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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

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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

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

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.

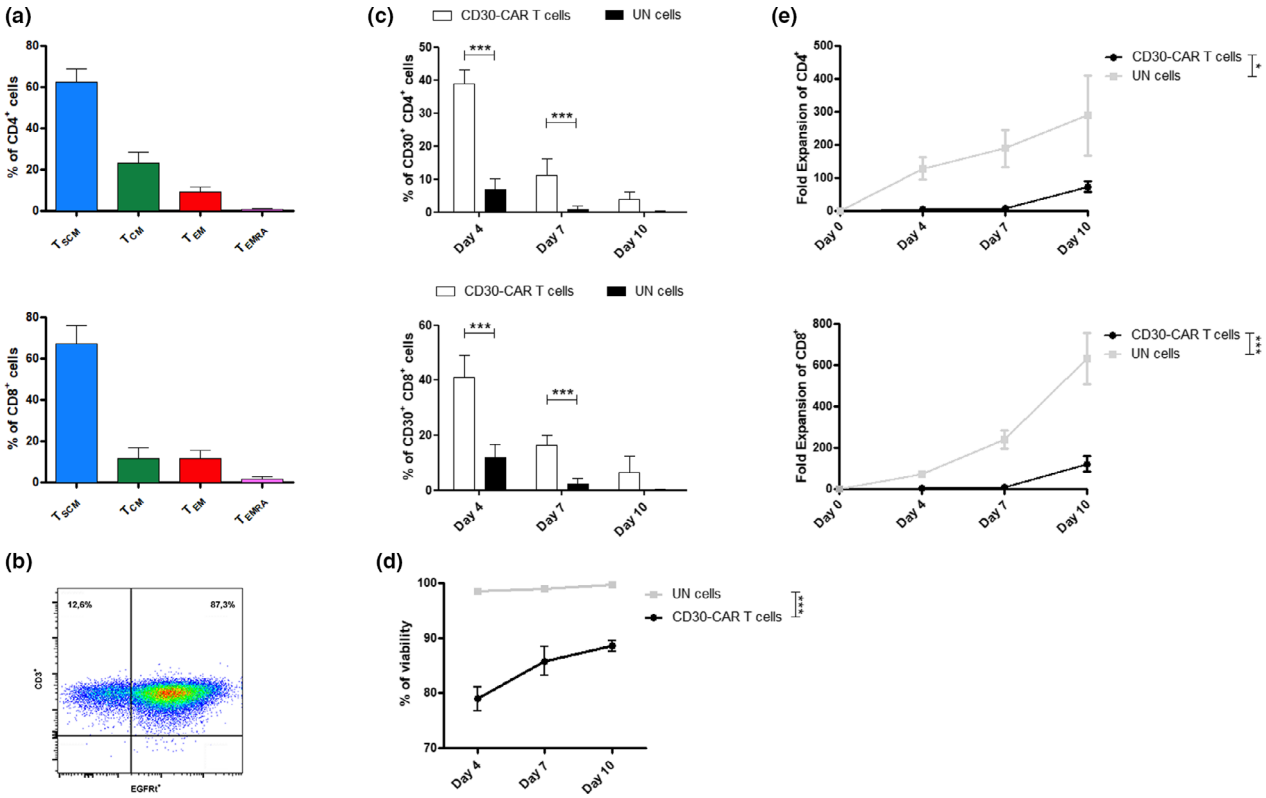

Figure 1. $T_{SCM\text{-}like}$ are highly enriched, CD30-CAR-transduced and expanded *in vitro* despite CD30 expression. Naïve T cells from healthy donors (n = 7) were cultured with CD3/CD28 costimulation in the presence of IL-7, IL-15 and IL-21 during 10 days. **(a)** Frequencies of $CD4^+$ and $CD8^+$ 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.

