bioluminescence imaging (IVIS Spectrum *In Vivo* Imaging System; PerkinElmer, Waltham, Massachusetts, USA) in all cases and analysed with specialised software (Living Image, PerkinElmer, USA). Animals were followed every other day for survival and sacrificed when moribund.

Soluble CD30 protein detection

Soluble CD30 protein was measured in supernatants of L540 and L428 cell cultures by enzyme-linked immunoabsorbent assay (Human CD30 PicoKine ELISA Kit; Boster Biological Technology, Pleasanton, California, USA).

Statistical analysis

Inter-donor variability was assessed using standard deviation (SD). Results are expressed as the mean ± SD. The Kaplan–Meier plots were used to analyse mice survival, and the significant differences between survival curves were assessed by the log-rank test. For all other data, the *t*-tests were performed to analyse the differences between groups. All statistical analysis and graphics were performed using GraphPad Prism 6 (GraphPad Software Inc. California, USA).

ACKNOWLEDGMENTS

This work was supported in part by grants from La Marató TV3 (Exp. 20130710), Deutsche José Carreras Leukämie Stiftung (DJCSL 10R/2016), Fundación Científica Asociación Española Contra el Cáncer (AECC-AIO2017), Instituto de Salud Carlos III (PI15/1383 y PI18/01023), Fundación Bancaria 'La Caixa', TerCel (SG/11/2008), Ministerio de Economía y Competitividad (RETOS; RTC 2015-3393-1) and AGAUR (2017SGR1395).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Laura Escribà Garcia: Investigation. **Ana C Caballero:** Investigation. **Eva Escudero-lópez:** Investigation. **Cristina Ujaldón-Miró:** Investigation. **Rosanna Montserrat-Torres:** Investigation. **Paula Pujol-Fernández:** Investigation. **Jorge Sierra:** Investigation.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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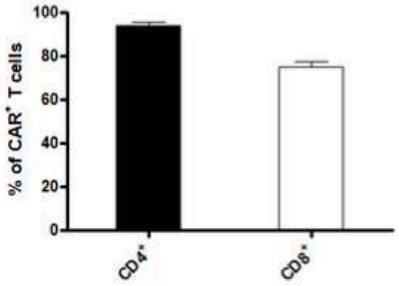
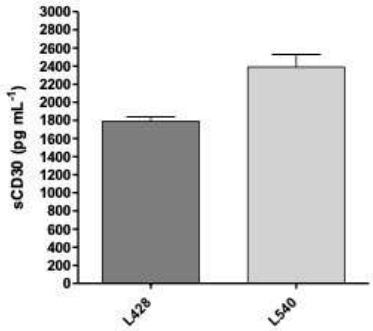
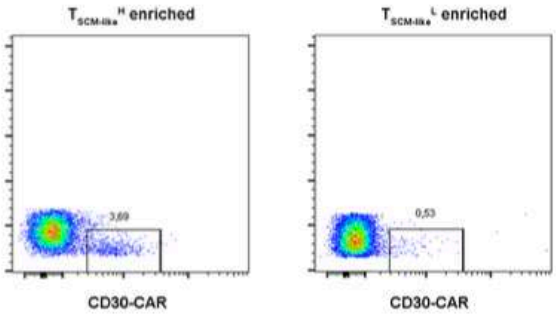
SUPPLEMENTARY FIGURES



Supplementary figure 1. Transduction efficiency of CD30-CAR in CD4$^+$ and CD8$^+$ T cells. CAR expression in CD4 and CD8 T cells was analysed at the end of culture by flow cytometry using an anti-EGFRt antibody (n = 7) (mean ± SD). Data are representative of seven independently repeated experiments.



Supplementary figure 2. CD30 soluble protein secretion by HL lines. CD30 secretion was analyzed in L540 and L428 48-h culture (n = 3). Absolute quantification of soluble CD30 was performed by ELISA (mean ± SD). Data are representative of three independently repeated experiments.



Supplementary figure 3. CD30-CAR T cells detection in blood from mice treated with TSCM-like^H enriched or TSCM-like^L enriched cultures. Blood of treated mice was obtained at the end of experiment and CD30-CAR T cells were analyzed by flow cytometry using an anti-EGFRt antibody. Plots represent one of the three analysis in each group.

4.2 Second research article

High CAR intensity of expression confers enhanced antitumor effect against lymphoma without functional exhaustion

Ana Carolina Caballero[1,2,3,4], *Laura Escribà-Garcia*[1,2,3], *Paula Pujol-Fernández*[1,2,3], *Eva Escudero-López*[1,2,3,4], *Cristina Ujaldón-Miró*[1,2,3,4], *Rosanna Montserrat-Torres*[1,2,3], *Jorge Sierra*[1,3,4], *Carmen Alvarez-Fernández*[1,2,3,5†] *and Javier Briones*[1,2,3,4,5†]

[†]Equal senior contributors

[1]Hematology Service, Hospital de la Santa Creu y Sant Pau, Barcelona, Spain

[2]Laboratory of Experimental Hematology-IIB, Institut Recerca Hospital de la Santa Creu y Sant Pau, Barcelona, Spain

[3]Jose Carreras Leukemia Research Institute, Barcelona, Spain

[4]Department of Medicine, Autonomous University of Barcelona, Barcelona, Spain

Cancer Gene Ther. 2022 Aug 29.

DOI: 10.1038/s41417-022-00518-6

DOI: 10.1038/s41417-022-00518-6

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5 OVERALL SUMMARY OF RESULTS

ACT with CAR19-modified T-cells has shown outstanding results for relapsed/refractory (R/R) B-cell malignancies, achieving its inclusion as standard of care for these patients. Despite the success of CART19 therapy, the clinical data that has been generated over the last few years reveals that there is much room for improvement, since at least 60% of those refractory patients don not benefit from that therapy. There is even greater potential for progress in hematologic malignancies that lack CD19 expression, such as cHL. In fact, available data on CART30 therapy for treatment of cHL shows modest antitumor efficacy with most patients remaining refractory or having early relapse.

Different factors influencing clinical outcome after CART19 therapy have been described with the patient and disease features being as important as the characteristics of the infusion product (46). It should be noted that properties of the infusion product are potentially modifiable and, therefore, could be the key to designing strategies that improve the efficacy of CART30 therapy.

With the purpose of enhancing antitumor effect of CART therapy against cHL, we have developed a novel second-generation CAR directed against CD30 (CAR30). This novelty will be widely described below. First, the recognition domain of the CAR is composed of a scFv targeting a membrane-proximal epitope (monoclonal antibody; clone T105). This has selected as previous studies have suggested superior antitumor effect from those scFv targeting proximal vs. distal membrane epitopes (129).

Second, the epitope recognized by the scFv is located within the non-soluble region of CD30 protein to avoid the potential blockage of CAR30 by the soluble CD30 protein, which is present in most of cHL patients and confer poor prognosis. The transmembrane domain is constituted by a CD8α hinge. The cytoplasmic tail includes both CD3ζ activation molecule and 4-1BB as co-stimulation. We have constructed a multidomain DNA sequence encoding the second-generation CAR30 and a truncated human epidermal growth factor receptor (EGFRt) separated by a T2A element. The EGFRt sequence lacks the ligand binding and tyrosine kinase activity domains, but retains an epitope fully recognized with the available antibody cetuximab as previously described (130). The EGFRt has been proven to be coordinately expressed with tumor-specific chimeric antigen, to provide a cell surface marker for *in vivo* tracking of adoptively transferred T-cells using both flow cytometry and immunohistochemistry and, potentially kill CAR T-cells (using cetuximab) *in vivo* if needed due to uncontrolled toxicities (130).

Third, and very important, we attempted to generate CAR T-cell products highly enriched in $T_{SCM\text{-}LIKE}$ T-cells to further improve antitumor capacity and extend persistence of $CAR30^+$ T-cells. To do this, we isolated $CD4^+$ and $CD8^+$ naïve T-cells from PBMCs of healthy donors, activated them with a very short CD3/CD28 co-stimulation (only 2 days), and cultured them in presence of interleukine-7 (IL-7), interleukine-15 (IL-15), and interleukine-21 (IL-21). The T-cells were efficiently transduced ($CAR30^+$T-cells: 87.03±1.69%), and the majority of $CD4^+$ and $CD8^+$ T-cells were $CAR30^+$ at the end of culture. We have managed to generate products that were homogeneous in terms of T-cell subsets and predominantly composed of $T_{SCM\text{-}LIKE}$ cells, constituting 62.4±6.49% in $CD4^+$ and 67.29±8.62% in $CD8^+$ T-cells.

The on-target off-tumor effect is one of the concerns of using anti-CD30 CARs. As previously described, CD30 is expressed on a subset of activated T-cells and therefore its clearance by $CAR30^+$ T-cells is possible (i.e., fratricide killing), thus affecting $CAR30^+$ T-cells expansion. Our data confirmed that there was a progressive decrease in the proportion of $CD30^+$ T-cells during culture, which was associated with an increase in $CAR30^+$ T-cell viability. Remarkably, the $CAR30^+$ T-cells expansion was substantial (≥50- fold expansion, both $CD4^+$ and $CD8^+$), despite the observed fratricide killing.

Another challenge of using the CD30 target is that HL patients exhibit high amounts of serum soluble CD30 protein (sCD30), which could blockade CAR30 and lead to defective antitumor activity (131). We evaluated e*x vivo* cytotoxic capacity of our $CAR30^+$ $T_{SCM\text{-}LIKE}$ enriched products against two different human HL tumor cell lines (L428 and L540). $CAR30^+$ $T_{SCM\text{-}LIKE}$ cells displayed a potent and specific cytolytic effect (tumor cell death at 5:1 E:T ratio; 89.92±5.95% in L540, and 71.11±8.91% in L428). We measured sCD30 in supernatants and verify that both HL cell lines produced high quantities of sCD30. To definitively prove that $CAR30^+$ T-cells function was sustained despite the presence of sCD30, a cytotoxicity assay was performed in the presence of saturating concentrations of recombinant sCD30 protein (equivalent to more than 10-fold the median sCD30 on HL patients) (131), and yet $CAR30^+$ $T_{SCM\text{-}LIKE}$ cells succeed to specifically kill HL tumor cell lines.

