

Engineered antibodies that stabilize drug-modified KRAS^{G12C} neoantigens enable selective and potent cross-HLA immunotherapy

Corresponding Author: Dr Lauren Stopfer

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)

In their current manuscript titled "Engineered antibodies that stabilize drug-modified KRAS^{G12C} neoantigens enable selective and potent cross-HLA immunotherapy," Lorenzo Maso and colleagues describe the development and functional characterization of antibodies that target chemically modified A3/KRAS^{G12C} and A11/KRAS^{G12C} epitopes for immunotherapeutic applications—specifically as components of CD3-engaging T-cell engagers (TcEs).

The core concept involves covalently modifying the free sulfhydryl group in the mutant KRAS^{G12C} peptide using the cell-permeable, SH-reactive drug sotorasib. This chemical modification generates a neoantigen that is selectively presented on KRAS^{G12C}-positive cancer cells. The bispecific TcE, designed to bind on the one hand CD3 and on the other hand the sotorasib-modified KRAS^{G12C} peptide-HLA complex (the latter with high affinity), then facilitates T-cell-mediated tumor cell killing.

The authors provide compelling evidence for:

1. Specific, high-affinity binding of their engineered AETX-R114 TcE.
2. The structural mechanisms enabling effective antibody targeting.
3. Stabilization of the KRAS^{G12C}-HLA complex upon AETX-R114 binding.
4. Potent, antigen-specific T-cell-mediated cytotoxicity in vitro and in vivo (using tumor-challenged NSG mice).
- 5.

In addition, the authors describe a second engineered cross-HLA-supertype antibody, R302, which recognizes a divarasib-conjugated cysteine-containing peptide presented by HLA molecules. Divarasib is another SH-reactive drug, supporting the modularity and potential generalizability of the platform.

This manuscript presents an innovative and well-executed study. The data are robust, the methodology is sound, and the conclusions are well-supported. Notably, the engineered system offers remarkable T-cell antigen sensitivity—critical for effectively killing tumor cells with low antigen levels.

I strongly recommend publication of this manuscript, pending inclusion of one final control experiment. Specifically, the authors should evaluate potential off-target effects by challenging primary T-cells isolated from healthy HLA-A3- and HLA-A11-positive donors with sotorasib-treated autologous EBV-transformed (or otherwise transformed) B cells in the presence of AETX-R114. This control is essential to assess the degree of T-cell cross-reactivity against other cysteine-containing A3- or A11-restricted self-epitopes as they may be produced during treatment.

Reviewer #2

(Remarks to the Author)

The authors present comprehensive and solid data on their strategy to target covalent k-Ras derived haptenylated peptides presented on HLA of cancer cells (lines). They provide convincing quantitative peptidomics data for the presentation of the peptide and show how a T cell engager by binding to the hapten-peptide-MHC complex the complex is stabilized and the copy number enhanced. Finally an intelligent strategy was chosen to target different haptenylated peptides in the context of different HLA supertypes and findings supported by cryo-EM data.

In my view the findings are very relevant and significant and the work presented supports the conclusion and is very well prepared

I only have few minor comments

- Include some benchmarking to hapten-peptide specific antibodies described in the literature and used for bispecifics in key experiments would be good to show the superiority
- Testing the cleanness of the TCE on whole blood or other tissue samples from HLA-typed donors or normal cells to show absence of unspecific binding/killing. Question: I am not sure whether the lack of weight loss in the mice can really be used as evidence for safety as the mice are not human HLA transgenic, in this context the question is whether mouse MHC molecules present the haptenylated peptides and whether they can be recognized by the pMHC binders optimized for different (human) HLAs?
- In general the chosen in vivo setting with co-mixed tumor and T cells is a low bar model and a PBMC engrafted or better humanized setting would be more realistic in terms of translation
- Provide additional data on the engineering of the TCE, which CD3 antibody is used, what is the affinity etc

Reviewer #3

(Remarks to the Author)

To the Authors:

Maso and colleagues report on an enhanced-engineered antibody (R114) targeting a haptenated KRAS G12C peptide presented by members of the HLA-A*03:01 super family (A3, A11, A68). Using A375 cells overexpressing KRAS G12C protein and HLA class I molecules, the authors performed immunopeptidomics to identify hap-KRAS G12C pep /HLA complexes and report these complexes to be present at >10p/MHC per cell. The authors demonstrate that binding of R114 to drug-treated cells enhances ½ life of p/MHC complexes suggesting the antibody promotes stability of complexes. Using R114 as a bispecific T cell engager, the authors evaluate in vitro and in vivo the ability of this agent to control tumor growth. Finally, the authors report on a distinct antibody, R302, that recognizes hap-KRAS G12C peptides of various AA sequences presented by unrelated HLA class I molecules, performed limited in vitro functional characterization using R302 as a T cell engager and structurally characterize R302 binding to HLA class I alleles. The study builds on previous reports from the authors, with antibody R114 presenting better affinity and selectivity for p/MHC and not free drug. Thus, AETX-R114 may represent an enhanced novel agent that targets haptenated p/MHC complexes resulting from soto, ada or diva treatment.

