nature portfolio

Peer Review File

Split-Design Approach Enhances the Therapeutic Efficacy of Ligand-Based CAR-T Cells against Multiple B-cell Malignancies

Corresponding Author: Dr Yu Cao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Li et al describe an adapter CAR system using myc-tagged APRIL and BAFF as antigen recognition domains and anti-myc_tag CAR as the universal receptor. This system is tested in vitro and in simple in vivo models. The work by Li Adds to the growing literature targeting BCMA, TACI and BAFFR, the literature whereby natural ligands are used for targeting and also adds to the literature of indirect or adapter CARS.

The context of this work is important: Highly effective CARs targeting BCMA, CD19 and CD22 are well described, with CARs against CD19 and increasingly BCMA becoming more established in clinical practise.

In terms of BCMA targeting: the BCMA CAR cilta-cel for instance results in a remarkable activity with deep remissions with prolonged EFS that extends for many years. It is noteworthy that in comparison, APRIL based therapeutics have had disappointing clinical data. Furthermore, for BCMA targeting, antigen negative escape seems very rare and not a significant cause of CAR T cell failure. Furthermore, toxicity with 41BB-Z CARs targeting BCMA is manageable using standard clinical protocols and is not considered limiting.

In terms of targeting of B-cell malignancies, CD19 CARs are increasingly established in routine clinical practice. CD19 loss is a limitation but there is increasing pre-clinical and clinical literature with approaches which simultaneously target CD19/CD22 or CD19/CD20. Notably, BCMA is not expressed on a high proportion of B-NHL, the expression of TACI on B-NHL is not fully explored but is likely limited to malignancies derived from more mature B-cells.

GENERAL COMMENTS:

* As regards Myeloma (BCMA) targeting, how is the proposed approach better than current state-of-the-art? Notably, Cilta-cel performance is quite remarkable and BCMA loss is rarely observed. Comparison with APRIL-CAR is insufficient since this has performed poorly in clinic and inferior performance to antibody based CAR is well described - Lee et al (PMID: 37399355). A detailed comparison vs state-of-the-art i.e. cilta-cel CAR should be performed.

* As regards B-NHL (BAFFR) targeting. Here, CD19 CAR T frequently leads to CD19 loss and the authors are correct in their citation that double antigen loss can occur with sequential therapies, but most efforts currently target CD19/CD22 or CD20 use mixtures or CAR T products, bi-cistronics, tanCARs and double transductions etc. How would this system which targets BCMA, TACI and BAFFR compare with the more standard approach of CD19, or CD19/CD20 or CD19/CD22 CARs? Direct functional comparisons of this current system with competing established approaches should be made so as to justify use of this approach and to benchmark it against current state-of-the-art.

* A clear hypothesis why use of these natural ligands as adapters would be superior to e.g. scFv or single domain antibody fragments as adapters is not stated. This hypothesis should be stated and some comparison made with e.g. an scFv adapter.

* The in vivo pharmacodynamics of antibody based proteins to act as adapters is well known. The pharmacodynamics of APRIL/BAFF as soluble system proteins are not well understood and adapter proteins can not be practically useful if they are too unstable in vivo. Notably, natural ligands can have proteolysis sites. Their in vivo half-life should be determined.

* A key challenge of BCMA/TACI targeting is that these are ultra low-density target antigens. The target cell lines used all have high density. A key evaluation should be of performance vs ultra-low density BCMA expressing cell lines particularly in comparison with current BCMA CAR state-of-the-art.

* A key assessment of CAR T cell function is function at low E:T ratio. Performance of these systems at low E:T ratios should be performed.

* Experiments with primary human tumors should be performed

SPECIFIC COMMENTS
* How efficient is trimerisation when attached to the CAR? What is the stoichometry of CAR to APRIL/BAFF? Does this result in CAR trimerization?

* Figure 3 - as stated above, comparisons with APRIL-CAR and BAFF-CAR are insufficient. Comparisons with standard BCMA and CD19 scFv based CARs should be made. Comparison should include target cells with pathologically relevant target antigen density.

* Figure 4 - as stated above, comparisons with standard BCMA CAR i.e. cilta-cel should be made in this experiment

* Figure 6 - authors perform comparison with CD19/CD22 loop CAR (Spiegel et al) which has performed poorly in clinic and appears to performed significantly worse than just a standard CD19 CAR. This then is not a suitable comparison - the authors should have gone with a standard CD19 CAR here - tisa-cel (fmc63-CD8stk-41BB-Z)

* Figure 7 - it is not clear what clinical scenario the authors are trying to simulate by first engrafting RPMI8226-Luc and then Nalm6-Luc sequentially in a mouse?

Reviewer #2

(Remarks to the Author)
Li et al report on findings on approaches to engineer split-design CAR (sCAR) T cells. They specifically highlight how ligand-based CAR T cells with APRIL and BAFF can be used to develop switchable CAR T cells with enhanced anti-tumor activity. Although BAFF and APRIL have been assessed as CAR T cell approaches by other investigators, the novelty of this manuscript is the switchable nature of the ligand-based design.