Additionally, we tested the performance of $CAR30^+$ $T_{SCM\text{-}LIKE}$ cells upon *ex vivo* re-challenge with tumor cells and confirmed that $CAR30^+$ $T_{SCM\text{-}LIKE}$ cells retained their robust and specific cytotoxic effect against L540 (tumor cell death at 5:1 E:T ratio; 88.75% after

three antigen expositions). The significant antitumor capability was surprisingly accompanied by an increase in the proportion of $PD1^{+}TIM3^{+}$ T-cells after 24 h from the first co-culture with L540, but that was followed by a significant decrease at 72 h.

Once we have demonstrated the *ex vivo* antitumor efficacy of $CAR30^{+}$ $T_{SCM\text{-}LIKE}$ cells, we evaluated the *in vivo* capacity to eliminate HL cells. We performed two mouse models, one of them intravenous (IV) with L428 to resemble advanced systemic disease, and the other subcutaneous (SC) with L540 to resemble extra nodal HL. Mice were treated with two different doses of $CAR30^{+}$ $T_{SCM\text{-}LIKE}$ cells ($5x10^{6}$ and $10x10^{6}$ $CAR30^{+}$ T-cells) and tumor growth was measured by *in vivo* bioluminescence imaging. Mice receiving $5x10^{6}$ $CAR30^{+}$ T-cells developed some control of the tumor but failed to eradicate it. In contrast, the highest dose induced a complete response in all mice treated, both in L428 and L540 models. Thus, the optimal dose for treatment was shown to be $10x10^{6}$ $CAR30^{+}$ T-cells.

A critical issue related to cellular immunotherapy efficacy is the development of T-cell persistence and long-lasting antitumor immunity (75,76,78). To test that our CAR30 T-cell product accomplished this, we performed a refined study in the systemic HL model, L428. Thus, mice in the L428 model that remained tumor-free after receiving the optimal dose of $CAR30^{+}$ T-cells were challenge with a second IV injection of lethal-dose of tumor cells. Interestingly, this confirmed the functional persistence of $CAR30^{+}$ T-cells *in vivo* as they were able to eradicate the tumor again in 100% of the mice that persisted tumor-free 81 days after second tumor challenge (end of experiment). Furthermore, $CAR30^{+}$ T-cells were detected in bone marrow and lymph nodes at this time, and we found that $T_{SCM\text{-}LIKE}$ was the dominant T cell population (43.75±0.07% in bone marrow and 41.8±7.21% in lymph nodes). Collectively, our data suggest that $CAR30^{+}$ products enriched in $T_{SCM\text{-}LIKE}$ cells have long-term persistence after a single infusion and provide long-lasting antitumor effect.

To gain insight into the critical importance of $T_{SCM\text{-}LIKE}$ cells in the enhanced antitumor efficacy, we decided to compare *in vivo* therapeutic antitumor activity of two different CAR30 T-cell products; one with a high proportion of $T_{SCM\text{-}LIKE}$ ($T_{SCM\text{-}LIKE}^{H}$) and another with a low proportion of this population ($T_{SCM\text{-}LIKE}^{L}$). $T_{SCM\text{-}LIKE}^{H}$ was composed of 70.95±0.06% in $CD4^{+}$ and 82.25±10.1% in $CD8^{+}$ of $T_{SCM\text{-}LIKE}$ cells. $T_{SCM\text{-}LIKE}^{L}$ included 28.45±12.1% in $CD4^{+}$ and 18.11±11.14% in $CD8^{+}$ $T_{SCM\text{-}LIKE}$ cells. We chose the L428 mice model with the suboptimal dose ($5x10^{6}$ $CAR30^{+}$ T-cells) and delayed the treatment administration time regarding the tumor challenge. These unfavorable conditions, designed

to highlight differences in efficacy, revealed that mice treated with $T_{SCM\text{-}LIKE}^{H}$ CAR30 T-cell products had a significant higher tumor growth control and a superior survival compared with those treated with $T_{SCM\text{-}LIKE}^{L}$.

Overall, we were able to produce T-cell products enriched in $T_{SCM\text{-}LIKE}$ cells and efficiently transduced them with a novel CAR30, which retains its function despite saturating concentrations of sCD30. The $CAR30^{+}$ $T_{SCM\text{-}LIKE}$ cells achieved a good expansion, overpassing fratricide killing of CD30-expressing T-cells. Moreover, $CAR30^{+}$ $T_{SCM\text{-}LIKE}^{H}$ products have shown greater *in vivo* antitumor activity than $CAR30^{+}$ $T_{SCM\text{-}LIKE}^{L}$, highly suggesting, for the first time, that a higher proportion of $T_{SCM\text{-}LIKE}$ cells in infusion products may contribute to improve the efficacy of CART30 therapy for cHL.

Next, pursuing the goal of recognizing and combining product features that are critical to optimize the function of CART30 therapy, we decided to study the impact of CAR30 expression intensity on T-cell function, cell exhaustion and antitumor efficacy. Again, we generated $CAR30^{+}$ products enriched for $T_{SCM\text{-}LIKE}$ cells, but this time $CAR30^{+}$ T-cells were sorted according to their mean fluorescence intensity (MFI) in two distinct subpopulations: CAR^{HI} subgroup with MFI $\geq 5\times10^{3}$ and CAR^{LO} with MFI $\leq 3\times10^{3}$. To notice, MFI of $CAR30^{HI}$ was 7598 ± 1183, in contrast to MFI of $CAR30^{HI}$ that was 1064 ± 124.7 ($p < 0.0001$).

At the end of culture, $CAR30^{LO}$ had a slightly higher proportion of $T_{SCM\text{-}LIKE}$ than $CAR30^{HI}$ and, in contrast, $CAR30^{HI}$ include a greater percentage of T_{CM} cells. Anyway, $T_{SCM\text{-}LIKE}$ was the most frequent subpopulation in both groups, and memory T-cell subsets ($T_{SCM\text{-}LIKE}$ + T_{CM}) comprised more than 90% of the total. We analyzed early (CD69), intermedium (CD25) and late (HLA-DR) T-cell activation markers. CD69 was barely detected, CD25 was expressed in most of $CAR30^{+}$ T-cells, being slightly more frequent in $CD8^{+}CAR30^{HI}$ than $CD8^{+}CAR30^{LO}$. HLA-DR expression was significantly higher in $CAR30^{HI}$. Meanwhile, CD30 expression was almost absent in both $CAR30^{HI}$ and $CAR30^{LO}$. On the other hand, PD1 and TIM-3 co-expression tend to be higher in $CD4^{+}CAR30^{HI}$ compared to $CD4^{+}CAR30^{LO}$, but no differences were observed between both groups in the $CD8^{+}$ subpopulation.

We evaluated *ex vivo* cytotoxic function of $CAR30^{HI}$ and $CAR30^{LO}$ against L540 HL tumor cell line. Remarkably, $CAR30^{HI}$ showed a higher specific cytotoxicity compared to $CAR30^{LO}$ at different E:T ratios ($p < 0.0001$); that was sustained and even more pronounced

at lower E:T ratios (e.g., tumor death at 1.25:1 E:T ratio: 77.57 ± 5.15% vs. 33.23 ± 14.95%; $p < 0.05$, $CAR30^{HI}$ and $CAR30^{LO}$, respectively).

We examined T-cell activation and inhibition patterns after antigen encounter. Both $CAR30^{HI}$ and $CAR30^{LO}$ underwent a significant increase of HLA-DR and CD69 expression at 24 h after antigen encounter. Furthermore, $CAR30^{HI}$ and $CAR30^{LO}$ exhibited a substantial increase in expression of CD30 in $CD4^+$ subset. However, in $CD8^+$ subset there was a significant boost of CD30 expression only in $CAR30^{HI}$ but not in $CAR30^{LO}$. There was a transient increase in the co-expression of PD1 and TIM-3 in the $CD4^+$ subpopulation, both in $CAR30^{HI}$ and $CAR30^{LO}$. In contrast, $CD8^+$ T-cells showed similar $PD1^+TIM\text{-}3^+$ co-expression over time. A higher proportion of $T_{SCM\text{-}LIKE}$ cells were detected in $CAR30^{HI}$ subgroups after exposure to tumor cells compared to baseline. Thus, the differences in T-cell composition between $CAR30^{HI}$ and $CAR30^{LO}$, described at the end of culture, disappeared after exposition to tumor cells.

Importantly, the intensity of CAR30 expression was periodically checked to confirm that $CAR30^{HI}$ and $CAR30^{LO}$ were clearly different. This allowed to detect that CAR30-MFI was greatly increased after 24 h of exposure to $CD30^+$ tumor cells and returned to baseline at 72 h.

