nature portfolio

Peer Review File

Improved safety of chimeric antigen receptor T Cells indirectly targeting antigens via switchable adaptors

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript entitled "Safety control of switchable chimeric antigen receptor T cells using dose-adjustable adaptors and suicidal drug conjugates" presents data that the lethal toxicity of conventional anti-CD40 CAR T cells derives mainly from CD40 expression in non-hematopoietic tissues (lung / liver / spleen). The authors also report the design of an anti-cotinine switchable CAR that, by adjusting the dosage of cotinine-conjugated CD40 adaptor, can ameliorate the lethal toxicity that plagues the conventional anti-CD40 CAR T cells. In addition, the authors have conjugated cotinine with a toxin (saporin) which can be exploited to eliminate the switchable CAR T cells in case GvHD is observed. A few suggestions/observations:

1. The experiments seem to be appropriately performed, the manuscript is well written, and the results are logically presented. The data clearly demonstrate the effectiveness and safety of their switchable CAR T cells.
2. The strategy of employing a switchable or universal CAR T cell to mitigate toxicity has been previously reported by Lee, Y.G. et al. (reference 22) using an anti-FITC CAR design, so the idea is not novel. However, the authors have taken a step further to identify the cause of the toxicity of the conventional CAR T cell with the same specificity. They have also proposed a brief mechanism to explain how the switchable CAR can alleviate this toxicity.
3. While the idea of generating a targeted toxin to eliminate the CAR T cells is interesting, but one wonders why the authors used a protein toxin instead of small molecule toxin (e.g. an auristatin or maytansine, etc.) to kill the CAR T cells?
4. The authors report infiltration of CD40 CAR T cells into the lungs, liver, and spleen, but do not disclose which cell types in these organs express CD40. Do the authors have any idea which cells in these healthy organs express CD40 and why their attack by the CAR T cells is so toxic? The protein atlas reports expression of CD40 on healthy macrophages, B cells, basal respiratory cells, secretory cells, dendritic cells, adipocytes, endothelial cells and many other cell types. Do the authors know whether elimination of any of these cell types causes the observed toxicity? One wonders why the authors selected a tumor antigen that is expressed so widely on healthy cells?
5. Did the authors observe cytotoxicity of Cot CAR T cells and CD40 CAR T cells on macrophages when performing the co-culture studies?
6. To control the GVHD, why didn't the authors simply discontinue administering the tumor-targeting adapter?
7. Does the use of saporin toxin have any immunogenicity concerns when administered to humans?

Overall, the manuscript is okay to publish elsewhere, but not novel enough to publish in Nature Communication.

Reviewer #2 (Remarks to the Author):

There is an increasing effort in the field to generate better CAR-Ts to treat cancer. In this manuscript, the authors assess whether a switchable CAR-T with a tumor- targeting adaptor can reduce off-tumor toxicity. There construct also includes a suicidal drug-conjugated tag that can eliminate the CAR-Ts to reduce long-term tox.
The authors use CD40 as antigen and clearly show the advantage of the switchable CarT.
The idea is original and important as this will allow the design of new CAR-Ts against Ag that are not tumor-specific without increasing adverse side-effects.
The manuscript is well written, has a logical flow, presents clean data and conclusions are sound.

While the strategy aiming at targeting CD40pos A20 cells shows therapeutic results in fig4, one could wonder to which extend the CD40+ macrophages and DCs are being targeted as well. There

are indeed many clinical trials using anti-CD40 agonist with promising results (when used in combination therapies). Which cells exactly will be targeted by the CARs?

Since BalbC mice are generally more Th2 polarized compared to C57Bl6 mice, it is important to recapitulate the therapeutic results found in the A20 in a C57Bl6 tumor model (for example B16F10 or MC38, which are still not very high-bar).

In figure 2, the authors assess the role of IL1b and IL6. However, anti-CD40 was shown to also induce IL-12 by both Mf and DCs (Murgaski et al, Cancer Res 2022). Do the CD40 Car induce IL-12 production by the splenic DCs? Moreover, it was recently shown that CD40-mediated IL12 production in mouse induces toxicity via the activation of Kupffer cells and neutrophils (Siwicki et al, Sci Immunol 2021). Would IL12 blockade lower the CD40 CAR toxicity more than IL6 blockade/anakinra (specifically looking at the early weight loss peak)?

Why do cot-Car-T bypass the lungs? What is the hypothesis?

There is still an ongoing debate on whether cotinine acts as a psychoactive in humans. This should briefly be discussed.

Some figures are difficult to read, for example suppl fig 3b: it's very hard to say what the dotted lines correspond to (the different dotted lines are very similar). Perhaps more colors could help?

Reviewer #3 (Remarks to the Author):