Major Concerns:

- 1- The authors have affinity mature R114 from R023. Any concerns that this enhanced antibody may cross-react with non-haptenated HLA class I alleles?
- 2- What is the levels of A3 and A11 expression on the cancer cell lines studied (Fig4a). Antibodies directed at these alleles are available. Interesting that engager shows better activity against A3 than A11 expressing cancer cells despite p7 exhibiting greater affinity for A11.
- 3- All the functional assays are performed with expanded T cells, what is observed with unactivated (freshly isolated from PBMC) T cells?
- 4- What is the rationale for mixing tumor and T cells at time of implantation. What is the long-term survival of AETX-R114 treated mice (Fig 4f)? The model has numerous deficiencies as it requires multiple T cell + engager injections to observed efficacy. Have the authors consider using huCD34 reconstituted NSG-FLT3 mice?
- 5- For the characterization of R302 and AETX-R302, the authors limit their analysis to cells pulsed with supra concentration of peptide. In the absence of evidence that R302 (and AETX-R302) recognizes processed and presented hap-KRAS G12C/HLA complexes, the relevance of this data is questioned. Although, a peptide derived from mutKRAS G12 has been reported to bind HLA-A*02:01, to date there is no evidence that such peptide is naturally processed and presented. The study will greatly benefit if functional characterization of AETX-R302 is conducted using KRASG12C/A2 cancer cell lines.

Minor Comments:

- 1-Fig 2d, in Table says AETX-R114 KD A*11 as160pM, text says 130pM.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The control experiment I suggested has been performed by the authors, and the results speak in favor of the specificity of the approach. I hence support publication of the ms. in its current revised form.

Reviewer #2

(Remarks to the Author)

The points raised during the review have been addressed sufficiently

Reviewer #3

(Remarks to the Author)
No additional comments.

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Point by point response to reviewers

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 5. In addition, the authors describe a second engineered cross-HLA-supertype antibody, R302, which recognizes a divarasil- conjugated cysteine-containing peptide presented by HLA molecules. Divarasil is another SH-reactive drug, supporting the modularity and potential generalizability of the platform.
- This manuscript presents an innovative and well-executed study. The data are robust, the methodology is sound, and the conclusions are well-supported. Notably, the engineered system offers remarkable T-cell antigen sensitivity—critical for effectively killing tumor cells with low antigen levels.

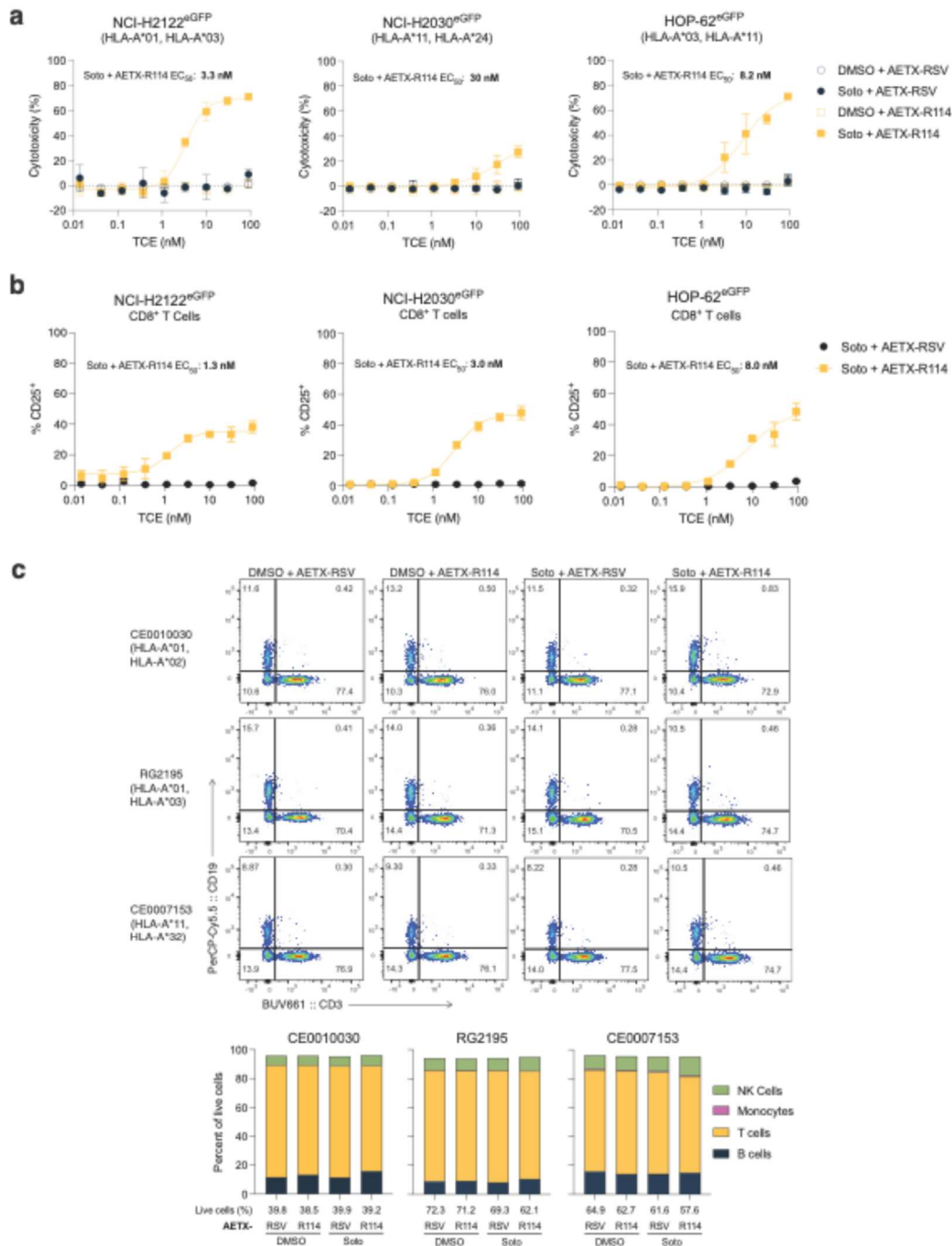
I strongly recommend publication of this manuscript, pending inclusion of one final control experiment. Specifically, the authors should evaluate potential off-target effects by challenging primary T-cells isolated from healthy HLA-A3- and HLA-A11-positive donors with sotorasib-treated autologous EBV-transformed (or otherwise transformed) B cells in the presence of AETX-R114. This control is essential to assess the degree of T-cell cross-reactivity against other cysteine-containing A3- or A11-restricted self-epitopes as they may be produced during treatment.