Comments
There is a clear outline of the data, including the text and figures. The authors also consistently note the replicates and statics of the studies.
-Please clarify the mention of electroporation as the reason for "poor outcomes" as this is not clear (lines 413-415).
-The authors do not report on the potential of non-specific binding of the BAFF or APRIL switches when they are injected in vivo as in Figure 7. Can the authors please also comment on the in vivo expression of the ligands?
-Can the authors please comment regarding the administration of the high doses of CAR T cells administered in their in vivo models – ranging from 10 to 30 million across the various in vivo experiments (Figures 4 to 7)? No clear rationale is provided for these differential doses. Did the authors monitor mouse weights or other clinical factors to assess for toxicity in the setting of these high doses?
-Further, did the authors perform a stress test of the CAR T cells and the tumor in their NALM6 and MM models with the respective BAFF and APRIL sCAR?
-In the context of the model of antigen escape (Figure 6), the recipients of the sCAR demonstrate prolonged survival. Yet, these mice ultimately die (Figure F). Did the authors characterize antigen loss in these mice as well as the other groups? Additionally, was CAR persistence assessed?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Dear authors. Thank you for addressing my comments and congratulations on an excellent manuscript.

The paper is more comprehensive now given there are comparisons with clinically established therapies which can act as a benchmark.

As a final comment please make sure the slightly lower cytotoxicity vs low BCMA density target cells is detailed and

discussed sufficiently in the discussion.

Reviewer #2

(Remarks to the Author)
Li et al. thoroughly addressed the reviewers' comments. In particular, the manuscript is enhanced by (1) the comparison of the split-design CAR (sCAR) T cells to tisa-cel and cilta-cel in vitro and the identification of the impact of antigen density on cytotoxicity, (2) "stress testing" of the sCAR in vivo, and (3) the activity of the BAFF-targeted sCAR T cells in the context of CD19 negative disease. These experiments contribute to the novelty and potential translation of this CAR construct. Overall, I am satisfied with the authors' careful attention to the initial review

Comment
1. For Figures 4 to 6, the authors assess sCAR T cells in vivo. However, the authors do not report on the persistence of these T cells in relation to the comparators in any of these in vivo models. For example, in Figure 5, did the authors assess persistence of the sCAR T cells versus conventional CD19- or BCMA-targeted CAR T cells? The enhanced survival data would suggest improved persistence. However, this is not mentioned by the authors. It would be ideal for this data to be added.

Reviewer #1 (Remarks to the Author): with expertise in CAR-T design/engineering, haematological malignancies

Li et al describe an adapter CAR system using myc-tagged APRIL and BAFF as antigen recognition domains and anti-myc_tag CAR as the universal receptor. This system is tested *in vitro* and in simple *in vivo* models. The work by Li Adds to the growing literature targeting BCMA, TACI and BAFFR, the literature whereby natural ligands are used for targeting and also adds to the literature of indirect or adapter CARS.

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In terms of BCMA targeting: the BCMA CAR cilta-cel for instance results in a remarkable activity with deep remissions with prolonged EFS that extends for many years. It is noteworthy that in comparison, APRIL based therapeutics have had disappointing clinical data. Furthermore, for BCMA targeting, antigen negative escape seems very rare and not a significant cause of CAR T cell failure. Furthermore, toxicity with 41BB-Z CARs targeting BCMA is manageable using standard clinical protocols and is not considered limiting.

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We thank the reviewer for the comprehensive summary and valuable suggestions.

GENERAL COMMENTS:

* As regards Myeloma (BCMA) targeting, how is the proposed approach better than current state-of-the-art? Notably, Cilta-cel performance is quite remarkable and BCMA loss is rarely observed. Comparison with APRIL-CAR is insufficient since this has performed poorly in clinic and inferior performance to antibody based CAR is well described - Lee et al (PMID: 37399355). A detailed comparison vs state-of-the-art i.e. cilta-cel CAR should be performed.

We appreciate the reviewer's insights. In the initial submission, our focus was limited to whether the split-design of CAR-T cells could overcome the obstacles faced by ligand-based CAR-T-cell strategies. To this end, we performed a series of experiments comparing conventional and split-design APRIL/BAFF CAR-T cells without comparing them to FDA-approved therapies. To address this concern, for myeloma (BCMA) targeting, we generated BCMA CAR-T cells (Cilta-

cel) and compared their antitumor efficacy with that of APRIL-based sCAR-T cells *in vitro* and *in vivo*. By introducing cell lines expressing variant BCMA expression, we compared the cytotoxic activity of the two types of CAR-T cells and found no significant differences in BCMA/TACI double-positive and BCMA high-to-middle-expressing cell lines, even at very low E:T ratios (Fig. 3j-k). However, APRIL-based sCAR-T cells were slightly less potent than BCMA CAR-T cells against BCMA-low-expressing cell lines (Supplementary Fig. 12b-d), possibly because Cilta-cel targets two epitopes of the BCMA antigens.

We further explored the *in vivo* efficacy in an orthotopic multiple myeloma (MM) model by comparing APRIL-based sCAR-T and BCMA CAR-T-cell therapy. As shown in Fig. 5a-e and Supplementary Fig. 21, both types of CAR-T cells quickly and completely cleared the tumor lesions, with no tumor recurrence observed during the observation period, and both types of CAR-T cells prolonged the survival time of the mice.