The authors demonstrate the ability to combat the major issue in the field of CAR T cells of on-target off-tumor toxicities by applying a universal adaptor CAR T cell approach. While several universal adaptor CAR T cell systems have been created, this is the first system that shows that dosing of the adaptor can lead to eliminating this type of toxicity. The authors first establish a syngeneic mouse model targeting CD40 antigen that is expressed on leukemia cells and on normal cells leading to lethal ON-target/OFF-tumor toxicities. As an alternative approach to a standard CAR, the authors employ their previously reported "Cot-CAR" that binds to tumor the nicotine metabolite cotinine on tumor targeting antibody adaptors, to ultimately target tumor cells. The authors show that in contrast to the standard anti-CD40 CAR, the adaptor CAR is able to specifically target high expressing tumor cells and not normal cells both in vitro and in vivo in a mouse model by tuning the adaptor dose. While the issue of ON-target/OFF-tumor toxicity has been addressed using several alternative methods including sensing of antigen combinations using combinatorial CAR systems, or avidity tuning via small molecule control as summarized by the authors, this is the first report of using a universal adaptor CAR technology to treat this major issue that plagues the targeting of many antigens especially in the solid tumor setting. The authors go into the mechanism of lack of toxicity for the adaptor CAR showing that the T cells are not undergoing the same stimulation in OFF-tumor sites (lung, spleen, liver) as compared to the standard anti-CD40 CAR. Finally, the authors generate a suicide adaptor containing the toxin saporin fused to the cotinine tag that rapidly kills off the Cot-CAR cells. They demonstrate activity in vitro as well as in vivo in mouse models including in an allogeneic CAR T/GVHD model. While it is more common for allogeneic CAR T cell therapy to have issues with T cell persistence, allogeneic cells can lead to GVHD toxicities in which case a targeted killing approach like this would be advantageous.
Overall the paper is well-written with logical experimental flow, and the data are robust with a major novel findings for the field. There are several concerns to be addressed before the manuscript is suitable for publication:
Concerns:
- Fig 2 add data showing confirmation of successful depletion of the macrophages
- Include text explaining why IL-6 production is used as a read-out for normal cell toxicity. Additionally, include in addition direct measurements of macrophage cell toxicity
- Check statistics throughout paper. In several figures where multiple comparisons are being performed, ANOVA testing should be used instead of t tests. Additionally, description of what the

error bars are describing (stdev or sem) and number or samples/replicates is lacking in several figure legends.
- In Fig 1 can A20 tumor be cleared in CD40KO mouse by standard anti-CD40 CAR T cells? This would establish that the standard anti-CD40-CAR is functional and capable of treating A20 tumor
- Use Greek micro symbol ‘μ’ instead of ‘u’
- “Taken together, the switchable Cot CAR-T cells were able to eliminate CD40-expressing tumors without on-target off-tumor toxicity, which cannot be avoided in conventional CD40 CAR-T cell therapy.” It looks like there is still some residual tumor in some mice, so tone down this language to something like “…significantly reduced the size of CD40 expressing tumors without on-target…”
- “Similar to the murine CAR-T cell model, hCot CAR-T cells co-infused with hCD40 adaptor effectively eliminated tumor cells compared to hCot CAR-T cells in the absence of adaptor (Fig. 5f, g).” similar comment to above, to tone down the language here.
- “The result of this study is one of the examples demonstrating that an optimal therapeutic window of a CAR-T cell can be established to maintain anti-tumor efficacy with minimal normal tissue toxicity with a single CAR system, obviating the need for dual CAR systems such as SynNotch, Split or iCARs.” While the CD40 adaptor system works to reduce on-target off tumor toxicities here, there remain myriad applications where single antigen targeting even using adaptor tuning would not be sufficient, such as cases where the antigen expression level on tumor is not significantly higher than on normal cells, cases of antigen downmodulation on tumor cells, or antigen expression level heterogeneity. Please edit this and add some more nuanced discussion of the technologies and trade-offs.
- “The epitope tag for the switchable adaptor should be carefully selected. It needs to be biologically inert, non-toxic, and non-immunogenic. In this sense, cotinine seems to be one of the ideal chemical tags for the adaptors.” While the cotinine on its own may be non-immunogenic, as part of a drug-protein adduct it is likely that it will be immunogenic. Unless you can provide data supporting lack of anti-cotinine adaptor antibody responses in the mice (this would be great data to add if you have it), then you cannot make this claim. Overall, it may not be an issue to have some immunogenicity against the adaptor, but lack of immunogenicity to the adaptor would be unexpected and the lack of immunogenicity to the metabolite alone is not sufficient evidence.
- Discuss general applicability of the strategy to other antigens as well as the issue of reduced antigen expression on tumor cells, and possible strategies, eg: potentially target a second tumor-high antigen

Response to the reviewers' comments

We appreciate the new experiments suggested by the reviewers and their constructive comments. Below, we provide a point-by-point response to all of the reviewers' comments. In the manuscript file, all changes are highlighted in blue.

REVIEWER COMMENTS

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We are grateful for the reviewers' commendation of our work.

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3. While the idea of generating a targeted toxin to eliminate the CAR T cells is interesting, but one wonders why the authors used a protein toxin instead of small molecule toxin (e.g. an auristatin or maytansine, etc.) to kill the CAR T cells?

We understand the reviewer's concern that saporin may not be directly available for clinical use at this time, although some investigators are trying to optimize it for clinical use (*Toxins* 2018, 10, 82). The reason we used saporin as a toxin is that saporin-conjugated streptavidin plus biotinylated antibodies or biotinylated MHC tetramers have been reported several times as effective immunotoxins for T cells and hematopoietic stem cells (Refs 41 - 44), as described in the following sentence on page 9.

"...streptavidin-saporin has been used for several immunotoxins to deplete hematopoietic stem cells and T cells in preclinical studies[41, 42, 43, 44]".

Based on these reports, saporin was used here as a model toxin for a proof-of-concept study rather than as a real toxin for clinical application.

Nevertheless, we have further evaluated the potential of small molecule toxins for use as T-cell killing toxins, as suggested by the reviewer. When we used different forms of

cotinine-conjugated duocarmycin and emtansine (DM1) in *in vitro* T cell cytotoxicity assays, the selective killing effect on Cot CAR-T cells was similar to that observed with Cot-saporin. Thus, this strategy has the potential to be translated into the development of clinical grade immunotoxins. These experiments are now presented as a new Supplementary Fig. 17 and mentioned in the Results section on page 9.