The functional superiority of $CAR30^{HI}$ was repeatedly demonstrated in *ex vivo* cytotoxicity assays, but the higher percentage of PD1 and TIM-3 co-expression in $CD4^+$ T-cells raised the concern that $CAR30^{HI}$ may reach functional exhaustion more rapidly than $CAR30^{LO}$. Therefore, we evaluated $CAR30^{HI}$ and $CAR30^{LO}$ antitumor activity under stressful circumstances. To this, $CAR30^{HI}$ and $CAR30^{LO}$ T-cells were exposed to $CD30^+$ tumor cells (L540) during serial tumor challenges (up to 5 sequential antigen encounters). Once again, $CAR30^{HI}$ significantly outperformed $CAR30^{LO}$ after sequential re-exposition to tumor cells. From the first exposure, $CAR30^{HI}$ showed higher cytotoxicity, which was preserved even after a fifth antigen encounter. In contrast, $CAR30^{LO}$ showed progressive loss of cytotoxic activity and failed to eliminate tumor cells after sequential challenge (donor 1: tumor death at E:T 5:1 after 4 exposures: 0% vs. 91.96%, $CAR30^{LO}$ and $CAR30^{HI}$, respectively; donor 2: tumor death at E:T 5:1 after 5 exposures: 0% vs. 93.7%, $CAR30^{LO}$ and $CAR30^{HI}$, respectively). In addition, we found that the enhanced and persistent antitumor effect of $CAR30^{HI}$ was associated with a greater expansion over time. In contrast, we saw that $CAR30^{LO}$ progressively failed to proliferate. PD1 and TIM-3 co-expression was assessed

prior to each antigen exposure, and double-positives T-cells were almost null, both in $CAR30^{HI}$ and $CAR30^{LO}$, highlighting the fact that despite higher levels of CAR expression, these cells were not exhausted.

Then, we proved that the superior *ex vivo* antitumor effect of $CAR30^{HI}$ was correlated with a greater *in vivo* efficacy. To this, we employed the L540 mice model with the suboptimal dose, since this was the same tumor cell line with which the *ex vivo* experiments were performed. Briefly, mice were injected SC with L540 and, when tumors were well-established, mice were treated with either $CAR30^{HI}$ or CAR^{LO} T-cells (5×10^6 $CAR30^+$ T-cells per mouse). Mice treated with $CAR30^{HI}$ T-cells exhibited a significant reduction in tumor growth compared to those receiving $CAR30^{LO}$ T-cells who had progressive disease ($p < 0.01$). Moreover, $CAR30^{HI}$ achieved clinically meaningful tumor control that translated into better survival. At the end of the experiment (day 52 post-challenge), mice treated with $CAR30^{LO}$ had died from tumor progression, in contrast to 80% of those receiving $CAR30^{HI}$ who were still alive.

Finally, as a proof of concept, *ex vivo* experiments were replicated with a different CAR. We used a second-generation CAR directed against CD19 (CAR19). The recognition domain consisted of a scFv from the FMC63 antibody that was cloned into the same backbone described for CAR30. $T_{SCM\text{-}LIKE}$ T-cells enriched products were generated as explained above, under the same culture conditions as used for CAR30. T-cells were genetically engineered to express CAR19, and then sorted based on their MFI in two distinct subpopulations: $CAR19^{HI}$ with an MFI of 10810 ± 1486 and $CAR19^{LO}$ with an MFI of 1610 ± 187 ($p < 0.01$). $T_{SCM\text{-}LIKE}$ T-cells comprised the vast majority of $CAR19^+$ T-cells, both in $CAR19^{HI}$ and in $CAR19^{LO}$, with no differences between them. We assessed the antitumor effect *ex vivo* against Raji tumor cell line ($CD19^+$ human B-cell lymphoma). Remarkably, $CAR19^{HI}$ T-cells displayed superior antitumor efficacy than $CAR19^{LO}$ at different effector:target ratios (e.g., tumor death at 5:1 E:T ratio: 59.9 ± 8.72% vs. 28.8 ± 8.7%, and 21.6 ± 11.4% vs. 2.9 ± 2.9% at 1.25:1 E:T ratio, $CAR19^{HI}$ and $CAR19^{LO}$, respectively ($p < 0.0001$). These data support our findings obtained with CAR30 and suggest that they may be generalizable to other antigen-targeted CARTs.

In summary, we have designed a new second-generation CAR30 targeting an epitope in the non-cleavable membrane-proximal region of CD30, generated $CAR30^+$ T-cell products enriched for $T_{SCM\text{-}LIKE}$ cells and demonstrated that enrichment of CAR30 products in $T_{SCM\text{-}}$

$_{LIKE}$ cells led to improved antitumor efficacy. Furthermore, we documented that CAR30^{+} T-cell products enriched for $T_{SCM\text{-}LIKE}$ cells that also display high intensity of CAR30 expression have an even more enhanced antitumor effect compared to those with low intensity of CAR30 expression. This functional advantage is sustained even after unfavorable conditions without functional exhaustion or greater differentiation.

6 OVERALL SUMMARY OF THE DISCUSSION

Classical Hodgkin lymphoma (cHL) is a hematologic malignancy of germinal center B-cells, characterized by the presence of the Hodgkin Reed-Sternberg (HRS) cells, although these cells typically account for <1% of cells in the affected tissue (132,133). cHL is the most commonly diagnosed cancer among adolescents aged 15 to 19. In developed countries, there is a bimodal age distribution for cHL with a peak in the adolescent/young adult population and again after age 55 years. Older adults (i.e., 45-55 years) often present with more advanced disease and have a worse prognosis (134).

cHL is a highly treatable malignancy with 85% to 90% of early-stage and 70% to 80% of advanced-stage patients cured after initial therapy (135–137). Yet about 10%–30% of patients develop relapsed or refractory (R/R) cHL with poor prognosis (137). Standard of care for those patients who are considered candidates includes salvage chemotherapy followed by autologous stem-cell transplantation (ASCT). This strategy reported five-year OS of 55% and PFS of 44% (138). This high-intensity treatment is frequently associated with late-onset treatment-related complications that can limit long-term survival and affect quality of life, such as heart disease, pulmonary toxicity, and second malignancies (117,134). Moreover, patients who relapse or progress within 1 year after ASCT have an extremely poor prognosis with a median survival of approximately 1.3 years (139). Thus, R/R cHL constitute an unmet medical need, as do elderly patients and those not eligible for ASCT.

One of the options to optimize cHL treatment and avoid adding long-term toxicity is immunotherapy. In this way, the HRS of cHL are characterized by overexpression of CD30 protein (29). In addition, CD30 expression is restricted to a subset of activated T-cells, virally infected T-cells, B-cells and NK-cells, and eosinophils (30). The high expression of the CD30 protein in cHL tumor cells and its limited presence in normal tissues make it an ideal target for immunotherapy. In fact, CD30 has been widely investigated as a target for antibody-based therapy (140–145).

The first attempts at immunotherapy with anti-CD30 monoclonal antibodies showed disappointing results with almost no clinical response (142,143). A different approach with an antibody-drug conjugate (ADC), comprising cAC10 monoclonal antibody conjugated by a protease cleavable linker to monomethyl auristatin E (MMAE) showed *ex vivo* and *in vivo* antitumor activity against cHL (141). This ADC, Brentuximab Vedotin (BV), confirmed that cHL may be sensitive to targeted immunotherapy. R/R cHL patients treated with BV frequently achieve some type of clinical response, but complete responses are less common

(CR rate 34%) (119,120). Moreover, response is often short-lived, with the median PFS of 5.6 months and only 22% of patients being disease-free after 5 years (119–121).

On the other hand, cHLs include rare malignant HRS cells within an extensive ineffective immune cell infiltrate, that includes macrophages, eosinophils, neutrophils, mast cells, and T-cells (146). The HRS cells escape immune surveillance by expressing large amounts of inhibitory surface receptors, one of them PD-L1 (115). PD-L1 interact with PD1 and induce T-cell "exhaustion", a reversible inhibition of T-cell activation and proliferation (147). Thus, cHL may be vulnerable to PD-1 blockade that could potentially reactivate tumor microenvironment (TME) immune cells against HRS cells. In fact, nivolumab and pembrolizumab are PD-1 blocking antibodies that have shown similar antitumor activity, reaching a median PFS of around 1 year in patients with R/R cHL (124,148). Although all these results are not negligible, the short duration of clinical response highlights that it is necessary to design new therapeutic alternatives.

In recent years, clinical trials with CART19 therapy have shown high efficacy in heavily previously treated B-NHL patients, leading to its inclusion in the current treatment of R/R B-NHL patients (53–59,67–70). CART therapy directed to CD30 has also been tested in clinical trials including R/R cHL patients(118,128). Unfortunately, the initial experiences showed very modest results with the majority of patients being refractory to treatment with CART30 (118,127). The introduction of LD before CART30 administration improved results with preliminary data showing that more than half of patients achieved an initial complete response, but only 20% of them remain disease free at 2 years (128). Hence, CART therapy directed against CD30 is a promising approach to treat R/R cHL, but some challenges must be addressed to obtain long-lasting clinical responses in a larger proportion of patients; some of them are to achieve greater expansion, longer persistence, and more efficient trafficking of CAR T-cells within the tumor (118,119,128).

In this regard, many lessons have been learned from mouse and humanized anti-CD30 naked antibodies and immunotoxins that have failed to develop clinical responses despite their original preclinical success (142,143,149–152). For example, the expression of the target limited to HRS cells has been related to the failure. Since most of the tumor masses of cHL do not express CD30, there is limited access of the antibody to its target, decreasing the antitumor activity (143). Theoretically, $CAR30^{+}$ T-cells have advantage over inert molecules, due to their ability to circulate within the tumor and produce effector cytokines,

which activate and attract more immune-effector cells. The presence of CAR30$^+$ T-cells has been detected in some tumor samples from patients treated with CART30 therapy, despite having been refractory to treatment (118). Even so, some preclinical data indicates that CAR30$^+$ T-cells trafficking is suboptimal due to HRS-produced chemokines (such as TARC) that attract regulatory T-cells (153,154), suggesting that CAR30$^+$ T-cells may need additional mechanisms to migrate inside the tumor (125,126).

The absence of disease control despite finding CAR30$^+$ T-cells within tumor tissues could be associated with an ineffective receptor-target binding. In this regard we should keep in mind that CD30 is transmembrane glycoprotein member of the TNF receptor superfamily (30,155,156), its extracellular domain is released from the cell as a soluble 85-90 kDa protein by a membrane-anchored metalloprotease (33,157,158). This is an important consideration for immunotherapy because the soluble form can neutralize antibody-based therapy before they reach their target on the cell membrane (156). sCD30 is released from activated T-cells and a number of malignant lymphocytes (i.e., HRS cells) (33,158,159). In many CD30$^+$ lymphomas, serum levels of sCD30 are elevated and correlate with clinical features such as tumor burden (149).