Conventional APRIL-based CAR-T cells were originally proposed to overcome the limitations of scFv-based BCMA CARs, namely, the low antigen density of BCMA results in suboptimal signaling and tumor escape through the loss of BCMA. Although BCMA antigen loss is considered a rare event that mediates MM resistance to BCMA CAR-T-cell therapy, cases of relapse are still observed clinically and are driven by BCMA-negative clones harboring biallelic deletions at the TNFRSF17 locus[1]. Therefore, dual-target CAR-T-cell therapy is necessary for MM. While scFv-based CAR-T cells have shown great success in the clinic, ligand-based CAR designs have been proven to be superior to antibody-based designs in some cases due to their low immunogenicity, high affinity, and multi-target binding ability[2]. Previous studies have demonstrated that conventional APRIL CAR-T cells have performed poorly in the clinic and have shown inferior performance to antibody-based CAR-T cells, potentially due to the reduced target-binding ability of cell surface-anchored ligands[3]. The difference between conventional CAR-T cells with cell surface-anchored ligands and split-design CAR-T cells is due primarily to the native conformation of the ligands, which was well explained in our study. Our updated data confirmed that the efficacy of the optimized APRIL-based sCAR-T cells was comparable to that of the current state-of-the-art BCMA CAR-T cells (Cilta-cel). We have updated the "Methods" and "Results" sections accordingly (Fig. 3j-k, Fig. 5a-e, Supplementary Fig. 12a-d, and Supplementary Fig. 21).

* As regards B-NHL (BAFFR) targeting. Here, CD19 CAR T frequently leads to CD19 loss and the authors are correct in their citation that double antigen loss can occur with sequential therapies, but most efforts currently target CD19/CD22 or CD20 use mixtures or CAR T products, bi-cistronics, tanCARs and double transductions etc. How would this system which targets BCMA, TACI and BAFFR compare with the more standard approach of CD19, or CD19/CD20 or CD19/CD22 CARs? Direct functional comparisons of this current system with competing established approaches should be made so as to justify use of this approach and to benchmark it against current state-of-the-art.

Thank you for the kind comments. In addition to the comparison of BAFF-redirected sCAR-T cells with CD19/CD22 bispecific CAR-T cells, which were described in detail in the original manuscript, we also conducted *in vitro* and *in vivo* efficacy studies by comparing BAFF-redirected sCAR-T cells with Tisagenlecleucel (CD19 CAR-T). As shown in Fig. 3m and Supplementary Fig. 12g, both types of CAR-T cells demonstrated similar killing activity in the BAFFR/BCMA/TACI triple-positive IM9 cell line. However, BAFF-redirected sCAR-T cells showed inferior activity to CD19 CAR-T cells in other cell lines with clinically relevant antigen densities. This disparity is likely due to the significantly lower density of BAFFR/BCMA/TACI antigens than of CD19 antigens (Supplementary Fig. 12e-f). Notably, although the efficacy of BAFF-specific sCAR-T cells is inferior to that of CD19 CAR-T cells in these contexts, BAFF-specific sCAR-T cells have advantages in cell lines lacking CD19 targets. To simulate the clinical scenario of CD19 loss, we performed *in vivo* comparisons in Nalm6 (B-ALL) and Raji (NHL) immune escape models. In immune escape models established with a cell mixture of Nalm6-WT and Nalm6-19KO cells or Raji-WT and Raji-19KO cells, BAFF-mediated sCAR-T cells demonstrated superior tumor clearance, increased cytokine release, and significantly prolonged survival (Fig. 5f-k, Supplementary Fig. 22-23). These results further confirm that the split-design ligand-based CAR-T-cell system exhibits multitargeting capabilities, broad-spectrum efficacy, and clinical relevance compared with FDA-approved CAR-T-cell therapies. We have updated the data in the "Results" section accordingly (Fig. 3l-m, Fig. 5f-k, Supplementary Fig. 12e-g, Supplementary Fig. 22-23).

* A clear hypothesis why use of these natural ligands as adapters would be superior to e.g. scFv or single domain antibody fragments as adapters is not stated. This hypothesis should be stated and some comparison made with e.g. an scFv adapter.

We thank the reviewer for this comment. In general, in comparison with scFvs or single-domain antibodies, the use of natural ligands as adapters offers several advantages, such as low immunogenicity, flexible multi-target design, and relatively simple construction.

1. Low immunogenicity: Antibodies are generally derived from mice or alpacas and can elicit an immune response during long-term exposure to the human immune system. The process of antibody humanization is also time-consuming and expensive. In contrast, natural ligands are generally of human origin and lack immunogenicity. The use of natural ligands can reduce the incidence of side effects in patients and improve the safety of treatment.

2. Multiple targets with simple designs: Antigen loss presents a major challenge for CAR-T-cell therapy, leading to tumor relapse. Although various bispecific and trispecific CAR-T strategies have been developed, these approaches carry the potential risk of chain mispairing, leading to CAR aggregation and triggering T-cell-exhausting signaling cascades. Alternatively, CAR designs incorporating natural ligands, which inherently bind to multiple markers on malignant cells,

present a potential solution for antigen escape. In this study, we designed natural ligand-based CAR-T cells via the natural ligands APRIL and BAFF, both of which are members of the TNF ligand superfamily that target multiple receptors, including BAFFR, BCMA, and TACI. Therefore, natural ligands, which possess simple and compact properties, could simplify the design of multispecificity and solve the current problem of immune escape.

In summary, the utilization of natural ligands as adapters for CAR-T cells, although relatively new in clinical applications, may have advantages in improving the safety, flexibility, and cost effectiveness of adoptive cell therapy. With a deeper understanding of ligands and advances in technology, we believe that this design strategy could play an increasingly important role in cancer immunotherapy. We have included these comments in the "Introduction" and "Discussion" sections.

Finally, owing to limited resources and conditions, we did not conduct the designs and comparisons of natural ligand- and scFv-based CAR-T-cell therapies in this study. The screening of different scFv clones specific to BAFFR, BCMA, and TACI, as well as the structural optimization of combinatorial scFvs for CAR designs, is very time-consuming. Further comprehensive comparative studies of multispecific CAR-T cells based on natural ligands and scFvs are necessary to determine the design and applicable advantages of this concept.