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4. The authors report infiltration of CD40 CAR T cells into the lungs, liver, and spleen, but do not disclose which cell types in these organs express CD40. Do the authors have any idea which cells in these healthy organs express CD40 and why their attack by the CAR T cells is so toxic? The protein atlas reports expression of CD40 on healthy macrophages, B cells, basal respiratory cells, secretory cells, dendritic cells, adipocytes, endothelial cells and many other cell types. Do the authors know whether elimination of any of these cell types causes the observed toxicity? One wonders why the authors selected a tumor antigen that is expressed so widely on healthy cells?

As described on page 5 for Figure 2i and j, CD40 CAR-T cells accumulated in the lung prior to lethality. And we observed severe perivasculitis on histologic examination. We also observed moderate immunostaining of the lung endothelium with our CD40 adaptor (Supplementary Fig. 8b). Therefore, we think that the endothelial cells in the lung would be the main target of CD40 CAR-T cells in this model. During the revision, we found literature that reported single cell mRNA sequencing data in mouse and human lungs (Ref 63). When we analyzed CD40 expression on different cell types in the lung using these data, vascular cell types expressed high levels of CD40 mRNA compared to other cell types (new Supplementary Fig. 19). These data confirm that CD40 mRNA-rich lung endothelium could be a major target of CD40 CAR-T cells. However, we also found in the literature that CD40 protein expression was not readily detected by conventional immunohistochemistry, but could be detected by highly sensitive radiolabeled anti-CD40 antibodies (Ref 61, 62). We also confirmed that CD40 protein could not be detected by immunohistochemistry using commercially available anti-CD40 antibodies (data not shown).

Thus, it appears that although CD40 mRNA is abundantly expressed in lung endothelium, actual CD40 proteins are expressed at low levels in this cell type. We propose that CD40 CAR-T cells could recognize this low level of CD40 protein in the lung endothelium and induce severe vascular inflammation. The endothelial damage may have led to acute lung injury and high mortality as described in the literature (Ref 64). In contrast, Cot CAR-T cells coupled to adaptors may have ignored this low CD40 expression in the lung endothelium and did not induce lung inflammation, consistent with the avidity tuning effect proposed in this study. Coherently, switchable CAR-T cells showed much lower cytotoxicity on other CD40-low cells such as macrophages and dendritic cells, probably in the spleen, as described below.

This rationale for CD40 CAR-T cell toxicity on normal tissues is now newly described in the Discussion section on pages 11 and 12. The analysis of CD40 mRNA expressing cell types using the scRNAseq database is now presented as a new Supplementary Fig. 19.

The reason we chose CD40 as the target antigen is that CD40 has long been a known tumor antigen targeted by anti-CD40 antibodies, as described in the Introduction section on page 3 as follows.

"The tumor antigen used is CD40, which is known to be expressed in various tumors, such as lymphoma, multiple myeloma, and acute myelocytic leukemia[23]. CD40 is also expressed on various immune cells, such as monocytes, macrophages, and dendritic cells, for which it acts as a stimulatory receptor[24]. Hence, antagonistic or agonistic anti-CD40 antibodies have been tested as anti-tumor immunotherapeutic modalities[25, 26]."

However, anti-CD40 antibodies did not show clinical benefit in clinical trials. Therefore, we believed that CD40-targeting CAR-T cells would enhance the therapeutic efficacy of CD40-targeting immunotherapy as described in the Discussion section on page 11 as follows.
"Although the high expression of CD40 in tumors and the stimulatory role of CD40 in dendritic cells and macrophages have led to the extensive development of both antagonistic and agonistic antibodies, the therapeutic efficacy of anti-CD40 antibodies in clinical trials has not been very impressive[59]. Therefore, CD40 may be a target for CAR-T cell development to improve the efficacy of the antagonistic strategy."

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5. Did the authors observe cytotoxicity of Cot CAR T cells and CD40 CAR T cells on macrophages when performing the co-culture studies?

We agree with the reviewer that we need to investigate whether Cot CAR-T cells have less cytotoxicity on macrophages than CD40 CAR-T cells. Therefore, we performed the cytotoxicity assay on macrophages with the two types of CAR-T cells. As expected, CD40 CAR-T cells showed full cytotoxicity on macrophages similar to that on A20 tumor cells. In contrast, Cot CAR-T cells showed less cytotoxicity on macrophages than CD40 CAR-T cells at all adaptor dose ranges. This reduced cytotoxicity was also seen on another CD40-expressing innate immune cell, dendritic cells.
These experiments are now shown as new Supplementary Fig. 11a and b, and described in the Results section on page 7.

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6. To control the GVHD, why didn't the authors simply discontinue administering the tumor-targeting adapter?

We apologize for not describing the reason more clearly. Allogeneic GVHD by donor CAR-T cells is the result of the allogeneic reaction between the T cell receptor of the donor cells and the MHC of the recipient tissues. Thus, even in the absence of CAR recognition of CAR targets on recipient tissues, GVHD can occur via T cell receptor on donor CAR-T-cells. Therefore, simple removal of adaptors cannot eliminate allogeneic GVHD.
For clarification, we have revised the relevant sentence in the Results section on page 10 as follows.
"This GVHD toxicity cannot be controlled by simply removing the anti-tumor adaptors, even for switchable CAR-T cells, because GVHD is induced by donor T cell recognition of recipient MHC through donor T cell receptor, not donor CAR."