It has been proven that sCD30 binds to CD30L with high affinity and can block transmembrane signaling by CD30 (157). To notice, high levels of sCD30 are required to neutralize the biological activity of CD30L, and such amounts might be localized within the TME of cHL (157). Many studies using therapeutic antibodies have emphasized the importance of selecting an appropriate epitope to avoid the susceptibility to competition by soluble forms of target antigens (156,160). Numerous monoclonal antibodies directed against CD30 distal epitopes have been reported to be blocked by the action of sCD30 (i.e., Ki-2, Ber-He2, Ki-4, SGN-30, MDX-060) (33,142–144,156).

Although the effectiveness of CAR30 targeting a distal epitope on CD30 in previous preclinical studies was apparently not affected by sCD30 (116,125), results of clinical trial with a CAR30 construct including an HRS-3 scFv, found an inverse correlation between sCD30 levels at the time of infusion and the subsequent expansion of CAR30$^+$ T-cells (127). As previously mentioned, the other clinical trial with a CAR30 with an AJ878606.1. derived scFv, have reported refractoriness of patients to CART30 therapy with ineffective CAR30$^+$ T-cells in cHL tissues (118). Both of these anti-CD30 scFvs recognize distal epitopes within

the cleavable part of CD30 molecule and may have a high binding affinity to sCD30, which could explain their limited antitumor activity.

In our study, we have designed a second-generation CAR targeting a membrane-proximal epitope in the non-soluble part of CD30 protein, to increase the antitumor effect and prevent CAR30 inhibition caused by the presence of sCD30 protein.

In addition, we have learned from CART19 therapy that B-NHL patients have substantial differences in their frequencies of peripheral blood T-cell subsets (naïve, T_{CM} or T_{EM}) (58,59), due to factors such as age and chemotherapy regimens received (94), and these variabilities have an impact on the apheresis cell composition, which are used as starting material for the manufacture of CAR T-cell products and, consequently, on the infused products. Even more, data from different clinical studies demonstrate that a high peak of CAR T-cell expansion and persistence in peripheral blood correlate with a durable response in B-cell neoplasms (70,75–80).

So far, CART19 products infused into patients are quite heterogeneous in terms of T-cell composition, but recent studies have been looking for the most appropriate T-cell subsets for CART therapy, and available data indicate that the use of less differentiated T-cells, results in greater antitumor efficacy (58,88,92,93,161,162), in contrast to unselected bulk T-cells (58,118,163). As T_{SCM} cells have the ability to self-renew and can produce $T_{CM,}$ T_{EM} and T_{EF} cells (89,91). To notice, T_{SCM} cells have been originally identified as $CCR7^{+}CD45RO^{-}CD45RA^{+}CD27^{+}CD95^{+}$. Then, it has been shown that when T_{SCM} are cultured *ex vivo*, they acquire the expression of CD45RO, while preserving the expression of CD45RA, CCR7, CD27, and CD95, this was defined as $T_{SCM\text{-}LIKE}$ phenotype (96).

We have postulated that, by being able to generate the entire repertoire of T-cells, $T_{SCM\text{-}LIKE}$ cells would have a greater capacity for expansion and longer persistence. Therefore, in addition to the new construction of our CAR30, we have generated CAR30 T-cell products comprised mostly by $T_{SCM\text{-}LIKE}$ cells to enhance efficacy and persistence of $CAR30^{+}$ T-cells.

In previous work, our group described *ex vivo* T-cell culture conditions to increase the frequencies and expansion of $T_{SCM\text{-}LIKE}$ cells. Specifically, we showed that if T_N cells are cultured in presence of IL-7, IL-15, and IL-21, after a very short CD3/CD28 co-stimulation, they have a higher proportion of $T_{SCM\text{-}LIKE}$ both in $CD4^{+}$ and $CD8^{+}$ T-cells (97). Here, we applied those culture conditions that we had described (97), additionally, we transduced the

T-cells with a third-generation lentiviral vector encoding our second-generation CAR30 co-stimulated with 4-1BB.

According to previous studies (125,164), the potential elimination of activated T-cells expressing CD30 (on-target off-tumor effect) by our CAR30$^+$ T-cells is not a limitation for $T_{SCM\text{-}LIKE}$ cells *ex vivo* expansion. Our CAR30$^+$ $T_{SCM\text{-}LIKE}$ enriched products exhibited an optimal cell viability and CAR30 expression. We observed a correlation between a progressive decrease in CD30 expression and a gradual increase in CAR30$^+$ T-cell viability during culture; these variations were not observed in non-transduced T-cells, which showed a superior expansion. All suggesting fratricide killing of CD30$^+$ T-cells in CAR30 T-cell culture, without adequate expansion impairment. Further research is needed to elucidate the mechanisms involved in the observed reduction of CD30 expression; internalization, or sequestration of CD30 protein in the cytoplasm of T-cells may play a role, as has been shown with other CAR targets expressed on activated T-cells (165).

We have shown great *ex vivo* effectiveness of our CAR30$^+$ $T_{SCM\text{-}LIKE}$ cells against human HL tumor cell lines. It has been described that patients with HL produce sCD30 in the serum at levels of 15–2,020 unit/ml (corresponding to 1.8–283 ng/ml CD30) (156). Remarkably, we demonstrated that our CAR30 was not blocked by the presence of high amounts of sCD30 protein (20 μg/mL, equivalent to 12 000 units/mL). We have used this elevated amount of sCD30 to ensure saturated concentrations, considering that sCD30 in TME may be even greater than in the peripheral blood of cHL patients.

Moreover, epitope location itself appears to be a determinant of CART efficacy, also for other targets, with membrane-proximal epitopes being better than those located distally (129). Thus, our CAR30 could be advantageous since is not affected by sCD30, but also it may have an enhanced cytolytic activity because of the proximal location of the targeted epitope.

Next, after having confirmed that our CAR30 *ex vivo* functionality was maintained under adverse conditions, we tested different doses of CAR30$^+$ $T_{SCM\text{-}LIKE}$ cells in two different HL *in vivo* models and found that CAR30$^+$ $T_{SCM\text{-}LIKE}$ cells products had a potent and specific antitumor efficacy against cHL, since at the appropriate dose, they could completely eliminate the tumor. Importantly, both nodal and extra nodal diseases were cleared, suggesting that CAR30$^+$ $T_{SCM\text{-}LIKE}$ cells were able to migrate efficiently to different organ

sites. We observed a long-term persistence of CAR30$^+$ $T_{SCM\text{-}LIKE}$ cells *in vivo*, that were able to fully eradicate the tumor after a second tumor challenge. After HL was eliminated for the second time, we confirmed the persistence of our CAR30$^+$ T-cells, as we were able to detect them in the lymph nodes and bone marrow of the mice. Remarkably, $T_{SCM\text{-}LIKE}$ cells remained the most frequent cellular subpopulation.

A few clinical findings have shown that, in patients who received bulk CAR19 T-cells, *in vivo* expansion has been correlated with the frequency of a T-cell subset closely related to T_{SCM} within the infusion product (99,166,167), despite being a minority population in CART19 products used so far (99,168). The idea that the memory T-cell subpopulations are more efficient in fighting cancer has been tested in animal models (92), but we have decided to prove whether a higher proportion of $T_{SCM\text{-}LIKE}$ cells is a determinant of greater *in vivo* antitumor efficacy. To do this, we challenged two different groups of CAR30$^+$ T-cells products, the first with more than 50% of $T_{SCM\text{-}LIKE}$ cells ($T_{SCM\text{-}LIKE}^H$), and the second with less than 30% of them ($T_{SCM\text{-}LIKE}^L$). We found higher persistence, trafficking to tumor and frequency of circulating CAR30$^+$ T-cells in mice receiving $T_{SCM\text{-}LIKE}^H$ products than in those treated with $T_{SCM\text{-}LIKE}^L$, which translated into a significant survival advantage. Collectively, although we did not attempt to quantify the minimum threshold of $T_{SCM\text{-}LIKE}$ cells proportion in infused CAR30 T-cell products that confer maximum antitumor activity, our data suggest that an elevated frequency of this cell subset expressing the CAR could significantly improve the outcome of CART30 therapy.

As mentioned above, we found a greater frequency of CAR30$^+$ T-cells in peripheral blood and a larger proportion of tumor-infiltrating CAR30$^+$ T-cells in mice treated with $T_{SCM\text{-}LIKE}^H$ than in those treated with $T_{SCM\text{-}LIKE}^L$, revealing an increased CAR30$^+$ T-cell traffic within the tumor. These findings corroborated our prior assumptions about the efficient migration of CAR30$^+$ $T_{SCM\text{-}LIKE}$ cells through the different tumor sites. In part, this encouraging result can be explained by previous studies showing that T-cells generated under IL-7 and IL-15 show increased expression of CXCR4 (169), a chemokine that promote T-cell migration to peripheral tissues and tumors. Under those conditions, T-cells also express CXCR3 that binds CCL5 (117,170), a chemokine secreted by HL cells. CAR T-cells capacity to migrate into the tumor, as well as their ability to overcome immunosuppressive TME, influence in CART therapy antitumor effectiveness. To get over these obstacles, other groups are working in alternative strategies such as the use of T-cells engineered to express both CAR30

and CCR4 to increase tumor homing (126), however, it is interesting to note that T-cell subpopulations within the infusion products can promote cell migration.