Response: We appreciate the reviewer's complimentary feedback on our work. To address the reviewer's recommendation of an additional experiment to address off-target effects, we utilized primary PBMCs from 3 different donors: one with HLA-A*11, one with HLA-A*03, and one without an HLA-A matched allele to AETX-R114. We elected to use primary PBMCs instead of transformed B cells for a translationally relevant readout of autologous cytotoxicity across multiple hematopoietic cell types. PBMCs were cultured with 300 nM sotorasib or DMSO and 10 nM AETX-RSV or AETX-R114 in the absence of target cells for 48 h. Immune cell subset percentages were quantified, showing no depletion following AETX-R114/sotorasib treatment in either allele-matched or non-matched donors. A minor (~5%) decrease in viability was observed in matched donors with sotorasib + AETX-R114, possibly due to known off-target effects of sotorasib and not indicative of significant safety concerns. These new data are included as **Supplementary Fig. 10c**, along with a new PBMC cytotoxicity experiment (**Supplementary Fig. 10a,b**) and the following additional text in the Results of the revised manuscript:

"AETX-R114 also elicited cytotoxicity and T cell activation using PBMCs as effector cells. Importantly, there was no evidence of fratricide observed against HLA-matched or unmatched donor cells, providing further evidence of TCE specificity (Supplementary Fig. 10)."

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Supplementary Figure 10: PBMC activity with sotorasib and AETX-R114 treatment.

a, IncuCyte analysis of AETX-R114-induced cytotoxicity against cell lines endogenously expressing KRAS^{G12C} in the presence of sotorasib (soto) or vehicle (DMSO). Cell lines were plated at a 5:1 E:T ratio of PBMCs (donor ID RG3564) to target cells and cytotoxicity was assessed at 72 h. **b**, Flow cytometry analysis of CD8⁺ T cell activation in cytotoxicity assays performed as in Fig. 4a with eGFP-expressing cell lines and a dose response of TCEs at 72 h. All dose response plots show mean $\pm$ standard deviation of 3 technical replicates per datapoint and are representative of 3 independent experiments. **c**, Flow cytometry analysis of live immune cell proportions in PBMCs cultured with 300 nM sotorasib (soto) or equivalent volume of DMSO and 10 nM AETX-RSV or AETX-R114 in the absence of target cells for 48 h. Three different PBMC donors were tested including 2 with on-target alleles (RG2195, HLA-A*03 and CE0007153, HLA-A*11) and one (CE0010030) with no on-target alleles. Representative flow cytometry plots show B cell (CD19⁺) and T cell (CD3⁺) proportions at 48 h and the graphs below show total cell viability and the percentages of each immune cell subset.