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7. Does the use of saporin toxin have any immunogenicity concerns when administered to humans?

Saporin may be immunogenic in the human body. As explained above, the use of saporin as a toxin here is for a proof-of-concept study rather than for clinical use. However, since cotinine-drug conjugates will be administered for a short period of time to deplete Cot CAR-T cells rather than for long-term use, immunogenicity may not be a major issue for this purpose.

Overall, the manuscript is okay to publish elsewhere, but not novel enough to publish in Nature Communication

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We agree that the concept of controlling cytokine release syndrome with switchable CAR-T cells is not new. However, as we wrote in the Introduction section and as reviewer #3 also acknowledged, the control of "on-target off-tumor toxicity" of CAR-T cells by this switchable CAR system has never been reported before and may have a major impact on the CAR-T field, particularly on the expansion of CAR target antigens. We hope that the reviewer appreciates the novelty of our work.

Reviewer #2 (Remarks to the Author):

There is an increasing effort in the field to generate better CAR-Ts to treat cancer. In this manuscript, the authors assess whether a switchable CAR-T with a tumor- targeting adaptor can reduce off-tumor toxicity. There construct also includes a suicidal drug-conjugated tag that can eliminate the CAR-Ts to reduce long-term tox.
The authors use CD40 as antigen and clearly show the advantage of the switchable CarT.
The idea is original and important as this will allow the design of new CAR-Ts against Ag that are not tumor-specific without increasing adverse side-effects.
The manuscript is well written, has a logical flow, presents clean data and conclusions are sound.

We thank the reviewer for recognizing our work.

While the strategy aiming at targeting CD40pos A20 cells shows therapeutic results in fig4, one could wonder to which extend the CD40+ macrophages and DCs are being targeted as well. There are indeed many clinical trials using anti-CD40 agonist with promising results (when used in combination therapies). Which cells exactly will be targeted by the CARs?

We agree with the reviewer that since Cot CAR-T cells have low cytotoxicity to macrophages and dendritic cells (new Supplementary Fig. 11a, b), the CAR-T cells may have stimulated them via CD40 adaptors, thereby indirectly facilitating the anti-tumor

immune response. To investigate this possibility, we co-cultured these innate cells with Cot CAR-T cells plus CD40 adaptors and examined the upregulation of their activation markers such as MHC, CD80 and CD86 on the cell surface (new Supplementary Fig. 11c, d). Consistent with the reviewer's suggestion, upregulation of CD80 and CD86 was observed on dendritic cells, although upregulation of these markers was not observed on macrophages. Therefore, Cot CAR-T cells coupled with CD40 adaptors partially stimulate antigen-presenting cells *in vitro*.

However, Cot CAR-T cells with CD40 adaptors are fundamentally cytotoxic to tumor cells *in vitro* and thus may also directly kill tumor cells *in vivo*. To measure the impact of this direct tumor cytotoxicity of CAR-T cells, we generated CD40-deleted A20 cells using the CRISPR/Cas9 method and evaluated the therapeutic efficacy of Cot CAR-T cells on these tumor cells *in vivo* (new Supplementary Fig. 12). As a result, Cot CAR-T cells showed no therapeutic effect on CD40-deleted A20 cells, suggesting that the anti-tumor effect of Cot CAR-T cells in this model is mainly due to direct tumor cytotoxicity rather than indirect immunostimulatory effects. Nevertheless, we believe that the immunostimulatory effect of these switchable CAR-T cells does exist and will pursue this effect in future studies.

These experiments are now presented as new Supplementary Figs. 11 and 12 and described in the Results section on pages 7 and 8 and in the Discussion section on page 12.

Since BalbC mice are generally more Th2 polarized compared to C57Bl6 mice, it is important to recapitulate the therapeutic results found in the A20 in a C57Bl6 tumor model (for example B16F10 or MC38, which are still not very high-bar).

The reviewer's point is well-taken. Since the tumor model used in this study is a lymphoma (A20), we chose EL4, another lymphoma on B6 background. As EL4 does not express endogenous CD40, we generated a CD40-overexpressing EL4 cell line (EL4-mCD40) for this experiment. When Cot CAR-T cells were injected into EL4-mCD40-bearing albino B6 mice with CD40 adaptors, as done for Fig. 4, we observed moderate anti-tumor efficacy as measured by bioluminescence assay, recapitulating the result with A20 in Balb/C mice. However, the potency of this therapeutic effect was somewhat lower than expected (please see "Additional Information for Reviewers" at the end of this letter). There could be several explanations for this low potency. We used a new lymphodepleting irradiator (X-ray irradiator) at our campus, which is different from the one used for Balb/C mice (Cesium irradiator). Although we used the same radiation dose we usually use for B6 mice (3 Gy), we felt that the degree of lymphodepletion for the X-ray irradiator was lower than for the Cesium irradiator, which reduces the therapeutic efficacy. Or, because the EL4 cells infused intravenously are usually very aggressive in our experience, the Cot CAR-T cells may not have been able to show a profound therapeutic effect.

We felt that although these data may support the efficacy of Cot CAR-T cells in mice other than Balb/C, the experimental setting has not yet been optimized. However, since the publication of this paper has been significantly delayed, instead of adding this result to the paper, we would like to provide the result to the reviewers only as "Additional Information for Reviewers".

If the editors or reviewers feel that this result should be included in the publication, we are happy to provide these data as a Supplementary Figure.