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

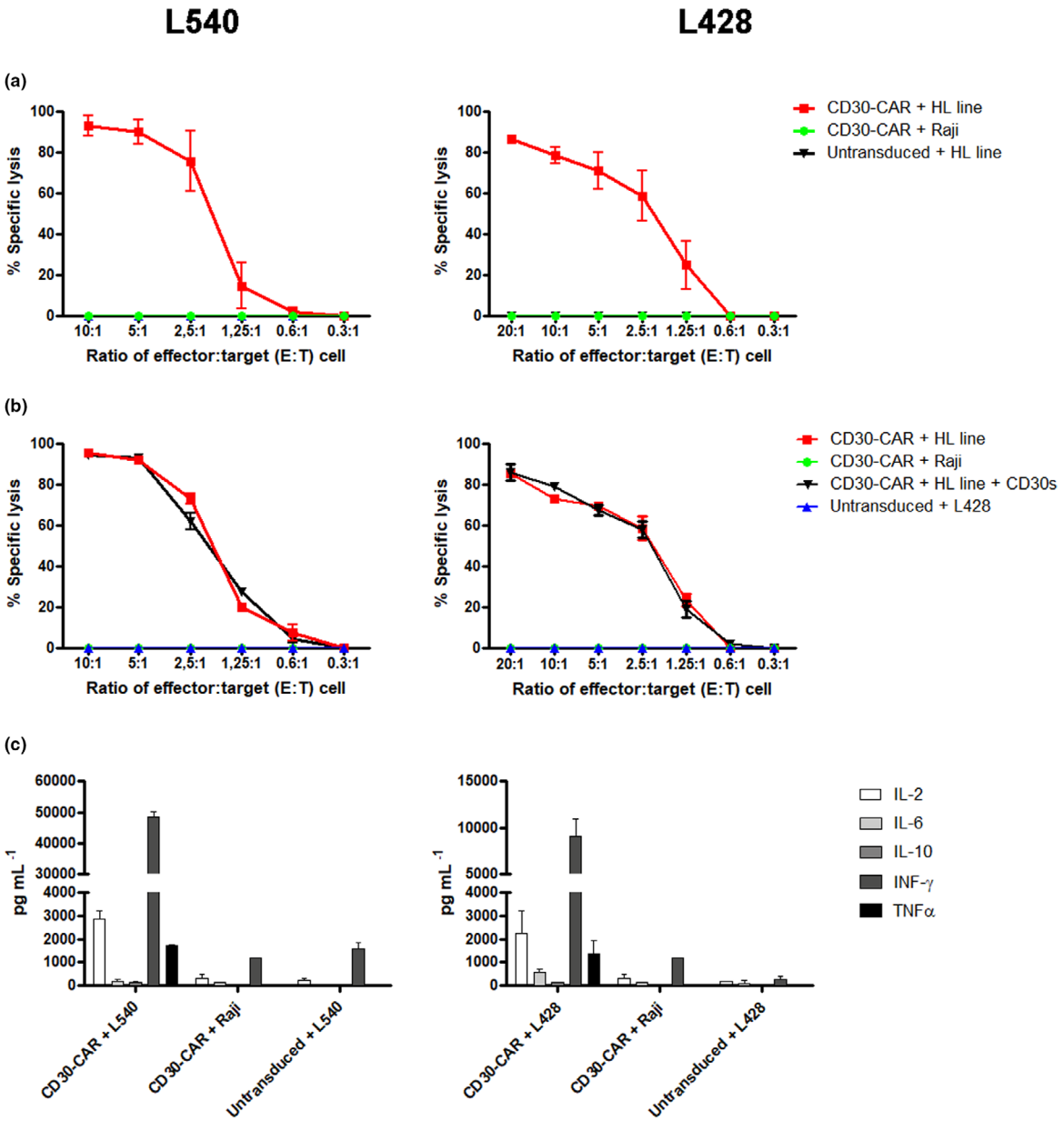

Figure 2. CD30-CAR $T_{SCM\text{-}like}$ enriched culture efficiently eradicates HL *in vitro*. **(a)** $T_{SCM\text{-}like}$ enriched cells expressing CD30-CAR (n = 7) were exposed to $CD30^+$ target cells (L540 or L428 tumor cells) and control $CD30^-$ cell line (Raji) at different effector:target (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