The observed functional differences, driven by different proportions of $T_{SCM\text{-}LIKE}$ cells, highlight the potential impact of different product characteristics on clinical outcome. Indeed, analysis of CART treatment failures has disclosed a number of possible mechanisms. Several features of the infusion product were described to be associated with antitumor effect, including T-cell exhaustion, differentiation, or memory T-cell subset (99,100,167). Also, primary resistance of tumor cells to CAR19 T-cells have been disclosed as a mechanism to explain failures in B-cell acute lymphoblastic leukemia (B-ALL) (171).

Moreover, heterogeneous types of relapses have been described after CART19 therapy (172). In some of them, tumor cells continue expressing tumor-associated antigen (TAA) and failures are mostly related to poor T-cell function or early disappearance of CAR T-cells. In others, the cancer evolves by modulating TAA expression, including loss of detectable antigen, diminished expression of TAA or changes in TAA conformation to hide or remove epitope that is recognized by CAR T-cells. These findings raised up interest in understanding CAR-TAA interaction and led to propose low antigen density of expression as a cause of CAR T-cell resistance (101,172–175).

To explain these findings, recent studies have disclosed important differences between CARs and TCRs regarding signaling and T-cell function after antigen-binding (176,177). Specifically, CARS are less efficient to promote T-cell signaling when binding to low expression TAA. Thus, CARs require larger number of ligands to promote adequate T-cell antitumor response, suggesting that an adequate balance between antigen and CAR densities would have a substantial impact on antitumor efficacy (178).

Most published data have focused on the study of tumor antigens, but we have considered that in order to obtain a powerful and functional immune synapse, the intensity of CAR expression would be as important as density of antigen to achieve a high-quality interaction. Therefore, we performed a systematic functional study of the CAR intensity of expression impact on anti-tumor efficacy and we have demonstrated that T-cells with increased CAR expression have greater antitumor effect compared to those with low CAR expression, without undergoing T-cell functional exhaustion.

We generated $T_{SCM\text{-}LIKE}$ enriched subpopulations and transduced them with our CAR30, having a good efficiency of transduction allowed us to separate $CAR30^{+}$ T-cells in two significantly different groups based on CAR30-MFI: $CAR30^{HI}$ and $CAR30^{LO}$. $CAR30^{HI}$ and $CAR30^{LO}$ were comparable regarding T-cell composition but showed dramatic differences in CAR expression intensity.

The activation profile before exposure to tumor cells showed a high expression of late activation markers with nearly absence of CD69 in both $CAR30^{HI}$ and $CAR30^{LO}$, reflecting the continuous stimulation due to culture conditions designed to promote T-cell proliferation (179). Furthermore, the expression of CD30 by some activated T-cells would have exposed our $CAR30^{+}$ T-cells to their TAA during culture, but CD30 protein expression was barely detected at the end of culture in both, $CAR30^{HI}$ and $CAR30^{LO}$. HLA-DR was more frequently expressed in $CAR30^{HI}$, suggesting a potentially more efficient cell-mediated immune response (179). Even more, HLA-DR expression in T-cells has also been reported to be related to proliferation, suppression of regulatory T-cell function and provision of necessary signals to induce responsiveness of previously resting T-cells (179–182).

We found that $CD4^{+}$ $CAR30^{HI}$ T-cells had a higher expression of inhibitory markers before antigen-exposure. Immune checkpoints (e.g., PD1 and TIM-3) are molecules shown to have immunomodulatory properties and, although initially associated to T-cell exhaustion, it has become clear that they are expressed during the normal course of T-cell activation (11,23,183). In a normal immune response, the first signal (TCR) confers specificity and a second (costimulatory signal) maintains activation, while negative co-inhibitory signals are necessary to maintain homeostasis (11,18,184). Thus, $CD4^{+}$ and $CD8^{+}$ subsets expressing inhibitory markers during an immune response are not necessarily functionally compromised (11,23). Supporting this idea, $CAR30^{HI}$ outperformed $CAR30^{LO}$ in killing HL cells *in vitro*, showing impressive cytotoxic activity even at very low ratios and demonstrating that antitumor effect was specific since no death was observed in $CD30^{-}$ tumor cells. Despite being highly activated, increased CAR30 expression *per se* did not induce further differentiation of CAR30 T-cells, as $T_{SCM\text{-}LIKE}$ remained the major subset within $CAR30^{HI}$ after tumor-antigen exposure, with no differences compared to $CAR30^{LO}$.

It is crucial to note that $CAR30^{HI}$ and $CAR30^{LO}$ were sorted only by CAR30-MFI without having been tested or stimulated by tumor-target cells, thus, this better functional performance displayed by $CAR30^{HI}$ can be accurately attributed to its higher expression of

CAR. In contrast, a previous *ex vivo* study showed that highly activated T-cells expressing high levels of CAR19 after exposure to tumor cells developed a greater cytotoxic effect against tumor cells in a second encounter, independently of their original CAR19 expression, since the separation has been performed after an antigenic exposure (185). Even more, selection was not limited to a higher expression of the CAR, but also to those with a higher CD25 expression, suggesting that the cell population was selected from those already fit for tumor killing.

But interestingly, we also found striking changes in cell surface CAR30 expression after antigen exposition with an increase of CAR expression 24 hours after antigen exposition followed by a return to baseline value 72 hours after exposure both in $CAR30^{HI}$ and $CAR30^{LO}$. These data seem to contrast with other previous studies that described a down-modulation of CAR cell surface expression after antigen binding (173), but methodological aspects including the efficacy of cytotoxicity by CAR T-cells and the kinetics of tumor-cell killing (i.e., disappearance or persistence of TAA) may explain these discordances. In fact, in agreement with our data, a transient increase in CAR expression following antigen recognition has also been reported (186,187).

These variations resemble those occurring in native TCR surface cell expression during the resting-activation state transition of T-cells. Surface cell expression of native TCR is influenced by dynamic processes such as synthesis and transport of newly assembled receptors, endocytosis and recycling to the plasma membrane of internalized receptors (endosomes) and degradation of TCR (lysosomes). These processes balance each other to ensure efficient T-cell responses while preventing autoimmunity (188,189), most of them could also influence CAR expression supporting those changes observed in CAR30. Indeed, it has been described that CAR suffers ligand-induced downmodulation as mechanisms to regulate lymphocyte function (176,177,190). Moreover, the engagement of tumor antigens induced rapid ubiquitination of CARs, followed by lysosomal degradation (190). The CAR T-cell activation triggered by antigen exposure, followed by this ligand-induced downmodulation motivated by turning to resting state after tumor cell eradication, explain the changes seen in cell surface expression of the CAR.

Next, we developed an *ex vivo* stress model to test the fitness of $CAR30^{HI}$ and $CAR30^{LO}$. We evaluated $CAR30^{HI}$ and $CAR30^{LO}$ performance after repetitive encounters with tumor-target cells, using a previously reported methodology (191). Remarkably, $CAR30^{HI}$ showed

higher antitumor efficacy which was detected from the first exposure and persisted even after five sequential re-expositions. Proportion of CART30^{+} T-cells did not significantly change after sequential antigen exposure and they were comparable between both groups, CAR30^{HI} and CAR30^{LO}, suggesting that the lower antitumor efficacy of CAR30^{LO} was not due to a decrease in the proportion of T-cells expressing the CAR. Still, CAR30^{LO} couldn't exert antitumor activity after the last tumor challenge.

The incapacity of CAR30^{LO} to eliminate tumor cells *ex vivo* after sequential re-exposition to tumor cells, was related with lower IFNγ production and a trend for lower IL-6 detection compared to CAR30^{HI}. Theoretically, a greater amount of IL-6 could lead to a higher risk of cytokine release syndrome (CRS), but we postulate that to achieve the maximum antitumor efficacy with the minimum toxicity depends on several combined factors and contrasting with the increased potential risk of CRS due to high intensity of CAR expression, there are various features of our CART product, that have been associated with lower toxicity. For instance, clinical trials with CAR19 therapy that revealed that second-generation CAR19 co-stimulated with 4-1BB showed lower rates of serious CAR-related toxicities (70,192), the use of CD8α hinge that dramatically decrease appearance of severe toxicities and (193,194), data from clinical trials evaluating safety of second-generation CAR30s, both with CD28 or 41BB co-stimulation, reported only grade 1 CRS (118,128). Lastly, a recent study revealed that T-cell products enriched in T_{SCM} subpopulations showed to be intrinsically less prone to inducing severe CRS, display superior antitumor capacity both *ex vivo* and i*n vivo*, increased expansion rates and persistence (195).

To validate the relationship between high CAR expression and efficacy, we studied the antitumor effect of a high CAR expression using CAR19 T-cells. In agreement with what we found with CAR30, CAR19^{HI} showed superior *ex vivo* cytotoxic activity compared to CAR19^{LO}, and this was remarkable at low effector:target ratios. While expression of CD30 on cultured CAR30 T-cells precludes the study of tonic signaling, we have found no constitutive activation of CAR19^{HI} cells, suggesting that a high CAR expression on the cell surface does not cause tonic signaling *per se*. Previous studies showed that an increased cell surface CAR expression was one of the factors involved in constitutive, antigen-independent proliferation. However, a detailed analysis further suggested that this phenomenon (i.e., tonic signaling) was dependent on the scFv used and largely restricted to CD28-costimulated CARs (196,197). Our data extends and highlight this concept, since we have demonstrated

that CD30 or CD19-targeted CAR T-cells co-stimulated with 4-1BB that have a high CAR expression do not experience tonic signaling.