In figure 2, the authors assess the role of IL1b and IL6. However, anti-CD40 was shown to also induce IL-12 by both Mf and DCs (Murgaski et al, Cancer Res 2022). Do the CD40 Car induce IL-12 production by the splenic DCs? Moreover, it was recently shown that CD40-mediated IL12 production in mouse induces toxicity via the activation of Kupffer cells and neutrophils (Siwicki et al, Sci Immunol 2021). Would IL12 blockade lower the CD40 CAR toxicity more than IL6 blockade/anakinra (specifically looking at the early weight loss peak)?

We appreciate the reviewer's suggestions.
When we co-cultured the Balb/C macrophages or splenic dendritic cells with CD40 CAR-T cells, we could not detect IL-12 secretion as determined by ELISA (data not shown). We then performed an *in vivo* IL-12 neutralization experiment similar to that shown in Fig. 2 with anti-IL-12 antibody treatment alone or in combination with anti-IL-6 and anakinra (new Supplementary Fig. 5). At the high dose of CD40 CAR-T cells, neither anti-IL-12 antibody alone nor the triple blockade (anti-IL-12 + anti-IL-6 + anakinra) could rescue the mice from lethality. At the low dose of CD40 CAR-T cells, although anti-IL-12 antibody treatment alone showed a small degree of reduction in body weight loss, the triple blockade was not better than anti-IL-6 plus anakinra in preventing toxicity. Therefore, it appears that IL-12 blockade is not able to contribute to the prevention of acute toxicity induced by CD40 CAR-T cells in this model.
These results are now presented as a new Supplementary Fig. 5 and described in the Results section on page 5.

Why do cot-Car-T bypass the lungs? What is the hypothesis?

As we discussed the expression of CD40 on normal lung endothelium in response to reviewer #1, we propose that CD40 proteins are expressed at low levels on lung endothelium. CD40 CAR-T cells appear to be potent enough to recognize these low levels of CD40 and induce lethal perivascular inflammation. In contrast, Cot CAR-T cells may not recognize the low levels of CD40 expression on lung endothelium and leave the lung without inflammation, consistent with our avidity tuning hypothesis.
This interpretation is now discussed in the Discussion section on pages 11 and 12.

There is still an ongoing debate on whether cotinine acts as a psychoactive in humans. This should briefly be discussed.

We acknowledge the reviewer's point. Therefore, we have rephrased the relevant sentence in the Discussion section on page 12 with a new reference (Ref 67) as follows.
"As a nicotine metabolite, it has been shown to be safe for smokers although there is some debate about its mild neuroprotective or neuropsychiatric role[66, 67]."

Some figures are difficult to read, for example suppl fig 3b: it's very hard to say what the dotted

lines correspond to (the different dotted lines are very similar). Perhaps more colors could help?

We have replaced the original Supplementary Fig. 3 with the new Supplementary Fig. 4, which is better represented by colored markings.

Reviewer #3 (Remarks to the Author):

The authors demonstrate the ability to combat the major issue in the field of CAR T cells of on-target off-tumor toxicities by applying a universal adaptor CAR T cell approach. While several universal adaptor CAR T cell systems have been created, this is the first system that shows that dosing of the adaptor can lead to eliminating this type of toxicity. The authors first establish a syngeneic mouse model targeting CD40 antigen that is expressed on leukemia cells and on normal cells leading to lethal ON-target/OFF-tumor toxicities. As an alternative approach to a standard CAR, the authors employ their previously reported "Cot-CAR" that binds to tumor the nicotine metabolite cotinine on tumor targeting antibody adaptors, to ultimately target tumor cells. The authors show that in contrast to the standard anti-CD40 CAR, the adaptor CAR is able to specifically target high expressing tumor cells and not normal cells both in vitro and in vivo in a mouse model by tuning the adaptor dose. While the issue of ON-target/OFF-tumor toxicity has been addressed using several alternative methods including sensing of antigen combinations using combinatorial CAR systems, or avidity tuning via small molecule control as summarized by the authors, this is the first report of using a universal adaptor CAR technology to treat this major issue that plagues the targeting of many antigens especially in the solid tumor setting. The authors go into the mechanism of lack of toxicity for the adaptor CAR showing that the T cells are not undergoing the same stimulation in OFF-tumor sites (lung, spleen, liver) as compared to the standard anti-CD40 CAR. Finally, the authors generate a suicide adaptor containing the toxin saporin fused to the cotinine tag that rapidly kills off the Cot-CAR cells. They demonstrate activity in vitro as well as in vivo in mouse models including in an allogeneic CAR T/GVHD model. While it is more common for allogeneic CAR T cell therapy to have issues with T cell persistence, allogeneic cells can lead to GVHD toxicities in which case a targeted killing approach like this would be advantageous.
Overall the paper is well-written with logical experimental flow, and the data are robust with a major novel findings for the field. There are several concerns to be addressed before the manuscript is suitable for publication:

We appreciate the reviewer's acknowledgment of our work.

Concerns:
-Fig 2 add data showing confirmation of successful depletion of the macrophages

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We have now included the data showing effective depletion of macrophages in Supplementary Fig. 6 as suggested by the reviewer. The relevant sentence has been added to the Results section on page 5.