* The *in vivo* pharmacodynamics of antibody based proteins to act as adapters is well known. The pharmacodynamics of APRIL/BAFF as soluble system proteins are not well understood and adapter proteins can not be practically useful if they are too unstable *in vivo*. Notably, natural ligands can have proteolysis sites. Their *in vivo* half-life should be determined.

We thank the reviewer for this comment. As suggested, we conducted pharmacokinetics and tissue biodistribution analyses of APRIL- and BAFF-based switches using the near-infrared fluorescent dye IRDye800 to label the proteins as a tracker. The half-lives of Myc-APRIL and Myc-BAFF were 21.54 minutes and 23.07 minutes (Supplementary Fig. 13a-b), respectively, which are similar to the half-life of BAFF-based fusion reported in the literature[4]. Although the half-life of the ligand as an adapter is less than half an hour, we observed tumor distribution up to 48 hours post i.v. injection, indicating excellent tumor residence of these relatively small natural ligand molecules (Supplementary Fig. 13c-d). In other words, although the switch is metabolized rapidly in the peripheral blood, it can reside at the tumor site for a long time, further supporting the feasibility of ligands as adapters. We have included these results in the main text.

In addition, we analyzed the sequences of APRIL and BAFF used in the switch designs. APRIL is processed by a furin convertase at amino acids 101-104 (RKRR) [5], but we used the truncated APRIL from S116-L250, which is located outside the protease cleavage site. For BAFF, the furin consensus site is positioned at R133-A134[6], which is not included in the truncated BAFF (A134-

L285) used in this study. Therefore, we confirmed that the switch design is based on truncated natural ligands with no proteolysis sites, guaranteeing the stability and feasibility of APRIL- and BAFF-redirected CAR-T-cell therapy. We have also included this information in the "Methods" section.

* A key challenge of BCMA/TACI targeting is that these are ultra low-density target antigens. The target cell lines used all have high density. A key evaluation should be of performance vs ultra-low density BCMA expressing cell lines particularly in comparison with current BCMA CAR state-of-the-art.

Thank you for the kind suggestion. Accordingly, we generated a series of engineered cell lines with varying BCMA densities that comprehensively spanned the antigen densities observed in patient-derived tumor cells (Supplementary Fig. 12b-c). Importantly, the cell lines with low or medium BCMA expression levels were representative of the clinical range observed in this study. The cytotoxicity of APRIL-based sCAR-T cells was comparable to that of BCMA CAR-T cells in cell lines with medium and high BCMA densities but was slightly reduced in cell lines with low BCMA expression (Supplementary Fig. 12d). This difference may be attributed to the ability of BCMA CAR-T cells to target two epitopes of the BCMA antigen simultaneously. We have incorporated these findings into the "Results" section (Supplementary Fig. 12b-d).

* A key assessment of CAR T cell function is function at low E:T ratio. Performance of these systems at low E:T ratios should be performed.

We appreciate the reviewer's suggestions. To clarify CAR-T-cell functionality, we conducted cell-killing assays by evaluating switch dosages and E:T ratios for APRIL- and BAFF-redirected sCAR-T cells. As depicted in Fig. 2j-k, at an E:T ratio of 1:1, the IC50 values for both types of natural ligand-based sCAR-T cells ranged from 1–10 pM against various target cell lines. Moreover, when 1 nM APRIL or BAFF was used, sCAR-T cells demonstrated specific cytotoxic activities against these tumor cells even at E:T ratios as low as 1:32 or 1:16 (Supplementary Fig. 6a-b). These data have been integrated into the updated "Results" section and are depicted in Fig. 2j-k and Supplementary Fig. 6a-b.

* Experiments with primary human tumors should be performed.

We appreciate the reviewer's comments. In the original submission, we included experiments with primary human tumors. The data are presented in Supplementary Table 4, Supplementary

Fig. 9, Supplementary Fig. 10, and Supplementary Fig. 11a-c, with detailed descriptions provided in the "Results" section.

Owing to limited access to clinical samples, we did not include new data comparing ligand-based sCAR-T cells with FDA-approved CAR-T-cell therapies for primary human tumor cells.

SPECIFIC COMMENTS

* How efficient is trimerisation when attached to the CAR? What is the stoichometry of CAR to APRIL/BAFF? Does this result in CAR trimerization?

We appreciate the reviewer's comment. The efficient trimerization of APRIL and BAFF is crucial for their stability and functionality. Both ligands belong to the TNF family[7] and share a common structural feature in which they are released as soluble trimeric forms following furin protease processing[8]. APRIL and BAFF are the same as other TNF ligands exist in humans, all of which share a structural feature, an extracellular TNF homology domain (THD), which contains the contact sites mediating predominantly hydrophobic interactions between TNF ligand monomers and triggers the assembly of stable TNF ligand homotrimers[9]. APRIL and BAFF can maintain stable trimeric conformations and can only dissociate into monomers under extreme nonphysiological conditions such as low pH, high salt, high temperature, or the presence of reducing agents[8]. The trimeric structure of our switches is unlikely to be disrupted under physiological conditions, which is essential for their ability to bind to receptors. Our results confirm that ligand-based switches maintain their trimerization throughout purification and characterization, unlike membrane-bound ligands, which cannot adopt their natural trimeric structure. This underscores the importance of the split design approach for ligand-based CAR-T-cell strategies, as detailed in the "Discussion" section.