We tested CAR30^{HI} and CAR30^{LO} in our stress *in vivo* model in which animals were treated with a suboptimal dose of CAR30 T-cells. Once again, CAR30^{HI} treatment showed a significant *in vivo* better tumor control with increased survival compared to CAR30^{LO}. In agreement with our results, a study with a CAR19 co-stimulated with 4-1BB confirmed that CART failure was accompanied by preexisting T-cell intrinsic defects or dysfunction acquired after infusion and that CAR19 extinction is associated with decreased expression of the transgenic protein. In addition, they found that intensity of CAR19 expression was significantly higher in responding patients compared to that found in refractory patients (198). As stated above, engagement of tumor antigens caused CAR down-modulation followed by lysosomal degradation. Blockade of CAR ubiquitination has been shown to markedly repress CAR down-modulation while enhancing recycling of internalized CARs back to the cell surface, leading to superior persistence *in vivo* and, thus, promoting their long-term killing capacity (190). Moreover, a recent study negatively correlated CAR expression level with tumor burden in an *in vivo* model, suggesting that cell-surface CAR was downregulated in proportion to tumor antigen and proposed the link between CAR expression levels and tumor control. From this study emerges the statement that tight transcriptional regulation of CAR expression is critical for effective tumor eradication (199).

Further research is needed to determine the intrinsic mechanisms that cause CAR^{HI} enhanced antitumor efficacy but, it seems that having a greater amount of CAR molecules in cell surface allows to hold antitumor capacity despite ligand-induced downmodulation. In addition, CAR T-cells has been described to induce intracellular signaling pathways rapidly with the functional consequence of having a faster lytic granule recruitment to the immune synapse than TCR and, consequently, faster detachment from dying tumor cells (177). This allows CAR T-cells to sequentially kill target cells (200), meaning that several successive immune synapses must be stablished. Since CARs are internalized and degraded after antigen binding (176,190), the original number of CAR molecules available, could determine the final number of efficient immune synapses, being these higher in CAR^{HI}. This would have influenced the inability of CAR30^{LO} to achieve cytotoxicity after repeated encounters with tumor targets.

Collectively, we have demonstrated $T_{SCM\text{-}LIKE}$ cells can be efficiently transduced and *ex vivo*-expanded with a novel second-generation CAR30 directed against an epitope in the non-cleavable membrane-proximal region of CD30 and co-stimulated win 41BB. Our CAR30 is effective despite the presence of sCD30, surpasses fratricide killing of $CD30^+$ T-cells and confers potent *ex vivo* and *in vivo* antitumor efficacy against cHL. We have proven that products that are highly enriched for $T_{SCM\text{-}LIKE}$ cells have enhanced antitumor effect and tumor trafficking compared to those composed predominantly of differentiated T-cell subsets. Furthermore, we have systematically studied $CAR30^+$ $T_{SCM\text{-}LIKE}$ cells according their CAR30 intensity of expression and established that those having a high intensity of CAR30 expression have superior, long-lasting antitumor effect, even after unfavorable conditions, without functional exhaustion or further differentiation. Our preclinical studies support the use of $CAR30^+$ products enriched for $T_{SCM\text{-}LIKE}$ cells to improve effectiveness of CART30 therapy in cHL patients. Additionally, our data shows that CAR expression intensity should be considered as an additional factor to improve efficacy of CART30. We postulate that identification and combination of different favorable product features is the key to optimizing the efficacy of CART30 therapy.

7 CONCLUSIONS

1. It is feasible to generate an adoptive cell therapy product composed mainly of memory "stem" T-cells ($T_{SCM\text{-}LIKE}$) genetically modified to express a second-generation chimeric antigen receptor directed against a membrane-proximal epitope in the non-soluble part of the CD30 protein (CAR30) for the treatment of classical Hodgkin lymphoma (HL).

2. The products enriched for $T_{SCM\text{-}LIKE}$ cells engineered to express our CAR30 exhibit a significant *ex vivo* cytotoxic effect against human HL tumor cell lines that is maintained in the presence of saturating concentrations of soluble CD30 protein.

3. At an appropriate dose, our CAR30 T-cell products enriched for $T_{SCM\text{-}LIKE}$ cells were able to completely eradicate HL in two different *in vivo* models. Our $CAR30^{+}$ $T_{SCM\text{-}LIKE}$ cells achieved a good expansion, persistence and trafficking to different tumor sites allowing them to clear HL after a second *in vivo* challenge. CAR30 T-cell products highly enriched for $T_{SCM\text{-}LIKE}$ displayed a greater *in vivo* antitumor efficacy than those mainly comprised by effector T-cells.

4. CAR T-cell products enriched for $T_{SCM\text{-}LIKE}$ cells engineered to express a second-generation CAR co-stimulated with 4-1BB directed against CD30 (CAR30) exhibiting high intensity of CAR expression have superior specific antitumor *ex vivo* effect against tumor target cells, which is sustained after repetitive exposures to tumor cells and is not associated with T-cell exhaustion or differentiation. The impact of a high intensity of CAR expression on antitumor effect have been verified *ex vivo* using a second model (CAR19).

5. The higher antitumor efficacy of our CAR30 T-cell products with high intensity of CAR30 expression was also found in an *in vivo* model of HL, where those products achieve better control of tumor growth and increased survival.

8 FUTURE RESEARCH LINES

The encouraging pre-clinical data, that have been exposed throughout this doctoral thesis, have allowed us to propose the development of a clinical trial to test the safety and efficacy of our CAR30 T-cell product, enriched for $T_{SCM\text{-}LIKE}$ T-cells (HSP-CAR30), for the treatment of relapse or refractory classical Hodgkin lymphoma and $CD30^+$ T-cell lymphoma.

8.1 Production of HSP-CAR30 according to Good Manufacture Practices: validation process

At first, we verified that we were able to produce our CAR30 T-cells products in compliance with Good Manufacture Practices (GMP) conditions using a semi-automated and semi-closed system, the CliniMACS Prodigy (Miltenyi Biotech, Bergisch Gladbach, Germany). We obtained leukapheresis from three different healthy donors with prior informed consent. Fresh leukapheresis was used as starting material, $CD4^+$ and $CD8^+$ T-cells were positively selected and then, T-cells were activated with CD3/CD28 and transduced with a third-generation lentivirus encoding our second-generation CAR directed against CD30 and co-stimulated with 41BB. Transduced T-cells were cultured with IL-7, IL-15, and IL-21 until day 10.

We managed to make the three HSP-CAR30 products, thus validating the manufacturing process. Table 3 summarizes some of the product features that were considered as product specifications approved by the Spanish Agency of Medicines and Medical devices (AEMPS)

Table 3. HSP-CAR30 T-cell product general specifications

ASSESSMENT	SPECIFICATIONS
Dose	2×10^6 - 10×10^6 $CAR30^+$T-cells/Kg
Purity	≥ 20% $CAR30^+$T-cells
Viability	≥ 70 %
Potency	More than 20% positive specific cytotoxicity
Sterility	Sterile

8.2 Phase I clinical trial design

We have designed a phase I clinical trial to assess feasibility and safety of HSP-CAR30 products (identify dose-limiting toxicities) and, to determine maximum tolerated dose. Secondary endpoints included overall and complete response rates after HSP-CAR30 administration.

We conducted a phase 1 dose-escalation study with a classical 3+3 design. Three cell-dose levels were evaluated in 3 cohorts: DL1 ($3x10^6$ $CAR30^+$ T-cells/kg), DL2 ($5x10^6$ $CAR30^+$ T-cells/kg) and DL3 ($10x10^6$ $CAR30^+$ T-cells/kg. Patients were sequentially enrolled, and a 30-day period was established between each administration of HSP-CAR30 to assess adverse effects. The clinical trial scheme is described in Figure 16.

We have included patients with relapsed or refractory classical Hodgkin lymphoma who have been treated with chemotherapy, brentuximab vedotin and anti-PD1 monoclonal antibodies. In addition, we treated patients with relapsed or refractory non-Hodgkin T-cell lymphoma including: ALK+ and ALK- anaplastic large T-cell lymphoma and peripheral T-cell lymphoma (NOS and angioimmunoblastic) after confirming CD30 expression in more than 95%. of the tumor cells.

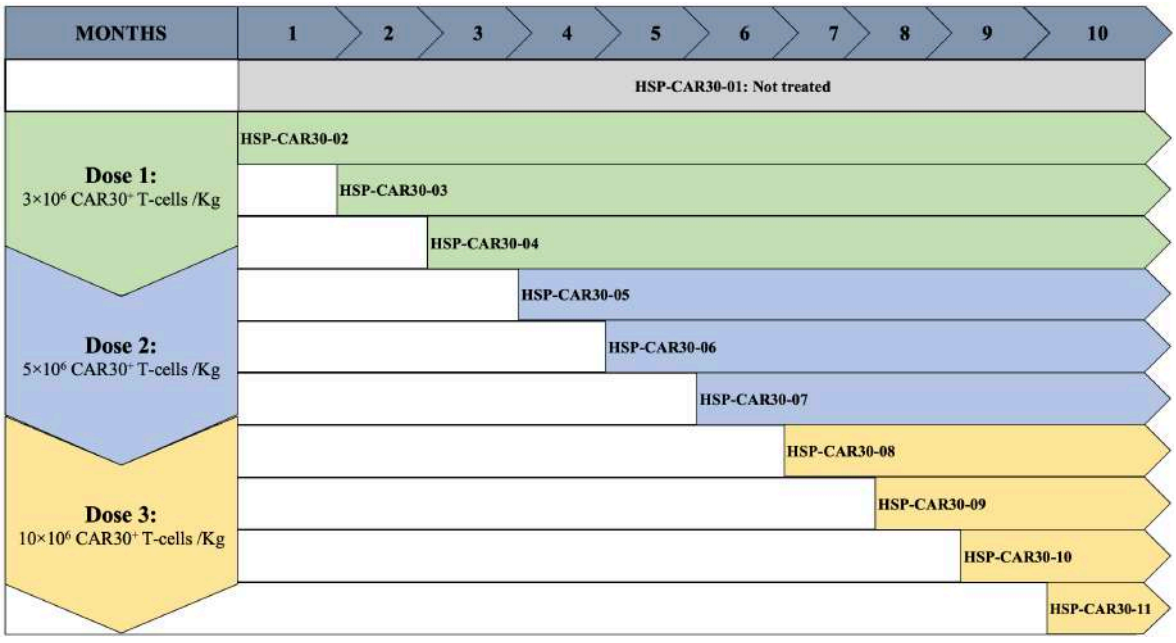

Figure 16: Phase I clinical trial design. Dose escalation clinical trial evaluating 3 different doses of HSP-CAR30. Patients were enrolled sequentially with a 30-day observation between them to assess for adverse events. They were distributed in 3 cohorts. In the first cohort, 3 patients were treated with $3x10^6$ $CAR30^+$ T-cells/kg. In the second one, 3 patients received $5x10^6$ $CAR30^+$ T-cells/kg. In the third cohort, $10x10^6$ $CAR30^+$ T-cells/kg were infused into 4 patients. One patient (HSP-CAR30-01) did not receive the treatment due to production failure.