-Include text explaining why IL-6 production is used as a read-out for normal cell toxicity. Additionally, include in addition direct measurements of macrophage cell toxicity

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We appreciate helpful suggestions.
We have added new sentences to explain the reason for choosing IL-6 as a toxicity parameter in the Results section on page 6 as follows.
"*To test this concept in vitro, we chose macrophages as a normal cell type because macrophages express CD40 and we have already observed that macrophages produce the toxic cytokine IL-6 upon co-incubation with CD40 CAR-T cells (Fig. 2a). In this case, IL-6 production by macrophages may be a sensitive indicator of CAR-T cell-mediated toxicity on macrophages in co-culture experiments.*"

We have also included data on differential cytotoxicity on macrophages and also on dendritic cells by CD40 CAR-T cells and Cot CAR-T cells as Supplementary Fig. 11a and b. These data are now described in the Results section on page 7.

-Check statistics throughout paper. In several figures where multiple comparisons are being performed, ANOVA testing should be used instead of t tests. Additionally, description of what the error bars are describing (stdev or sem) and number or samples/replicates is lacking in several figure legends.

We apologize for the lack of attention to statistics and detailed experimental description. We have consulted with a statistician and have now used appropriate statistical tools to analyze multiple comparisons, such as 1-way ANOVA (Fig. 2c and e, Fig. 6f, and new Supplementary Fig. 5b) or nonparametric Kruskal-Wallis test (Fig. 4e and Fig. 5g). These analyses are now described in the corresponding figure legends and in "Statistical analysis" in the Methods section on page 20.

New descriptions of the error bars used and the number of samples have been added to the legends of the figures and have been highlighted in blue.

-In Fig 1 can A20 tumor be cleared in CD40KO mouse by standard anti-CD40 CAR T cells? This would establish that the standard anti-CD40-CAR is functional and capable of treating A20 tumor

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We agree that the proposed experiment would be another way to demonstrate that CD40 can be a good target for CAR-T cells in the absence of on-target off-tumor toxicity. However, A20 cells could not be used for this purpose because the CD40 KO mice used in this study are on the B6 background, whereas A20 tumor cells are derived from Balb/C mice. Therefore, we used another lymphoma cell line EL4 from B6 mice for this experiment. Since EL4 do not express CD40, we generated a new CD40-overexpressing EL4 (EL4-mCD40) cell line. When we injected CD40 CAR-T cells into EL4-mCD40-bearing CD40 KO mice, acute lethal toxicity was not observed as expected. Instead, mice treated with CD40 CAR-T cells survived longer than untreated CD40 KO mice, suggesting that CD40 CAR-T cells can be effective for tumor treatment in the absence of

an off-tumor target (new Supplementary Fig. 2). However, statistical significance could not be reached due to the small number of mice used for each group (n=4), as we had difficulty obtaining sufficient numbers of CD40 KO mice through breeding during this revision period. We ask for the understanding of the reviewer in this matter.
This new result is now presented as new Supplementary Fig. 2 and described in the Results section on page 4.

-Use Greek micro symbol 'μ' instead of 'u'

We are grateful to the reviewer for correcting this typographical error. We have corrected this typo throughout the manuscript.

- "Taken together, the switchable Cot CAR-T cells were able to eliminate CD40-expressing tumors without on-target off-tumor toxicity, which cannot be avoided in conventional CD40 CAR-T cell therapy." It looks like there is still some residual tumor in some mice, so tone down this language to something like "…significantly reduced the size of CD40 expressing tumors without on-target…"

We think the reviewer's comment is valid. Therefore, we have corrected the sentence on page 8 as follows.
"Taken together, the switchable Cot CAR-T cells were able to significantly reduce CD40-expressing tumor burden without on-target off-tumor toxicity, which cannot be avoided in conventional CD40 CAR-T cell therapy."

-"Similar to the murine CAR-T cell model, hCot CAR-T cells co-infused with hCD40 adaptor effectively eliminated tumor cells compared to hCot CAR-T cells in the absence of adaptor (Fig. 5f, g)." similar comment to above, to tone down the language here.

The indicated sentence on page 8 has been corrected to read as follows.
"Similar to the murine CAR-T cell model, hCot CAR-T cells co-infused with hCD40 adaptor effectively reduced tumor burden compared to hCot CAR-T cells in the absence of adaptor (Fig. 5f, g)."

-"The result of this study is one of the examples demonstrating that an optimal therapeutic window of a CAR-T cell can be established to maintain anti-tumor efficacy with minimal normal tissue toxicity with a single CAR system, obviating the need for dual CAR systems such as SynNotch, Split or iCARs." While the CD40 adaptor system works to reduce on-target off tumor toxicities here, there remain myriad applications where single antigen targeting even using adaptor tuning would not be sufficient, such as cases where the antigen expression level on tumor is not significantly higher than on normal cells, cases of antigen downmodulation on tumor cells, or

antigen expression level heterogeneity. Please edit this and add some more nuanced discussion of the technologies and trade-offs.

We consider the reviewer's criticism to be valid. We have therefore rephrased the relevant part of the Discussion section on page 11 as follows.

"The switchable CAR-T system, targeting a single antigen, also has its shortcomings. Tumors with relatively low tumor antigen levels, tumors with down-regulated tumor antigens, or tumors with antigenic heterogeneity may not be efficiently eliminated by switchable CAR-T cells. In these cases, multi-antigen targeting by adding adaptors against a second or third tumor antigen, as originally proposed, may be useful[17, 18]. However, the practical hurdle would be that the adaptors would have to be produced as a separate protein drug for each individual target, increasing the cost of the overall treatment."