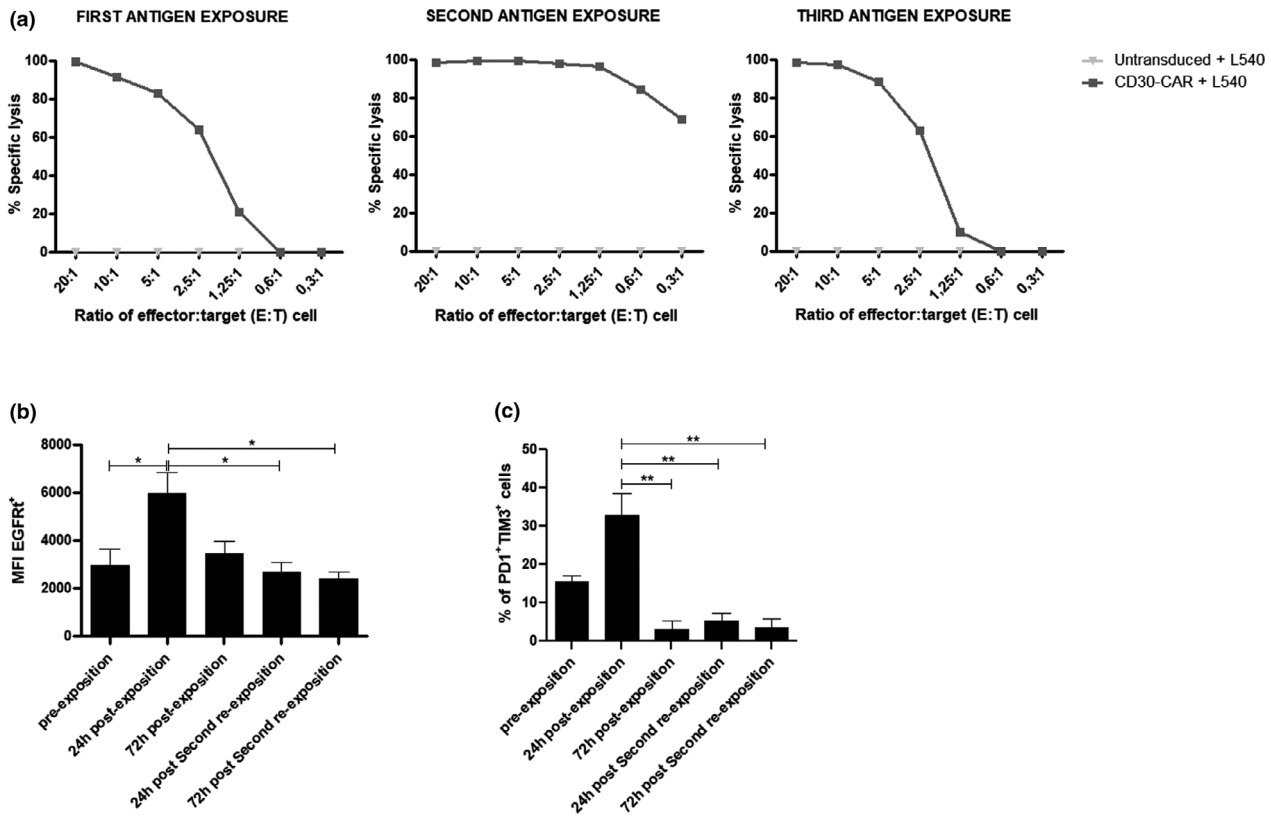

Figure 3. CD30-CAR $T_{SCM\text{-}like}$ function persists after $CD30^+$ antigen re-exposition. **(a)** Representative graphic of untransduced and CD30-CAR $T_{SCM\text{-}like}$ enriched cells exposed to $CD30^+$ target cells (L540) at different effector:target (E:T) ratios every 72 h after each antigen 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-CAR^+ in lymphoid organs of mice that survived after L428 tumor rechallenge. We detected the presence of CD30-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).