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To further acknowledge the reviewer’s point regarding the importance of considering off-target toxicity, we have added a section to the discussion elaborating on this concept, highlighting that the antibodies’ specificity relies heavily on the drug’s specificity and that the drug’s off-target profile should be integrated into the specificity analysis of p*MHC targeting antibodies. To this end, we included an example where we investigated whether 2 known sotorasib off-targets, RTN4 [C1101] and HMOX2 [C282], could be off-targets to AETX-R114. We took +/- 10 amino acids from the off-target cysteine and performed MHC-I binding predictions for HLA-A*03, HLA-A*11, and HLA-A*68 using 2 search algorithms, PanNetMHC4.1 and HLATHENA. Data from A*03 and A*11 of any epitope with a % Rank binding score <5% are summarized below. We also searched using the sequence containing the cysteine as well as substituting the cysteine for tryptophan to mimic the drug hapten, which often changes the prediction slightly. Peptides that did not contain the cysteine in an amino acid position recognized by R023 were filtered out (shown in grey below, recognition motif reported in [reference 33](#) (Maso et al., PNAS, 2024). Here, we assume the recognition motifs between AETX-R023 and AETX-R114 are essentially unvaried because only 3 mutations on the V_L differentiate the 2 antibodies, leaving the paratope largely unchanged. All remaining epitopes (shown in yellow) were not predicted to be binders by either prediction algorithm. As a result, neither off-target cysteine emerged as a threat for AETX-R114.

Gene/Cysteine	Sequence used for searching MHC presentation
HMOX2/C282	QDKGALEGGSSCPFTAMAVLR
RTN4/C1101	YSNSALGHVNCTIKELRRFL

RTN4 C1101						
Allele	Epitope w/ C	PanNetMHC % Rank	HLATHENA % Rank	Epitope w/ W	PanNetMHC % Rank	HLATHENA % Rank
HLA-A*03:01	ALGHVNCTIK	0.827	3.411	ALGHVNWTIK	0.735	1.446
HLA-A*03:01	SALGHVNCTIK	2.239	6.26	SALGHVNWTIK	2.14	1.702
HLA-A*03:01	HVNCTIKELR	6.3141	4.098	HVNWTIKELR	3.717	2.235
HLA-A*03:01	HVNCTIKELRR	7.005	6.406	HVNWTIKELRR	3.798	5.834
HLA-A*11:01	SALGHVNCTIK	1.167	0.508	SALGHVNWTIK	1.122	0.371
HLA-A*11:01	ALGHVNCTIK	2.432	3.69	ALGHVNWTIK	1.79	1.37
HLA-A*11:01	HVNCTIKELR	3.79	5.019	HVNWTIKELR	2.978	5.896
HLA-A*11:01	HVNCTIKELRR	5.844	3.505	HVNWTIKELRR	3.496	3.009
HMOX2 C282						
Allele	Epitope w/ C	PanNetMHC % Rank	HLATHENA % Rank	Epitope w/ W	PanNetMHC % Rank	HLATHENA % Rank
HLA-A*03:01	ALEGGSSCPFR	2.428	8.003	ALEGGSSWPFR	2.08	3.205
HLA-A*11:01	GALEGGSSCPFR	2.836	1.687	GALEGGSSWPFR	2.595	1.195
HLA-A*11:01	ALEGGSSCPFR	2.9	4.747	ALEGGSSWPFR	2.517	2.084
HLA-A*11:01	SSCPFTAM	4.922	17.89	SSWPFTAM	3.308	18.24

- = Epitopes filtered out based on motif
- = Predicted non-binders (%Rank >2%)
- = Epitopes predicted to be weak binders (<2% Rank)
- = Epitopes predicted to be strong binders (<0.5% Rank)

The additional text included in the Discussion of the revised manuscript is as follows:

*“While hapten modification uniquely enables high-affinity, cross-HLA recognition, the potential for off-target hapten modification and presentation of peptides on healthy tissue necessitates rigorous evaluation to avoid toxicity. Unlike conventional pMHC-targeting immunotherapies, standard cross-reactivity assays such as alanine scanning have limited suitability for p*MHCs, as antibody recognition is restricted to hapten-modified peptides. Thus, off-target reactivity of the antibody is dictated primarily by the specificity of the covalent modification. We addressed this issue by identifying peptide positions capable of presenting the hapten for antibody recognition and assessing whether off-target cysteine-containing sequences can bind relevant HLA alleles. Previously identified off-target cysteine sites—RTN4^{C1101} and HMOX2^{C282}—are not predicted to bind A3 supertype alleles or to be recognized by AETX-R114 due to hapten positional constraints³³, reducing the risk of off-target effects^{52,53}. Nevertheless, comprehensive profiling of a small molecule’s off-target liabilities remains critical for ensuring clinical safety and guiding the selection of antibody clones with that avoid unintended cross-reactivity.”*

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Point by point response to reviewers