Regarding the stoichiometry of CAR to APRIL and BAFF, it follows a general pattern observed between single-chain CAR (sCAR) and switches. Our previous studies suggested that compared to monovalent switches, bivalent constructs coordinate more effectively with dimerized IgG4m hinge-based sCAR[10]. In this study, size exclusion chromatography (SEC) analysis (Supplementary Fig. 3) demonstrated that both switches preserve the natural trimeric structure of APRIL and BAFF, indicating the presence of trivalent Myc tags fused to their N-termini. While we did not prepare APRIL/BAFF-based fusions with different valencies to determine the exact CAR-to-switch stoichiometry, we speculate that the bivalent Myc tags from trimerized switches are sufficient to maximize T-cell activation.

To address the final question, we conducted western blot analysis after co-culturing 9E10-IgG4m CAR-T cells with target cells in the presence of various switch concentrations. After one hour of incubation, we observed stable dimer formation of CAR molecules but no trimerization or

aggregation induced by APRIL- or BAFF-based switches, regardless of concentration. This finding suggests that the binding of trimerized APRIL- or BAFF-based switches to CAR-T cells does not induce CAR trimerization, which is consistent with previous findings[11]. The detailed results are presented in the "Results" section (Supplementary Fig. 7).

* Figure 3 - as stated above, comparisons with APRIL-CAR and BAFF-CAR are insufficient. Comparisons with standard BCMA and CD19 scFv based CARs should be made. Comparison should include target cells with pathologically relevant target antigen density.

We appreciate the reviewer's suggestion. To address this concern, we conducted comprehensive experiments comparing ligand-based sCAR-T cells with standard BCMA CAR-T cells (Cilta-cel) or CD19 CAR-T cells (Tisa-cel) on target cells with pathologically relevant antigen densities. For APRIL-based sCAR-T cells and BCMA CAR-T cells, cytotoxicity assays were performed on cell lines expressing pathologically relevant antigen densities, including BCMA/TACI double-positive and BCMA high/medium cell lines. Our data demonstrated that APRIL-based sCAR-T cells exhibited cytotoxicity similar to that of BCMA CAR-T cells against BCMA/TACI double-positive and BCMA high/medium cell lines but showed reduced effectiveness against BCMA low-expressing cell lines. These findings have been updated in the "Results" section (Fig. 3j-k and Supplementary Fig. 12a-d).

Additionally, we compared BAFF-based sCAR-T cells with CD19 CAR-T cells in cell lines with pathologically relevant antigen densities. Although BAFFR/BCMA/TACI antigen densities on these cell lines correlate with clinical pathology, the CD19 antigen density was significantly higher than that observed clinically. Our cytotoxicity results indicate that BAFF-based sCAR-T cells exhibited weaker killing ability than CD19 CAR-T cells did against these cell lines. However, BAFF-based sCAR-T cells effectively addressed immune evasion due to CD19 escape, showing stronger killing ability against CD19 knockout (CD19KO) cell lines than CD19 CAR-T cells. These results have been updated and revised in the "Results" section (Fig. 3l-m and Supplementary Fig. 12e-g).

* Figure 4 - as stated above, comparisons with standard BCMA CAR i.e. cilta-cel should be made in this experiment.

We agree with the reviewer's suggestions. Accordingly, we conducted additional experiments comparing APRIL-based sCAR-T cells with BCMA CAR-T (Cilta-cel) cells in a multiple myeloma model. The *in vivo* comparison demonstrated comparable tumor clearance rates, serum cytokine release, and mouse survival rates between both CAR-T-cell types, underscoring the potential of split-design APRIL-based CAR-T-cell therapy as a promising strategy for multiple myeloma

treatment. These results have been updated and revised in the "Results" section (Fig. 5a-e and Supplementary Fig. 21).

* Figure 6 - authors perform comparison with CD19/CD22 loop CAR (Spiegel et al) which has performed poorly in clinic and appears to performed significantly worse than just a standard CD19 CAR. This then is not a suitable comparison - the authors should have gone with a standard CD19 CAR here - tisa-cel (fmc63-CD8stk-41BB-Z).

We thank you for this helpful suggestion. The inclusion of clinical CD19/CD22 bispecific CAR (Spiegel *et al*.) as a comparative CAR-T-cell therapy aims to emphasize the enhanced efficacy of BAFF-based sCAR-T cells in addressing CD19/CD22 dual-antigen escape scenarios. In alignment with the reviewer's recommendation, we conducted new experiments using standard CD19 CAR-T cells (Tisa-cel) as an experimental control to illustrate the advantages of BAFF-based sCAR-T cells in immune escape models. While CD19 CAR-T cells failed to control disease progression in B-ALL and NHL models due to pre-existing CD19KO tumor variants, BAFF-based sCAR-T cells exhibited significantly enhanced treatment responses. They resulted in lower tumor burden, higher serum cytokine release, and prolonged survival in mice. Notably, on day 18 of the experiment, tumor recurrence from CD19-negative clones was observed in the peripheral blood of mice treated with CD19 CAR-T cells, whereas no tumor cells were detectable in the peripheral blood of mice treated with BAFF-based sCAR-T cells. These findings have been updated and integrated into the "Results" section (Fig. 5f-k and Supplementary Fig. 22-23).

* Figure 7 - it is not clear what clinical scenario the authors are trying to simulate by first engrafting RPMI8226-Luc and then Nalm6-Luc sequentially in a mouse?