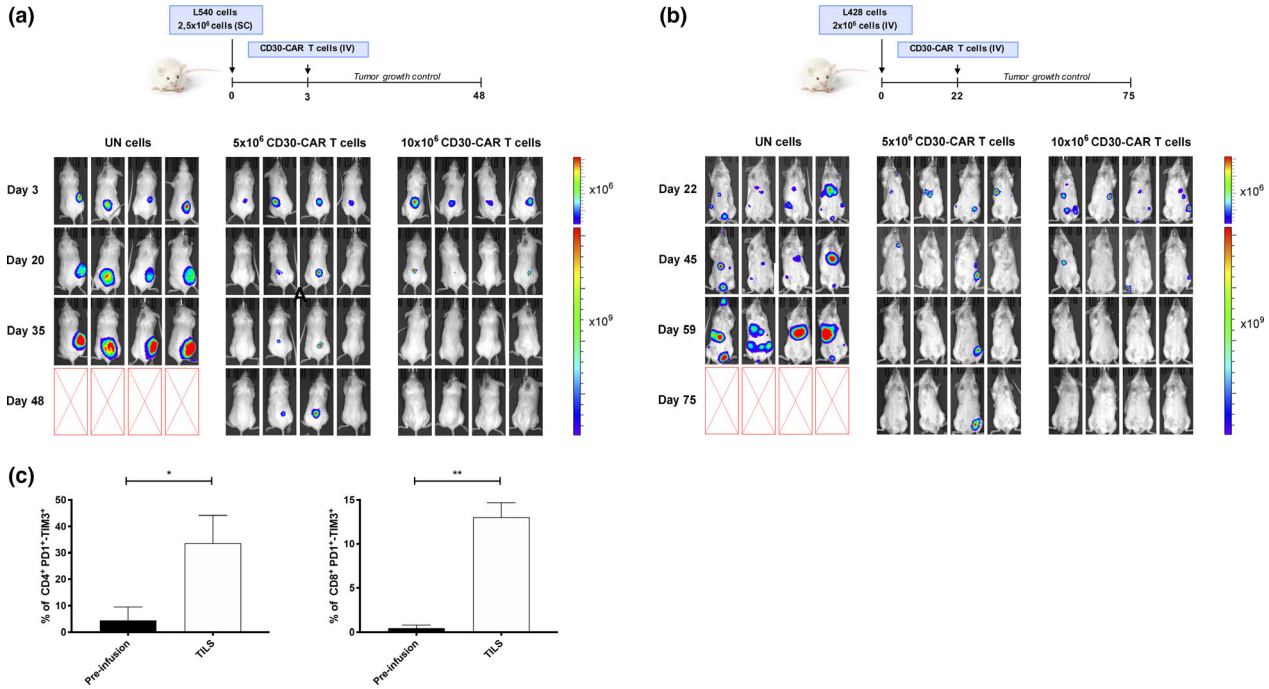

Figure 4. Therapeutic treatment with CD30-CAR $T_{SCM\text{-}like}$ enriched cell products induces a potent antitumor response against HL *in vivo*. NSG mice ($n = 4$ for both experimental and control groups) were injected with **(a)** 2.5×10^6 L540 tumor cells (sc) on day 0 and treated 3 days later 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