-"The epitope tag for the switchable adaptor should be carefully selected. It needs to be biologically inert, non-toxic, and non-immunogenic. In this sense, cotinine seems to be one of the ideal chemical tags for the adaptors." While the cotinine on its own may be non-immunogenic, as part of a drug-protein adduct it is likely that it will be immunogenic. Unless you can provide data supporting lack of anti-cotinine adaptor antibody responses in the mice (this would be great data to add if you have it), then you cannot make this claim. Overall, it may not be an issue to have some immunogenicity against the adaptor, but lack of immunogenicity to the adaptor would be unexpected and the lack of immunogenicity to the metabolite alone is not sufficient evidence.

We agree with the reviewer that it is inappropriate to claim that cotinine-labeled adaptors are non-immunogenic. Therefore, we have removed the non-immunogenicity claim and toned down the relevant part in the Discussion section on page 12 as follows.

"The epitope tag for the switchable adaptor should be carefully selected. It needs to be biologically inert and non-toxic. In this sense, cotinine seems to be one of the appropriate chemical tags for the adaptors. As a nicotine metabolite, it has been shown to be safe for smokers although there is some debate about its mild neuroprotective or neuropsychiatric role[66, 67]."

-Discuss general applicability of the strategy to other antigens as well as the issue of reduced antigen expression on tumor cells, and possible strategies, eg: potentially target a second tumor-high antigen

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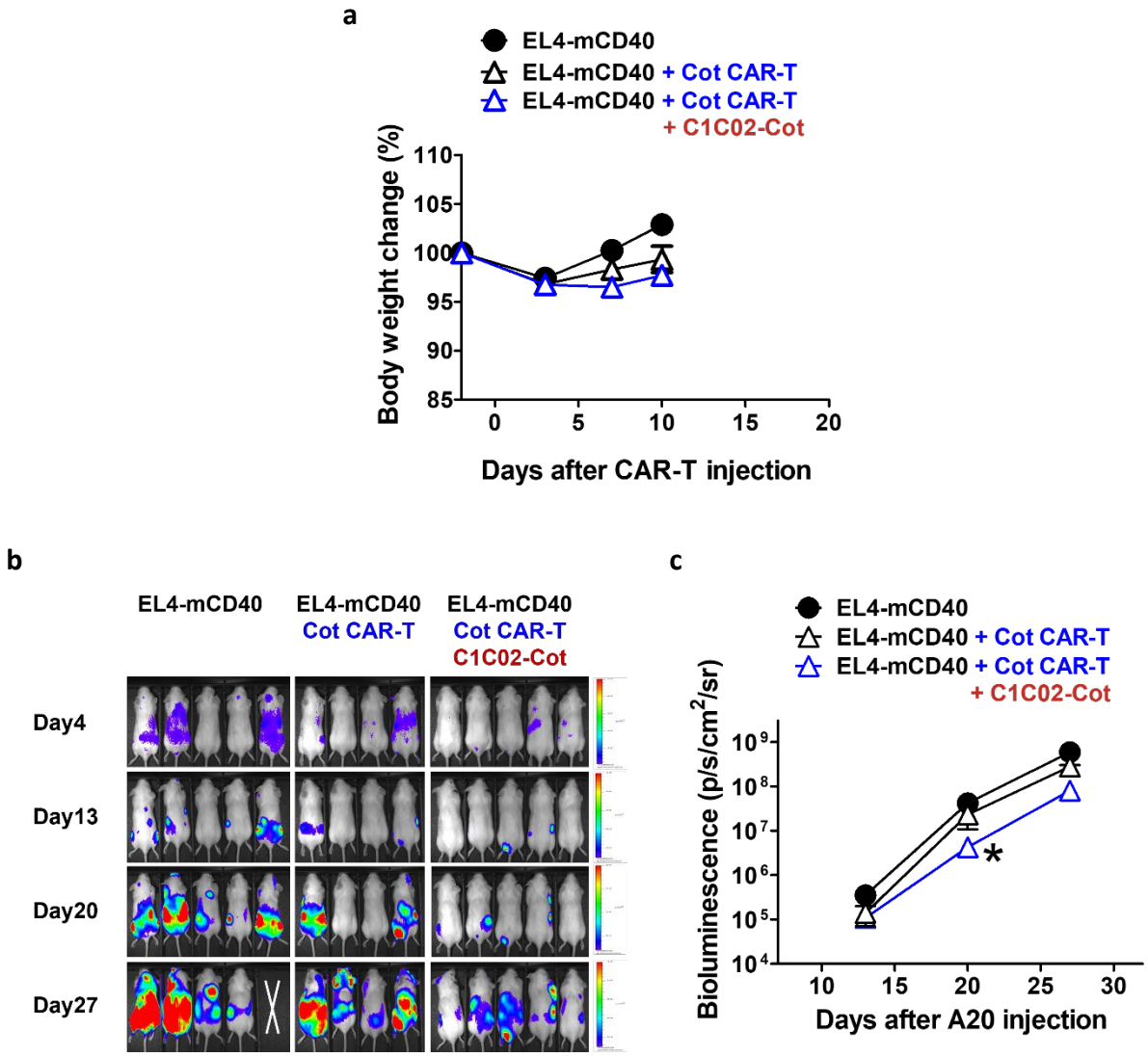
We have incorporated this strategy into the new statements we have written about this technology, as described above in the Discussion section on page 11, as follows.

"The switchable CAR-T system, targeting a single antigen, also has its shortcomings. Tumors with relatively low tumor antigen levels, tumors with down-regulated tumor antigens, or tumors with antigenic heterogeneity may not be efficiently eliminated by switchable CAR-T cells. In these cases, multi-antigen targeting by adding adaptors against a second or third tumor antigen, as originally proposed, may be useful[17, 18]. However, the practical hurdle would be that the adaptors would have to be produced as a separate protein drug for each individual target, increasing the cost of the overall treatment."