Thank you for the kind comment. While such clinical cases are highly improbable, our study aimed to validate the controllability and adaptability of sCAR-T cells redirected by different ligand-based switches across varying tumor burdens. Integrating antigen-specific approaches for precise tumor targeting can significantly enhance therapeutic efficacy. Directing single CAR-T-cell therapy toward diverse antigens offers a broad-spectrum alternative to the current challenges of tumor relapse. This approach contrasts with the conventional method of designing separate batches of CAR-T cells for different tumor antigen specificities, which can be costly. Therefore, the introduction of drug-gated sCAR systems activated by different drugs that target distinct antigens holds considerable promise. This rationale guided our study in which a split-design ligand-based CAR-T system targeting various tumors with diverse antigen preferences was used.

Similar experimental designs have been previously reported:

1. Hyrenius-Wittsten *et al.* demonstrated that synthetic Notch (synNotch) CAR circuits enhance the specificity and persistent antitumor activity of therapeutic T cells[12]. In Figure 7C, the authors illustrated the prolonged persistence of ALPPL2 synNotch CAR circuit T cells by inoculating mice with SK-OV-3 cells (ovarian cancer) on one side and K562 cells (chronic myeloid leukemia) on the other side after a period of treatment.

2. Li *et al.* developed a collection of CARs controllable with FDA-approved antiviral drugs[13]. In Figure 6, the authors demonstrated the orthogonal regulation of dual-gated CARs *in vivo* by inoculating (s.c.) mice with MSTO cells (lung cancer) followed by (i.v.) administration of Nalm6 cells (acute lymphoblastic leukemia) five days later.

These references underscore the strategic potential of engineered CAR-T systems with controlled activation mechanisms in diverse tumor settings.

Reviewer #2 (Remarks to the Author): with expertise in CAR-T, haematological malignancies

Li et al report on findings on approaches to engineer split-design CAR (sCAR) T cells. They specifically highlight how ligand-based CAR T cells with APRIL and BAFF can be used to develop switchable CAR T cells with enhanced anti-tumor activity. Although BAFF and APRIL have been assessed as CAR T cell approaches by other investigators, the novelty of this manuscript is the switchable nature of the ligand-based design.

Comments

There is a clear outline of the data, including the text and figures. The authors also consistently note the replicates and statics of the studies.

Thank you for your positive evaluation of our manuscript.

-Please clarify the mention of electroporation as the reason for "poor outcomes" as this is not clear (lines 413-415).

Thank you for your comment. Our study initially aimed to clarify that we employed the lentiviral transduction method to generate conventional BAFF CAR-T cells, which is distinct from the approach used by Wong *et al.* in their previous report, where they utilized the TcB transposon system for BAFF CAR-T-cell production[14]. Wong *et al.* systematically compared the TcB transposon system with lentiviral vectors and reported that, compared with lentivirus, TcB transposon tended to insert transposons into non-transcript regions more frequently. Moreover,

the frequency of exonic insertions in TcB transposon system was lower than that in lentivirus, and TcB transposon tended to insert DNA farther from transcriptional start sites. They concluded that TcB transposon system has an insertion profile similar to that of other transposon systems and may offer advantages over lentiviral transduction. In our study, we exclusively used lentiviral vectors for CAR-T-cell preparation and did not employ the TcB transposon system. Given the inferior performance of conventional BAFF CAR-T cells, which has been noted in some studies, we hypothesized that this effect could be influenced by the method of lentiviral CAR-T-cell preparation. We have clarified these points in the revised "Discussion" section.

-The authors do not report on the potential of non-specific binding of the BAFF or APRIL switches when they are injected *in vivo* as in Figure 7. Can the authors please also comment on the *in vivo* expression of the ligands?

We appreciate the reviewer's insightful comments. The primary objective of Fig. 7 (now referred to as Fig. 6 in the revised manuscript) was to demonstrate the controllability and switchability of sCAR-T cells redirected by APRIL- and BAFF-based switches across different tumor burdens. RPMI8226 cells exhibit high expression of BCMA and medium levels of TACI, whereas Nalm6 cells show high BAFFR expression but very low to negative expression of TACI and BCMA. APRIL ligands exhibit a strong binding preference for BCMA over TACI, whereas BAFF ligands bind to all three receptors with a pronounced preference for BAFFR[8]. On the basis of the *in vitro* data (Supplementary Fig. 19b-c) and the *in vivo* results (Fig. 4b), we concluded that Myc-BAFF efficiently redirected sCAR-T cells toward Nalm6 cells compared with Myc-APRIL, whereas Myc-APRIL showed a greater preference for RPMI8226 cells but not BAFF fusions. Therefore, we designed the experimental workflow depicted in the revised Fig. 6, where Myc-APRIL mediated sCAR-T redirection against RPMI8226 cells in the first phase, followed by Myc-BAFF mediation in the second phase after complete eradication of RPMI8226 tumors. Given the short half-life of both switches (20–30 minutes) and the 4-day interval between treatment switches, we believe that the administration of Myc-APRIL in the first phase did not interfere with the ability of sCAR-T cells to switch to Nalm6 tumors, especially since Nalm6 tumors appeared after Myc-APRIL depletion. We acknowledge the reviewer's suggestion to include control groups of RPMI8226 and Nalm6 cells treated with BAFF and APRIL switches, respectively, to verify any potential non-specific binding interference *in vivo*.

In terms of ligand expression, APRIL ligands are produced primarily by bone marrow cells, including monocytes, dendritic cells, and granulocytes, and are also expressed in various tissues, such as the intestine, tonsils, mammary glands, skin, and certain tumors. BAFF ligands are predominantly produced by bone marrow cells such as neutrophils, monocytes, dendritic cells, and follicular helper T cells in germinal centers and can also be produced by activated T cells, B cells,

and certain non-hematopoietic stem cells in the bone marrow. BAFF ligands are mainly distributed in immune-cell-rich areas such as bone marrow and lymph nodes.