REVIEWER #2

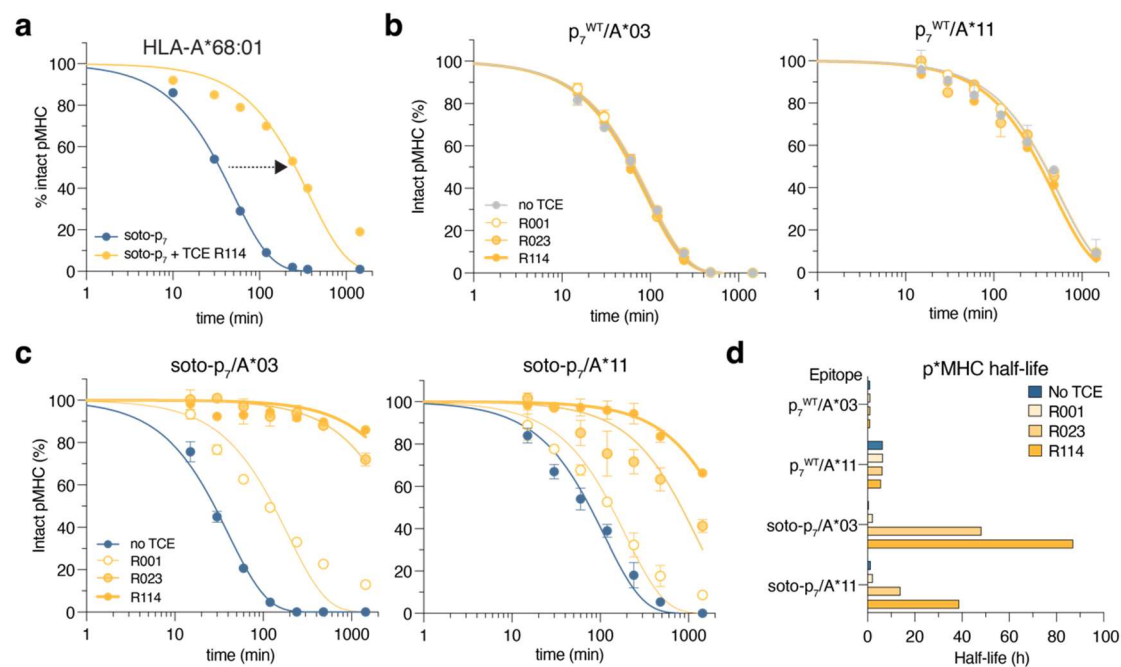
Reviewer: The authors present comprehensive and solid data on their strategy to target covalent k-Ras derived haptenylated peptides presented on HLA of cancer cells (lines). They provide convincing quantitative peptidomics data for the presentation of the peptide and show how a T cell engager by binding to the hapten-peptide-MHC complex the complex is stabilized and the copy number enhanced. Finally an intelligent strategy was chosen to target different haptenylated peptides in the context of different HLA supertypes and findings supported by cyro-EM data.

In my view the findings are very relevant and significant and the work presented supports the conclusion and is very well prepared.

I only have few minor comments

-Include some benchmarking to hapten-peptide specific antibodies described in the literature and used for bispecifics in key experiments would be good to show the superiority.

We appreciate the reviewer's suggestion. Included in **Supplementary Fig. 7** is a benchmarking analysis of the half-life extension on HLA-A*03 and HLA-A*11 using R001 (first generation) and R023 (third generation) antibodies, which were previously reported in [reference 18](#) (Hattori et al., Cancer Discov, 2023).



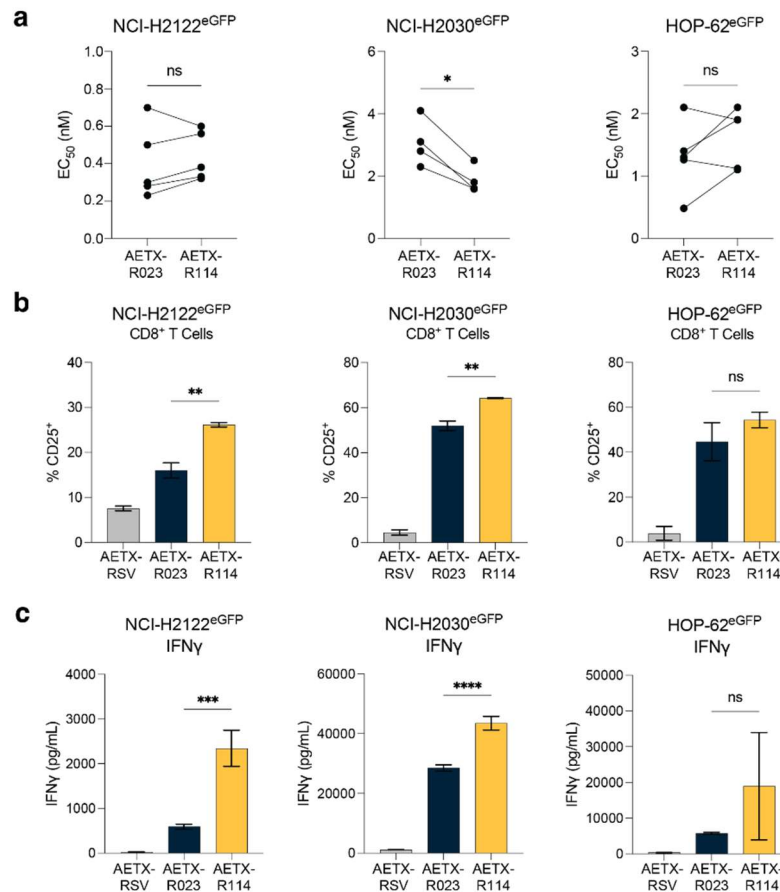
Supplementary Fig. 7: Antibody binding extends half-lives of p*MHCs.

a-c, Proportion of intact p/p*MHC remaining after incubation at 37° C from 0 to 24 h with or without TCE. Complex half-life was determined using a one-phase decay non-linear fit equation. Data points in b, c represent 3 technical replicates; error bars are standard deviations. R001 is the predecessor of R023 and has low affinity for soto-p₇ on A*03 and minimal binding on A*11. **d**, Calculated p/p*MHC half-lives measured at 37° C incubated with or without a TCE (3 technical replicates per peptide/antibody pair).