Additional Information for Reviewers. Cot CAR-T cells with CD40 adaptors show partial therapeutic efficacy in B6 mice. Albino B6 mice were injected *i.v.* with EL4-mCD40-Luc cells (5×10^5) on day 0, irradiated (3 Gy) for lymphodepletion on day 6, and injected with Cot CAR-T cells (5×10^6) on day 7. From the day of CAR-T cell injection, C1C02-Cot (20 μg/head) was injected *i.v.* every other day for a total of 8 times. **a** Body weight changes (*n*=5) were measured. **b** Bioluminescence imaging of tumor burden after Cot CAR-T cell plus C1C02-Cot treatment at indicated time points after EL4-mCD40-Luc cell injection. **c** Bioluminescence intensity was calculated as the mean flux (p/s/cm^2/sr, mean ± SEM) of a region of interest (ROI) in a single mouse (*n*=4 or 5). Statistical significance between groups at each time point was determined by the nonparametric Kruskal-Wallis test (*p<0.05, compared to EL4-mCD40 only group).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed the reviewer's questions satisfactorily. The manuscript can be accepted.

Reviewer #2 (Remarks to the Author):

The authors performed a substantial amount of experiments which enabled them to reply to all the major comments I had.
I would appreciate some minor changes:
- I believe it would be more transparent to add the results obtained in the EL4-mCD40 model as supplemental figure. I believe it's a good practice to also show "negative" data.
- In the discussion, in the part about the expression of CD40 on the lung endothelium, the authors mention two novel supplementary figures. Original data is typically not added in the discussion and ideally the parts referring to these original figures should be integrated in the result section
- Write "C57BL/6" mice instead of "B6"

I believe the quality of the manuscript increased and in principle I now recommend it's publication.

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed my previous concerns and comments. The reported data is sound and would make an important contribution to the CAR T literature. I have just a couple of minor comments regarding newly added data - 1. the "Additional Information for Reviewers" should be included as supplementary data, and 2. the discussion of supp fig 2 should instead use language such as "trending toward showing anti-tumor activity" as this result is not significant, 3. and the title of fig S2 should also be altered to reflect this point.

Response to the reviewers' comments

We thank the reviewers for their final recommendations to improve the manuscript. The changes are now marked by the 'Track Change' feature, as requested by the editor.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed the reviewer's questions satisfactorily. The manuscript can be accepted.

We are grateful for the reviewer's final approval.

Reviewer #2 (Remarks to the Author):

The authors performed a substantial amount of experiments which enabled them to reply to all the major comments I had.

I would appreciate some minor changes:

- I believe it would be more transparent to add the results obtained in the EL4-mCD40 model as supplemental figure. I believe it's a good practice to also show "negative" data.

The reviewer's point is well taken. We have now included this result as a new Supplementary Fig. 14 and mentioned it in the Results section on page 8 as follows: "*The absence of acute lethality of Cot CAR-T cells with the CD40 adaptor was also recapitulated in C57BL/6 (hereafter B6) mice inoculated with CD40-transduced EL4 lymphoma, although the therapeutic benefit in this model was moderate (Supplementary Fig. 14).*"

- In the discussion, in the part about the expression of CD40 on the lung endothelium, the authors mention two novel supplementary figures. Original data is typically not added in the discussion and ideally the parts referring to these original figures should be integrated in the result section

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As suggested by the reviewer, the original Supplementary Fig. 19 has now been moved to a new Supplementary Fig. 9. The results related to this figure are now described in the Results section on page 6 as follows:
"It was also found that CD40 mRNA was enriched in vascular cells among various cell types in the lung (Supplementary Fig. 9)."

- Write "C57BL/6" mice instead of "B6"

We have written the full name of this strain, C57BL6, when first used in the text, with a note to use its abbreviated name, B6, thereafter, as follows on page 8:
"The absence of acute lethality of Cot CAR-T cells with the CD40 adaptor was also

recapitulated in C57BL/6 (hereafter B6) mice inoculated with CD40-transduced EL4 lymphoma, although the therapeutic benefit in this model was moderate (Supplementary Fig. 14).”

I believe the quality of the manuscript increased and in principle I now recommend it’s publication.

We thank the reviewer for the final approval.

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed my previous concerns and comments. The reported data is sound and would make an important contribution to the CAR T literature.

We appreciate the final approval of the reviewer.

I have just a couple of minor comments regarding newly added data.
1. the “Additional Information for Reviewers” should be included as supplementary data, and

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As suggested by the reviewer, this result is now presented as a new Supplementary Fig. 14 and described in the Results section on page 8 as follows:
“The absence of acute lethality of Cot CAR-T cells with the CD40 adaptor was also recapitulated in C57BL/6 (hereafter B6) mice inoculated with CD40-transduced EL4 lymphoma, although the therapeutic benefit in this model was moderate (Supplementary Fig. 14).”

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2. the discussion of supp fig 2 should instead use language such as “trending toward showing anti-tumor activity" as this result is not significant,

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We have rephrased the relevant sentence in the Results section on page 4 as follows:
“In the absence of toxicity in these mice, CD40 CAR-T cells now tended to exhibit anti-tumor efficacy (Supplementary Fig. 2).”

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3. and the title of fig S2 should also be altered to reflect this point.

As suggested by the reviewer, the title of Supplementary Fig. 2 has been changed to read as follows:

“CD40 CAR-T cells tend to show antitumor efficacy without lethal toxicity in CD40 knockout mice.”