-Can the authors please comment regarding the administration of the high doses of CAR T cells administered in their *in vivo* models – ranging from 10 to 30 million across the various *in vivo* experiments (Figures 4 to 7)? No clear rationale is provided for these differential doses. Did the authors monitor mouse weights or other clinical factors to assess for toxicity in the setting of these high doses?

We appreciate the reviewer's valuable suggestions regarding the administration doses of CAR-T cells, which are crucial for both preclinical and clinical studies. To ensure a comprehensive experimental design, we reviewed and summarized relevant studies from our laboratory and others[10,11,15-17]:

1. (DOI: 10.1073/pnas.1524155113) Switch-mediated activation and retargeting of CAR-T cells for B-cell malignancies (40 million CAR-T-cell dosing).

2. (DOI: 10.1073/pnas.1524193113) Versatile strategy for controlling the specificity and activity of engineered T cells (40 million CAR-T-cell dosing).

3. (DOI: 10.1002/anie.201601902) Design of Switchable Chimeric Antigen Receptor T Cells Targeting Breast Cancer (30 million CAR-T-cell dosing).

4. (DOI: 10.1021/acssynbio.1c00007) Switchable CAR-T Cells Outperformed Traditional Antibody-Redirected Therapeutics Targeting Breast Cancers (15 million CAR-T-cell dosing).

5. (DOI: 10.1093/procel/pwae034) A novel loop-structure-based bispecific CAR that targets CD19 and CD22 with enhanced therapeutic efficacy against B-cell malignances (10 million CAR-T-cell dosing).

Additionally, to determine the optimal therapeutic dose for CAR-T cells, we referred to studies conducted by other laboratories[18-21]:

1. (DOI: 10.1126/science.ade0156) Multidimensional control of therapeutic human cell function with synthetic gene circuits (15 million CAR-T-cell dosing).

2. (DOI: 10.1002/advs.202205044) Nanomodified Switch Induced Precise and Moderate Activation of CAR-T Cells for Solid Tumors (10 million CAR-T-cell dosing).

3. (DOI: 10.1038/s41467-023-37863-5) Post-translational covalent assembly of CAR and synNotch receptors for programmable antigen targeting (10 million CAR-T-cell dosing).

4. (DOI: 10.1016/j.cell.2018.03.038) Universal Chimeric Antigen Receptors for Multiplexed and Logical Control of T Cell Responses (10 and 35 million CAR-T-cell dosing).

Furthermore, we considered other critical factors in our experimental design. For instance, our CAR-T cells have a positivity rate of 40–50%, meaning that the actual doses of CAR-positive cells administered are approximately half of those described in the publications. The expression levels of BAFFR, BCMA, and TACI were also accounted for. As depicted in Supplementary Fig. 12f, BAFFR, BCMA, and TACI exhibited relatively lower expression than the CD19 antigen did, posing greater therapeutic challenges in treating these tumors. Moreover, switchable CAR-T cells engage in dynamic processes involving complex immune synapse formation with tumor cells[20], which differ fundamentally from that of conventional CAR-T cells. To prevent any potential "hook effect" between CAR-T cells and tumors caused by excessive switches, thereby diminishing therapeutic efficacy, we opted for a relatively high CAR-T-cell dosage.

Throughout our *in vivo* experiments, we did not observe significant body weight loss in the mice during drug administration. Future studies will include detailed investigations into toxicity indicators such as histological staining, cytokine changes related to cytokine release syndrome (CRS), and biochemical markers of liver injury.

-Further, did the authors perform a stress test of the CAR T cells and the tumor in their NALM6 and MM models with the respective BAFF and APRIL sCAR?

We thank the reviewer for the suggestions. To further evaluate the antitumor efficacy of APRIL- and BAFF-based sCAR-T cells, we conducted stress tests in multiple myeloma (MM) and B-cell acute lymphoblastic leukemia (B-ALL) tumor models, respectively. For the MM model, NSG mice were intravenously inoculated with luciferase-expressing RPMI8226 cells. Twenty-one days post-tumor engraftment, we performed stress tests with CAR-T cells and Myc-APRIL. For the CAR-T-cell stress test, the mice were intravenously administered 1–10×10^6 9E10-IgG4m CAR-T cells, while the dose of Myc-APRIL was fixed at 1 mg/kg via intraperitoneal (i.p.) injection. For the switch stress test, the mice received i.p. injections of 0.3–3 mg/kg Myc-APRIL, with a fixed dose of 10×10^6 CAR-T cells per mouse. Our results showed that the majority of treatment conditions led to the complete elimination of tumor lesions. Notably, partial tumor recurrence was observed only in the lowest CAR-T-cell dose (10×10^6) and lowest switch dose (0.3 mg/kg) groups. We observed a positive correlation between serum cytokine release and the administered dose, and all the treatment groups exhibited significantly prolonged survival (Supplementary Fig. 14).