We have now also included additional data as **Supplementary Fig. 9**, comparing AETX-R023 and AETX-R114 across the 3 target cell lines featured in **Fig. 4**. Panel A shows AETX-R114 demonstrates a significant increase in potency on NCI-H2030 cells, which aligns with AETX-R114 having the biggest affinity enhancement towards HLA-A*11:01. Despite similar *in vitro* potency, AETX-R114 induces greater CD8+ T cell activation and IFN-γ release compared to AETX-R023 across all cell lines, suggesting it may deliver enhanced efficacy *in vivo*. Together, these data further highlight AETX-R114's superiority over AETX-R023.

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Supplementary Fig. 9: AETX-R114 demonstrates superiority over AETX-R023

a, Potency of AETX-R023 vs AETX-R114 in Incucyte-based T cell cytotoxicity assays comparing individual donors (ns, not significant or $P > 0.05$ and * $P < 0.05$ by paired t-test). **b**, Activation of CD8⁺ T cells and **c**, IFN γ release in T cell cytotoxicity assays after 48 h treatment with 300 nM sotorasib and 90 nM TCE (ns, not significant or $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons test). Data show mean $\pm$ standard deviation of 2-3 technical replicates per datapoint and are representative of 3-5 independent experiments.

-Testing the cleanness of the TCE on whole blood or other tissue samples from HLA-typed donors or normal cells to show absence of unspecific binding/killing.

This issue was also raised by Reviewer #1. Please see our response above.

Question: I am not sure whether the lack of weight loss in the mice can really be used as evidence for safety as the mice are not human HLA transgenic, in this context the question is whether mouse MHC molecules present the haptenylated peptides and whether they can be recognized by the pMHC binders optimized for different (human) HLAs?

Response: We agree that the lack of weight loss is not a comprehensive analysis addressing safety of the sotorasib/AETX-R114 combination in humans but does serve as an indicator that it does not induce general off-target events. Human-HLA transgenic mice are also not suitable for demonstrating safety as they present a murine immunopeptidome on the human HLA molecules. To further address the reviewer's comment on safety and off-target binding, we have added clarification in the discussion noting that antibody specificity is closely tied to the drug's specificity, and that the small molecule's off-target profile should be considered as part of the overall specificity assessment for p*MHC-targeting antibodies. Please also see our corresponding response to Reviewer #1.

The additional text included in the Discussion of the revised manuscript is as follows:

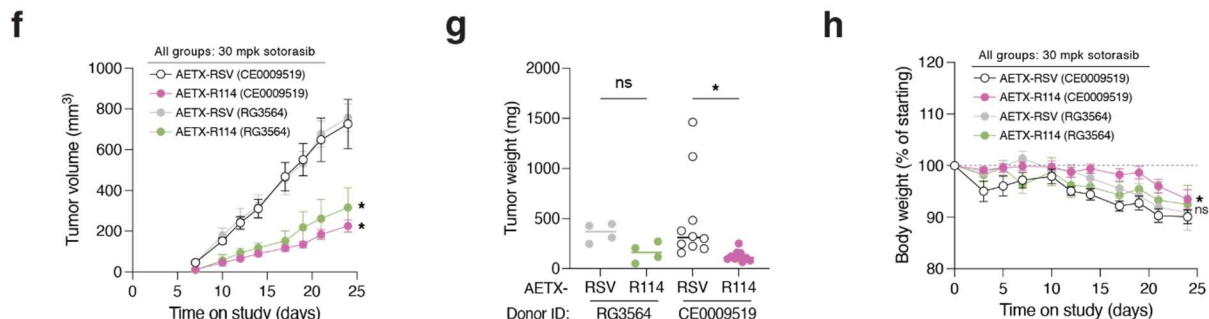
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-In general the chosen *in vivo* setting with co-mixed tumor and T cells is a low bar model and a PBMC engrafted or better humanized setting would be more realistic in terms of translation.

Response: To add additional rigor to the mouse model, we repeated the experiment with T cells from 2 additional donors and included these new *in vivo* data as **Supplementary Fig. 13**. We would like to argue that implementing a preventative model in a cell system with antigen densities of <10 copies-per-cell (CPC) presents significantly greater challenges than targeting surface antigens expressed at thousands of CPC. Nevertheless, we agree with the reviewer - and now have included in the discussion - that a preventative model is a lower bar than a therapeutic model. We would also like to point out that immunodeficient xenogeneic systems set a high bar for immunotherapeutic interventions that rely on orchestrating multiple components of the immune system well beyond T cells and PBMCs. As such, immunocompetent syngeneic systems based on HLA transgenic mice would be more relevant and have become a focus for our preclinical investigations, which we will report in due time.