To be include in the clinical trial patients had to meet all criteria detailed in Table 4, while not presenting any of the exclusion criteria summarized in Table 5.

Table 4. Main phase I clinical trial inclusion criteria

INCLUSION CRITERIA	
DISEASE	
Classical Hodgkin lymphoma	Anaplastic large T-cell lymphoma (ALK^+/ALK^-) and peripheral T-cell lymphoma (NOS and angioimmunoblastic):
- Relapsed after ASCT and who have received BV and anti-PD1 monoclonal antibodies, without achieving CR. - Primarily refractory patients (after first line of treatment) and who do not achieve CR after salvage treatments including BV and anti-PD1 monoclonal antibodies.	- CD30 expression determined by immunohistochemistry on >95% of tumor cells for peripheral T-cell lymphoma. - Relapsed after an ASCT - Primarily refractory patients (after first line of treatment including anthracycline) who do not achieve CR after salvage chemotherapy (i.e., ESHAP, ICE, DHAP, GemOx or BV type).
All patients must sign informed consent, before starting any procedure.	
All patients must have measurable disease (detected by PET-CT) at the time of inclusion.	
18 - 70 years old	
ECOG 0-1	
FEV1 > 39%; DLCO and FVC > 39% of normal theoretical values.	
Absence of significant ventricular dysfunction, left ventricular ejection fraction > 45%	
Total bilirubin and transaminases < 3 times the maximum normal value.	
Creatinine < 2 times the normal maximum value and glomerular clearance > 40 mL/min.	
Absence of clinically relevant active bacterial or viral infection.	

ASCT: autologous stem cell transplantation; BV: brentuximab vedotin; CR: complete response; NOS: not otherwise specified; ESHAP: etoposide, cytarabine, cisplatin and methylprednisolone; ICE: ifosfamide, etoposide, carboplatin; DHAP: cytarabine, cisplatin, dexamethasone; GemOx: gemcitabine, oxaliplatin; ECOG: Eastern Cooperative Oncology Group Performance Status Scale; FEV1: Forced expiratory volume during the first second, DLCO: diffusing capacity of the lungs for carbon monoxide, and FVC: Forced vital capacity.

Table 5. Main exclusion criteria of the phase I clinical trial

EXCLUSION CRITERIA
ECOG 2 – 4
Previous allogeneic hemopoietic transplant.
Active HBV or HCV infection.
HIV infection
Active bacterial, fungal, or viral infection
Active CNS infiltration by lymphoma.
Presence of cirrhosis or active hepatitis due to the HBV or HCV virus.
Concomitant severe neurological or psychiatric disease.
Active autoimmune or rheumatic disease that requires systemic treatment.
Major surgery 6 weeks prior to the inclusion.
Any concomitant antineoplastic treatment.
Pregnant patients.

8.3 Patients' characteristics

From October 2020 to January 2022, 11 patients (9 R/R cHL and 2 R/R NHL-T) were enrolled and underwent leukapheresis. Of these, 10 patients received HSP-CAR30: 3 at DL1, 3 at DL2 and 4 at DL3. One patient did not received treatment due to lack of T-cell expansion. Demographic characteristics and baseline disease features are summarized in Table 6.

Among infused patients, median age was 50 years (range 21–65). Median number of prior lines of treatment was 5 (range 3–7). Disease status before infusion was progression disease (PD) in all patients and most of them were in advanced stage according to Ann Arbor classification (7 stage IV, 1 stage III). Extra-nodal disease involved bone, lung, skin, subcutaneous tissue, muscle, and gastrointestinal tract (stomach). Six patients were refractory to BV treatment and 4 of them achieved objective response after BV (3 CRs and 1 PR). Seven patients with cHL were also refractory to anti-PD-1 monoclonal antibodies.

Table 6. Demographic characteristics and baseline disease features

COHORT		DL1			DL2			DL3			
Patient Nº	HSP-CAR30-01	HSP-CAR30-02	HSP-CAR30-03	HSP-CAR30-04	HSP-CAR30-05	HSP-CAR30-06	HSP-CAR30-07	HSP-CAR30-08	HSP-CAR30-09	HSP-CAR30-10	HSP-CAR30-11
SEX	M	M	F	M	M	M	M	M	M	M	M
Age (years)	38	42	65	49	48	43	38	63	65	65	21
Diagnosis	cHL	cHL	T-NHL	cHL	cHL	cHL	cHL	cHL	T-NHL	cHL	cHL
Stage	IIA	IVA	IVA	IIA	IIA	IVA	IVB	IVA	IVA	IVA	IIIA
Extra-nodal sites	-	SC tissue Muscle Bone	Stomach Lung	-	-	Bone Lung	Bone	Bone	Skin SC tissue Muscle	Bone	-
Prior lines Nº	8	4	7	5	4	5	4	5	3	5	4
Refractory to prior line	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No	No	Yes
Prior ASCT	Yes	No	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
Bridging therapy	Yes	No	Yes	No	No	No	No	No	No	Yes	No
Refractory to BV	Yes	No	Yes	No	No	Yes	Yes	Yes	Yes	Yes	No
Refractory to Anti-PD1	Yes	Yes	No	Yes	No	Yes	Yes	Yes	-	No	Yes

ASCT: autologous stem cell transplantation; BV: brentuximab vedotin; M: Male; F: Female; cHL: classical Hodgkin lymphoma; NHL-T: T-cell non-Hodgkin lymphoma; SC: subcutaneous.

8.4 Summary of procedures for HSP-CAR30 treatment

Figure 17 sums up the patient journey within the clinical trial from enrollment to HSP-CAR30 administration. After a screening period of maximum 30 days, all patients who met inclusion criteria underwent leukapheresis. As described above, HSP-CAR30 products were manufactured under GMP. First, $CD4^+$ and $CD8^+$ T-cells from fresh leukapheresis were selected and then, activated with CD3/CD28 and engineered with a lentiviral vector to express our CAR30. T-cells were cultured in the presence of IL-7, IL-15, and IL-21. Manufacturing was successful in 10/11 patients and all products reached the target dose by day 10 of culture. One patient did not received treatment due to absence of growth of T cells.

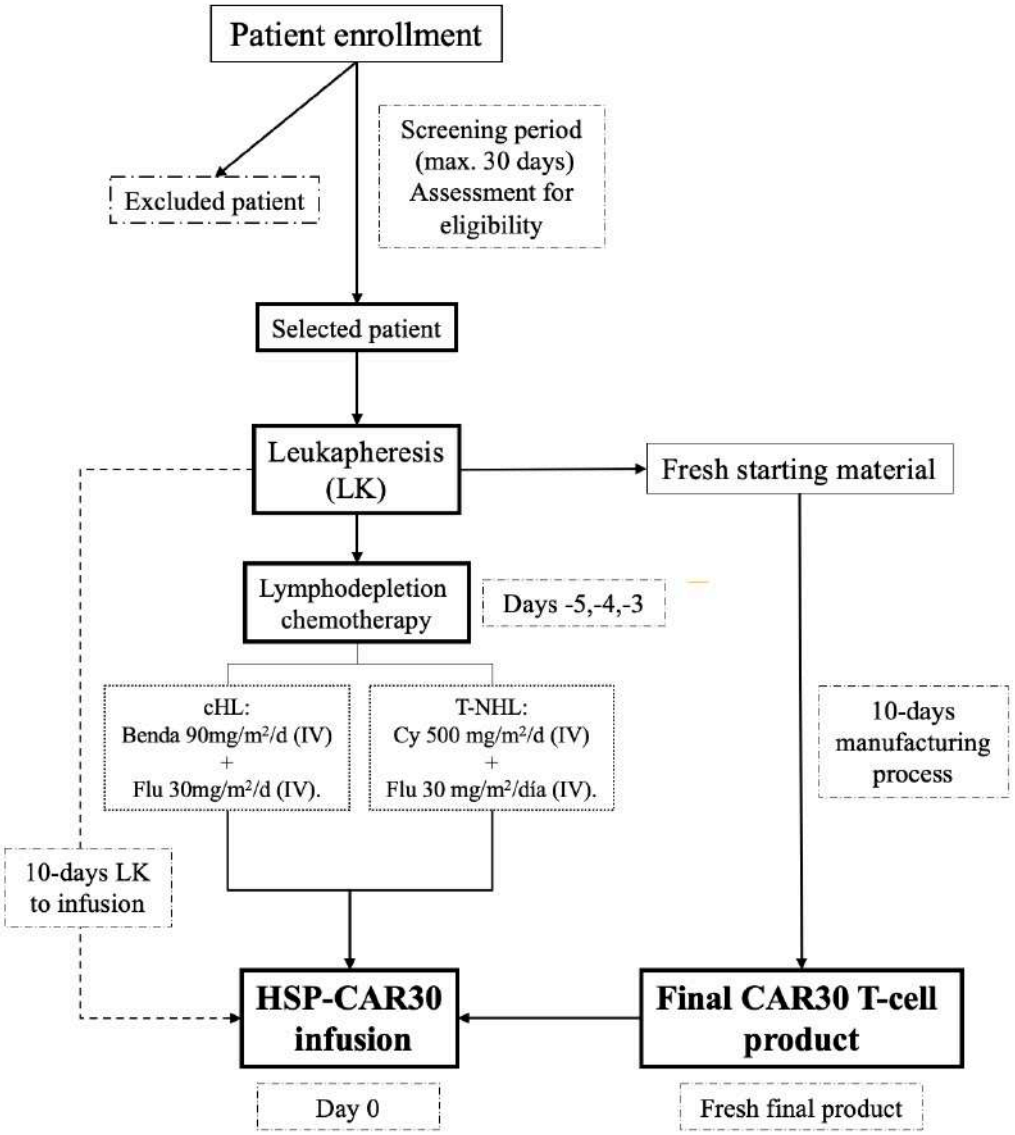

Figure 17: Flowchart summarizing procedures in the Phase I clinical trial with HSP-CAR30. LK: leukapheresis; cHL: classical Hodgkin lymphoma; T-NHL: T-cell non-Hodgkin lymphoma; Benda: Bendamustine; Flu: fludarabine; Cy: cyclophosphamide.