For the B-ALL model, NSG mice were intravenously inoculated with luciferase-expressing Nalm6 cells. Three days post-engraftment, we evaluated the dose stress of CAR-T cells and Myc-BAFF. For the CAR-T-cell stress test, the mice received an i.v. injection of 3–30×10^6 9E10-IgG4m CAR-T cells, with the dose of Myc-BAFF fixed at 1 mg/kg via i.p. injection. For the switch stress test, the mice received i.p. injections of 0.3–3 mg/kg Myc-BAFF, with a fixed dose of 30×10^6 CAR-T cells per mouse. Interestingly, we found that the ability of BAFF-redirected sCAR-T cells to

inhibit tumor growth was more dependent on the dose of CAR-T cells than on the switch dose. Reduction in CAR-T-cell dose significantly accelerated tumor recurrence, whereas changes in the switch dose had relatively minor effects on antitumor activity. Consistent with the MM model, there was a positive correlation between early-stage serum cytokine release and the administered dose (Supplementary Fig. 15). Due to the aggressive nature of B-ALL tumors, BAFF-redirected sCAR-T cells exhibited limited effectiveness in disease control, which may be attributed to low antigen expression levels. We have updated the "Methods" and "Results" sections accordingly and included Supplementary Fig. 14 and 15 to provide comprehensive data on these stress tests.

-In the context of the model of antigen escape (Figure 6), the recipients of the sCAR demonstrate prolonged survival. Yet, these mice ultimately die (Figure F). Did the authors characterize antigen loss in these mice as well as the other groups? Additionally, was CAR persistence assessed?

Thank you for the insightful suggestions. In our previous study (Fig. 6, now corresponding to Fig. 4h-m in the revised manuscript), we investigated the immune escape model to assess CAR-T-cell persistence *in vivo*. We observed CAR-T-cell expansion in the peripheral blood on day 21, which was dependent on switch administration. However, by day 28, CAR-T-cell expansion became undetectable following cessation of switch administration, indicating that sCAR-T-cell proliferation is switch dependent. Future studies may consider assessing immune-competent mice via a mouse sCAR-T system to further elucidate sCAR-T-cell persistence *in vivo*.

To address concerns about antigen loss during CAR-T-cell therapy, we redesigned experiments to compare BAFF-based sCAR-T cells and CD19 CAR-T cells (Tisa-cel) in an immune escape model (Fig. 5f-k). On day 18 of the experiment, peripheral blood analysis of the control group revealed a CD19+/CD19- tumor cell ratio of approximately 1:1. In the CD19 CAR-T-cell group, we detected only CD19- Nalm6 cells, while no tumor cells were detected in the mice treated with BAFF-redirected sCAR-T cells, indicating efficient inhibition of target tumor cells initially by both CAR-T-cell therapies (Fig. 5j, Supplementary Fig. 22b). However, by day 36, tumor relapse was observed in the mice treated with BAFF-based sCAR-T cells. Analysis of tumor cell antigen expression (Supplementary Fig. 22c) demonstrated that Nalm6 cells still expressed the BAFFR antigen on their surface, suggesting that recurrence was likely associated with insufficient CAR-T-cell persistence due to discontinuation of switch administration. We have updated the "Results" section and included characterization data in Fig. 4h-m, 5f-k and Supplementary Fig. 22 to reflect these findings.

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Reviewer #1 (Remarks to the Author):

Dear authors. Thank you for addressing my comments and congratulations on an excellent manuscript.

The paper is more comprehensive now given there are comparisons with clinically established therapies which can act as a benchmark.

We sincerely appreciate the reviewer's positive evaluation of our manuscript.

As a final comment please make sure the slightly lower cytotoxicity vs low BCMA density target cells is detailed and discussed sufficiently in the discussion.

We sincerely appreciate the reviewer's insightful suggestions. In response to the comment regarding the insufficient discussion of comparisons with FDA-approved CAR-T therapies, we have revised the "Discussion" section of the manuscript to include a more comprehensive analysis. This revision also addresses the observed slightly lower cytotoxicity in target cells with low BCMA density, providing additional context and clarification.

Reviewer #2 (Remarks to the Author):

Li et al. thoroughly addressed the reviewers' comments. In particular, the manuscript is enhanced by (1) the comparison of the split-design CAR (sCAR) T cells to tisa-cel and cilta-cel in vitro and the identification of the impact of antigen density on cytotoxicity, (2) "stress testing" of the sCAR in vivo, and (3) the activity of the BAFF-targeted sCAR T cells in the context of CD19 negative disease. These experiments contribute to the novelty and potential translation of this CAR construct. Overall, I am satisfied with the authors' careful attention to the initial review.

We sincerely appreciate the reviewer's thorough assessment and constructive feedback on our study.

Comment

1. For Figures 4 to 6, the authors assess sCAR T cells in vivo. However, the authors do not report on the persistence of these T cells in relation to the comparators in any of these in vivo models. For example, in Figure 5, did the authors assess persistence of the sCAR T cells versus conventional CD19- or BCMA-targeted CAR T cells? The enhanced survival data would suggest improved persistence. However, this is not mentioned by the authors. It would be ideal for this data to be added.

We greatly appreciate the reviewer's valuable suggestion, and we acknowledge our oversight in omitting this detection index. To improve the manuscript, we have repeated the experimental

procedure for Figure 5f and collected new data on the cell expansion curves of sCAR-T and CD19 CAR-T cells during treatment, now presented in Supplementary Fig. 22a. The expansion and contraction patterns of both CAR-T cell types in peripheral blood were consistent with the trends observed in previous experiments (Figure 4l). In the immune escape model, CD19 CAR-T cells became exhausted shortly after infusion due to a lack of targets. In contrast, 9E10-IgG4m sCAR-T cells showed robust proliferation and improved persistence. These findings further highlight the advantage of the BAFF-based sCAR-T strategy in overcoming immune escape compared to Tisa-cel.