Supplementary Fig. 13: **f**, NCI-H2122 tumor growth tested as described in Fig. 4d with AETX-R114 vs AETX-RSV treatment and 2 additional T cell donors (Donor IDs CE0009519, n=10/group and RG3564, n=4/group). Data points show mean $\pm$ SEM. **g**, Tumor weights after 26 d on study. **h**, Body weights of mice in **f** normalized to starting weight, with data shown as mean $\pm$ SEM (ns, not significant or $P > 0.05$ and * $P < 0.05$ by one-way ANOVA using Šidák's multiple comparisons test of AETX-RSV vs AETX-R114 for each donor).

The new text added to the Discussion of the revised manuscript is as follows:

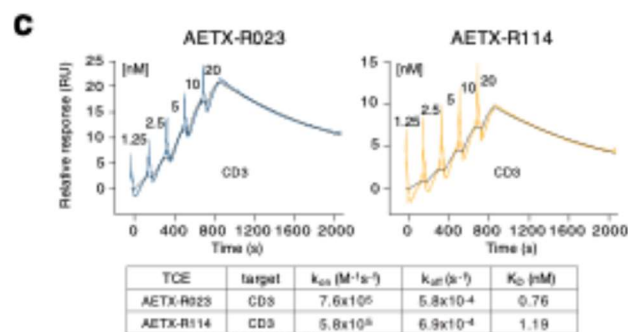
*“Future *in vivo* studies using more physiologically relevant models, such as humanized or syngeneic systems, will be important to refine the evaluation of therapeutic efficacy and translational potential of AETX-R114.”*

- Provide additional data on the engineering of the TCE, which CD3 antibody is used, what is the affinity etc

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Response: We added additional information to the section “Affinity maturation of antibody R023,” including the anti-CD3 clone hUCHT1.v9 (humanized mAb UCHT1, version v9, [reference 35](#)), the TCE format ([reference 36](#)), and the affinity of CD3 in **Supplementary Fig. 6c**.



Supplementary Fig. 6: c, SPR sensorgrams of the interaction between either AETX-R023 or AETX-R114 (analytes), and CD3 (immobilized ligand), using a single cycle kinetic experiment at 37° C. Kinetic values of fitted data are shown in the table below. Both AETX-R023 and AETX-R114 bind to CD3 through their hUCHT1.v9 effector arm with a K_D of about 1 nM.

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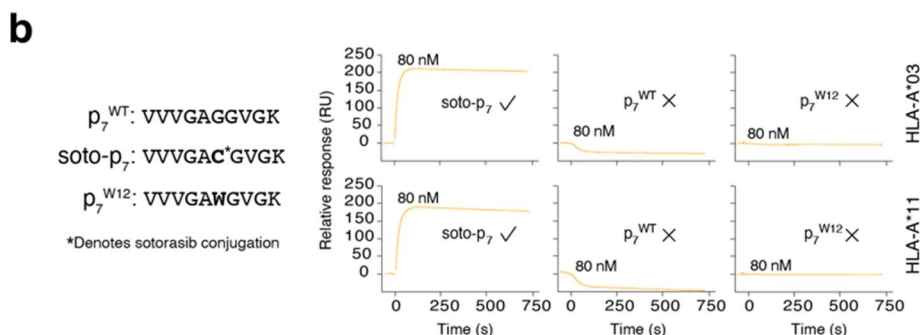
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Major Concerns:

1- The authors have affinity mature R114 from R023. Any concerns that this enhanced antibody may cross-react with non-haptenated HLA class I alleles?

Response: We have assessed the specificity of R114 by evaluating its binding towards the closest possible non-haptenated pMHCs (p_7^{WT}/A^*03-A^*11 and p_7^{W12}/A^*03-A^*11), data provided in **Supplementary Fig 6b**.



b, SPR sensorgrams of the interaction between AETX-R114 and the indicated p/p*MHC antigens. Biotinylated antigens were immobilized, and binding of soluble AETX-R114 samples was measured. On the left side of the sensorgrams are reported the sequences of the peptides used in the binding experiment. Explored off-target peptides include KRAS^{WT}- and KRAS^{G12W}-derived peptides (p_7^{WT} and p_7^{W12} , respectively). The latter was used to mimic haptenated peptides with a non-modified peptide, presenting a bulky tryptophan (W12) instead of the hapten of the p*MHC targets. For each sensorgram, next to the sample name is indicated if the antibody is binding to the target (check mark) or not (cross).

These data highlight the strict dependence of AETX-R114 on the hapten, as is also explained by previously published structural and mutational data (reference 33). This is further supported by cell-based experiments showing no activity in the absence of sotorasib treatment (e.g., **Fig. 4a**). However, we are aware of the possibility that covalent inhibitor-modified off-target proteins may be processed and presented by HLA molecules and recognized by p*MHC antibodies. To address this point, we have added commentary in the discussion noting that antibody specificity is closely tied to the drug's specificity, and that the small molecule's off-target profile should be considered as part of the overall specificity assessment for p*MHC-targeting antibodies.