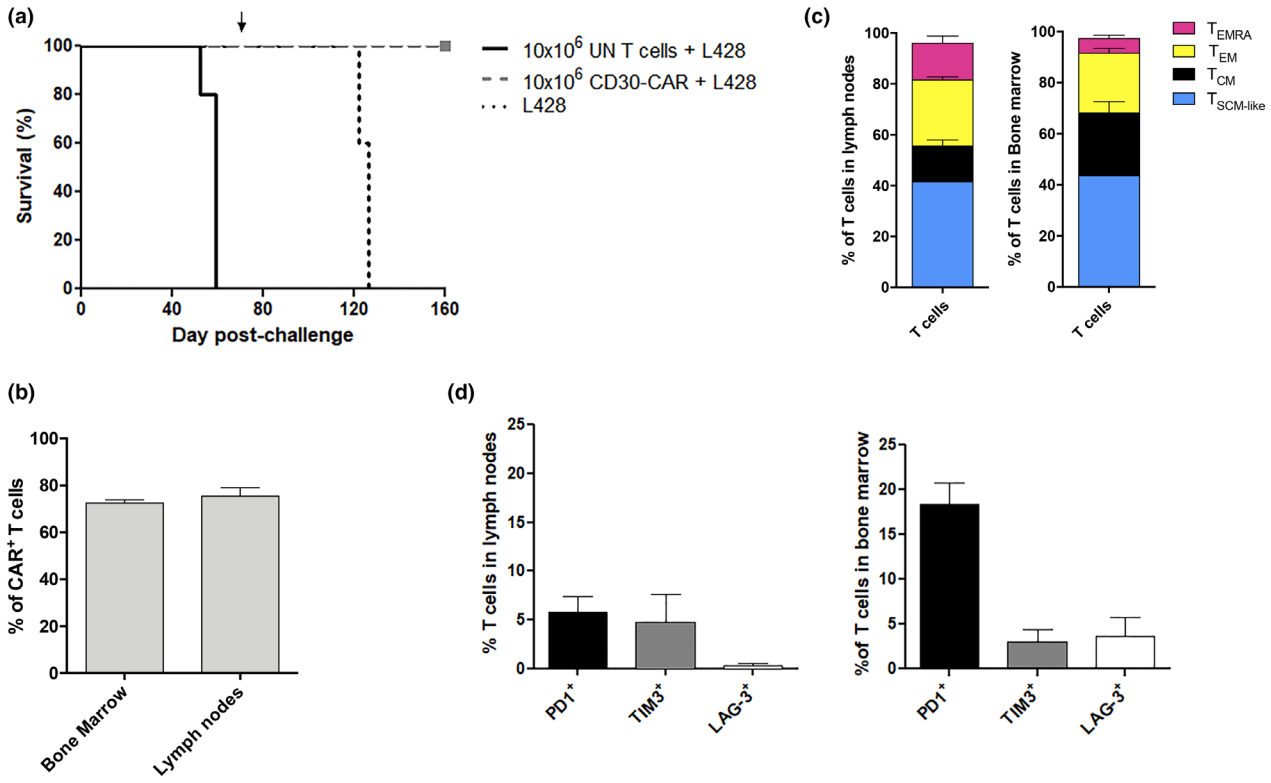

Figure 5. $T_{SCM\text{-}like}$ enriched cells expressing CD30-CAR have high persistence *in vivo* and confer long-lasting immunity against HL. **(a)** NSG mice surviving from L428 tumor administration ($n = 4$) were rechallenged again with the same tumor dose (2×10^6 cells per mouse, iv), 79 days (arrow) 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,

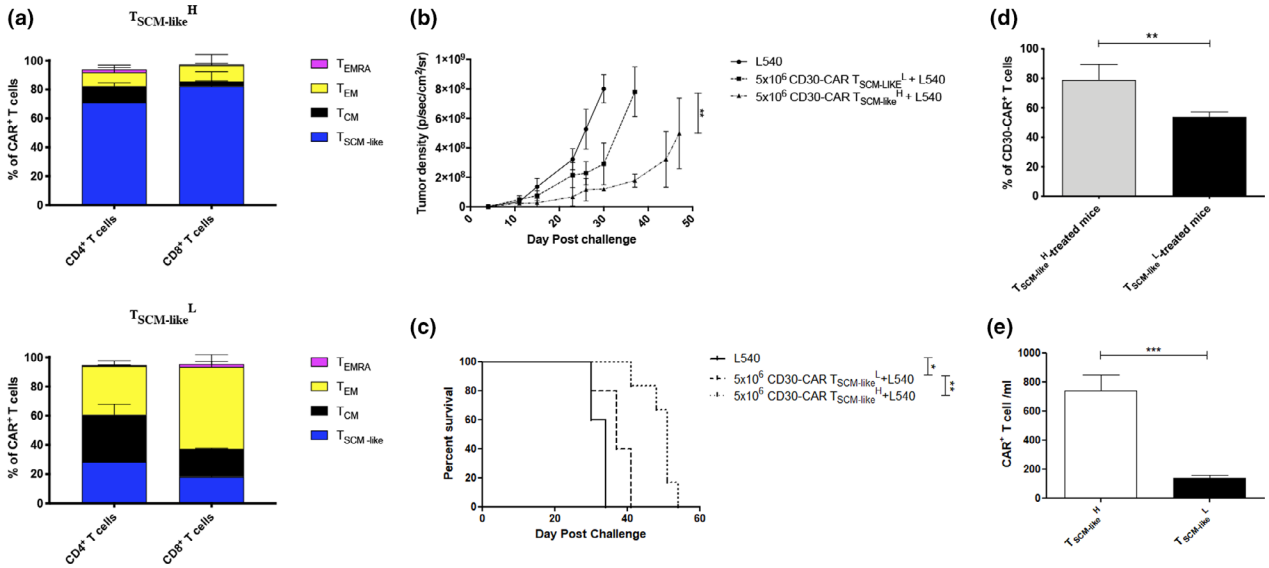

Figure 6. CD30-CAR $T_{SCM\text{-}like}^{H}$ products have higher antitumor efficacy and T-cell persistence than CD30-CAR $T_{SCM\text{-}like}^{L}$ products. **(a)** Composition of $T_{SCM\text{-}like}^{H}$ and $T_{SCM\text{-}like}^{L}$ cultures at the day of treatment (mean ± SEM). **(b, c)** NSG mice ($N = 4$/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

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

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

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 ($n = 4$)

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 $\pm$ 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).

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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.