All patients received lymphodepleting chemotherapy (LD) before infusion. Patients with cHL (n=8) received Bendamustine 90mg/m^2/d (IV), in combination with Fludarabine 30mg/m^2/d (IV) for 3 days. Cyclophosphamide 500 mg/m^2/d (IV) and Fludarabine 30 mg/m2/día (IV) for 3 days were used as LD for T-NHL patients (n=2). HSP-CAR30 products were infused without cryopreservation.

8.5 CAR30 T-cell products features

Mean viability of HSP-CAR30 product was 89.53±3.88% (±SD) and mean CAR30 expression was 94.79±3,38% (±SD). Infusion products had a high proportion of CAR30$^+$ memory T-cells: 26.09±6.06% memory stem (T_{SCM}) and 67.52±6.07% central memory (T_{CM}) in CD4$^+$, while T_{SCM} represented 42.2±5.4%, and T_{CM} 50.5±5.7% of CD8$^+$ CAR30$^+$ T-cells.

The main specifications of each infused HSP-CAR30 product are described in Table 7.

Table 7. Main specifications of HSP-CAR30 Products

Cohort (dose)	Patient Nº	Viability (%)	CAR30$^+$ T-cells (%)	Total CAR30$^+$ T-cells received (Nº)
DL1 (3x10^6/kg)	**HSP-CAR30-02**	92.18	97.41	234x10^6
	HSP-CAR30-03	91.2	93.3	204x10^6
	HSP-CAR30-04	93.22	96.76	360x10^6
DL2 (5x10^6/kg)	**HSP-CAR30-05**	91.04	92.54	495x10^6
	HSP-CAR30-06	86.17	88.36	385x10^6
	HSP-CAR30-07	92.91	90.62	435x10^6
DL3 (10x10^6/kg)	**HSP-CAR30-08**	90.06	96.26	970x10^6
	HSP-CAR30-09	92.12	97.92	720x10^6
	HSP-CAR30-10	83.22	98.11	730x10^6
	HSP-CAR30-11	83.18	96.58	940 x10^6

8.6 HSP-CAR30 safety assessment

HSP-CAR30 was well tolerated; there were no dose limiting toxicities (DLTs). Relevant adverse events are shown in Table 8. Cytokine release syndrome (CRS) was observed in 6 (60%) patients (all grade 1). No patient developed neurotoxicity. Self-limited skin rash was

seen in 4 (40%) patients. One patient with history of cytomegalovirus (CMV) infections had CMV pneumonia. Another patient developed pulmonary tuberculosis. Long-lasting cytopenias (>3 months) occurred in 2 patients, with a complete recovery at 5 months in one of them. The other was diagnosed with myeloid neoplasm post-cytotoxic therapy (MN-pCT) with complex karyotype, considered to be unrelated to CART30 therapy.

Table 8. Adverse events after HSP-CAR30 infusion

PATIENT	**CRS** *(Grade)*	**CRS onset day**	**CRS duration** *(days)*	**ICANS** *(Grade)*	**Infections**		**Rash** *(BS %)*	**Grade 3 or 4 cytopenias**		
					(Grade)	*(Type)*		*Anemia*	*Thrombopenia*	*Neutropenia*
HSP-CAR30-02	Yes (1)	1	1	No	Yes (4)	Mycobacteria (TB)	No	Yes (3)	Yes (4)	Yes (4)
HSP-CAR30-03	Yes (1)	21	2	No	Yes (1)	Viral (Rhinovirus)	Yes (27)	Yes (3)	No	Yes (4)
HSP-CAR30-04	No			No	Yes (1)	Viral (SarsCov2)	No	No	No	No
HSP-CAR30-05	No	-	-	No	No	-	No	No	No	Yes (4)
HSP-CAR30-06	No	-	-	No	No	-	No	No	No	Yes (4)
HSP-CAR30-07	Yes (1)	0	2	No	Yes (3)	Viral (CMV)	Yes (18)	Yes (3)	Yes (4)	Yes (4)
HSP-CAR30-08	No	-	-	No	No	-	Yes (45)	No	No	No
HSP-CAR30-09	Yes (1)	1	2	No	No	-	Yes (36)	No	No	Yes (4)
HSP-CAR30-10	Yes (1)	0	3	No	No	-	No	Yes (3)	No	Yes (3)
HSP-CAR30-11	Yes (1)	2	2	No	No	-	No	Yes (3)	Yes (4)	Yes (4)

CRS: cytokine release syndrome; ICANS: Immune effector Cell-Associated Neurotoxicity Syndrome; BS: body surface; TB: tuberculosis; CMV: cytomegalovirus.

8.7 Preliminary efficacy data for HSP-CAR30

Best objective response was 100%, including 5 (50%) patients with CR, all of them with cHL (DL1=1; DL2=3; DL3= 1). At data cutoff (December 23rd, 2022), the mean follow-up of living patients is 463 days (range 263 – 665) and median PFS was 372 days. All CRs were maintained at last follow-up. Four patients died, 3 of them due to progressive disease (2 T-NHL and 1 cHL) and one with refractory MN-pCT while in CR of his cHL.

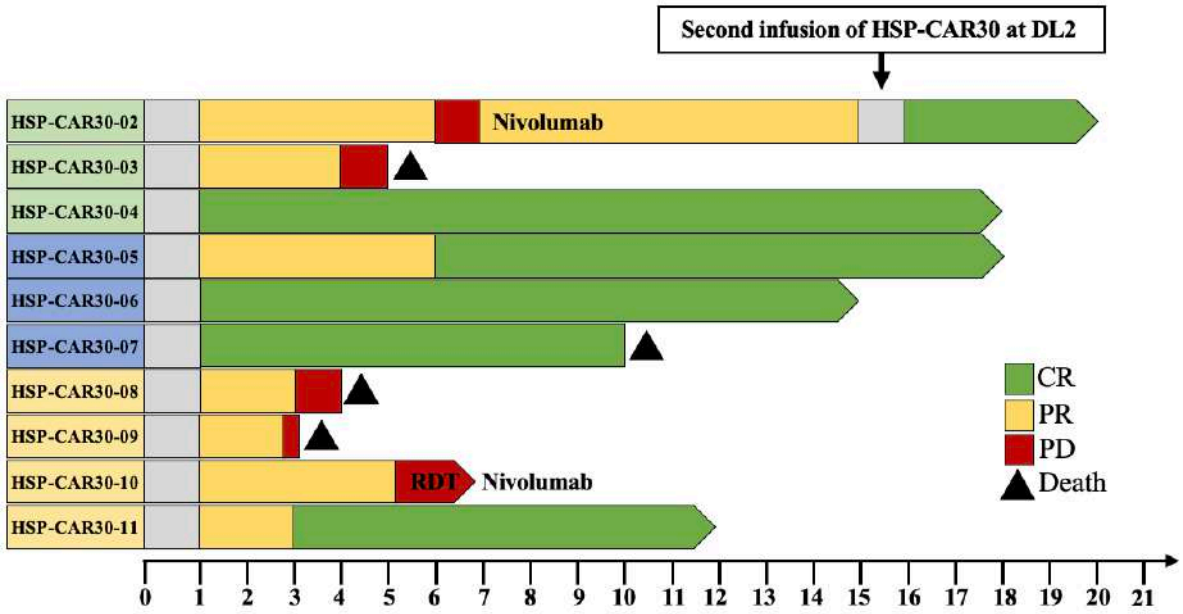

Figure 18: HSP-CAR30 efficacy assessment. All treated patients responded. Initially, we found that seven patients had PRs and three of them, CRs. Two of the PRs deepened during follow-up, becoming CRs. Thus, making a total of 50% of CRs. HSP-CAR30-02, HSP-CAR30-08 and HSP-CAR30-10 had cHL progression. While HSP-CAR30-03 and HSP-CAR30-09 had a reappearance of T-NLH. HSP-CAR30-02 received Nivolumab as rescue treatment achieving PR. Subsequently, he was treated with a second HSP-CAR30 infusion with $5x10^6$ $CAR30^+$ T-cells/kg and CR was achieved (5 moths follow-up). At first, HSP-CAR30-10 had localized disease progression and received targeted radiotherapy (RDT), but later developed systemic progression and Nivolumab was chosen as rescue treatment (ongoing). HSP-CAR30-03, HSP-CAR30-08 and HSP-CAR30-09 died due to disease progression (2 T-NHL and 1 cHL). One patient (HSP-CAR30-07) died of a myeloid neoplasm post-cytotoxic therapy while cHL CR was maintained. PR: partial response, CR: complete response, cHL: classical Hodgkin lymphoma; T-NHL: T-cell non-Hodgkin lymphoma; RDT: radiotherapy.

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