The additional text included in the Discussion of the revised manuscript is as follows:

"While hapten modification uniquely enables high-affinity, cross-HLA recognition, the potential for off-target hapten modification and presentation of peptides on healthy tissue necessitates rigorous evaluation to avoid toxicity. Unlike conventional pMHC-targeting immunotherapies, standard cross-reactivity assays

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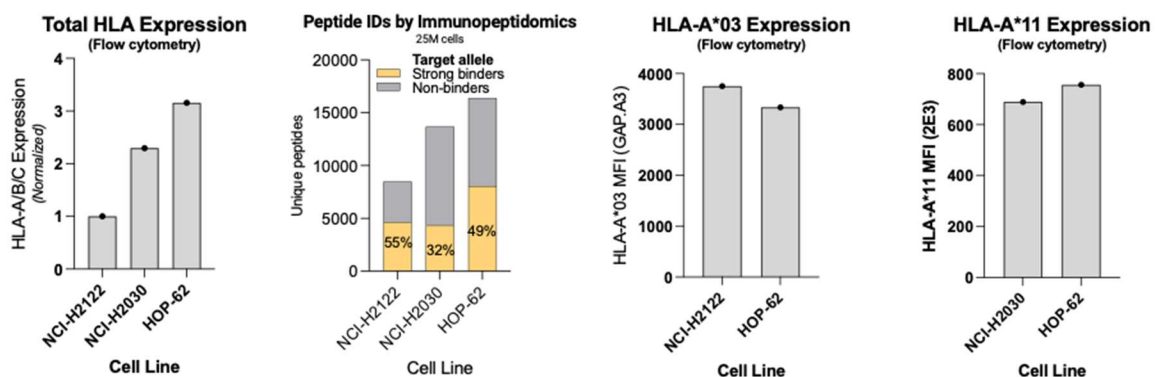
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*such as alanine scanning have limited suitability for p*MHCs, as antibody recognition is restricted to hapten-modified peptides. Thus, off-target reactivity of the antibody is dictated primarily by the specificity of the covalent modification. We addressed this issue by identifying peptide positions capable of presenting the hapten for antibody recognition and assessing whether off-target cysteine-containing sequences can bind relevant HLA alleles. Previously identified off-target cysteine sites—RTN4^{C1101} and HMOX2^{C282}—are not predicted to bind A3 supertype alleles or to be recognized by AETX-R114 due to hapten positional constraints³³, reducing the risk of off-target effects^{52,53}. Nevertheless, comprehensive profiling of a small molecule's off-target liabilities remains critical for ensuring clinical safety and guiding the selection of antibody clones with that avoid unintended cross-reactivity."*

Additional information on the analyses that were behind our specificity analysis is described above in our response to Reviewer #1, who raised a similar point.

2- What is the levels of A3 and A11 expression on the cancer cell lines studied (Fig4a). Antibodies directed at these alleles are available. Interesting that engager shows better activity against A3 than A11 expressing cancer cells despite p7 exhibiting greater affinity for A11.

Response: Provided below are the data we generated via flow cytometry on total HLA expression and allele-specific (HLA-A*03/HLA-A*11) expression on the NCI-H2122, NCI-H2030, and HOP-62 cell lines used as endogenous HLA/KRAS^{G12C} target cell lines in this study. We also include our immunopeptidomics characterization, which correlates well with the flow cytometry data showing highest HLA presentation in HOP-62 cells and lowest in NCI-H2122 cells. These data also provide an estimate as to the percentage of peptides on each cell line that are predicted to bind the target allele of interest (note that this analysis cannot distinguish between most HLA-A*03 and A*11 binders so the % represents the cumulative total of predicted binders). These data show that NCI-H2122 cells have a disproportionately high fraction of their immunopeptidome that is predicted to present HLA-A*03 peptides (55%), given that only one of the 6 HLA alleles in NCI-H2122 cells is a target allele of AETX-R114. This is even more than HOP-62, cells which have 49% of their immunopeptidome predicted to present HLA-A*03 or HLA-A*11:01 peptides.



Taken together, we agree that it is interesting that AETX-R114 exhibits the highest potency against NCI-H2122 cells, despite this cell line having the lowest total HLA expression. AETX-R114 is second most potent against HOP-62 cells, which have both HLA-A*03 and A*11 expression, and is least potent against NCI-H2030 cells (A*11 only). With many factors contributing to potency beyond what is described here, including KRAS expression, sotorasib sensitivity & MHC-I upregulation response to sotorasib, cytokine release profile, cell surface expression of ligands that regulate T cell activation, and more, it is impossible to draw a conclusive correlation between the potency of AETX-R114 and HLA expression, HLA allele, and/or peptide stability across these 3 cell lines. Therefore, we decided against including these data in the manuscript. We acknowledge with a broader panel of cell lines that match the endogenous profile, this would be interesting to explore. In our case, these were the only 3 cell lines available that had the target

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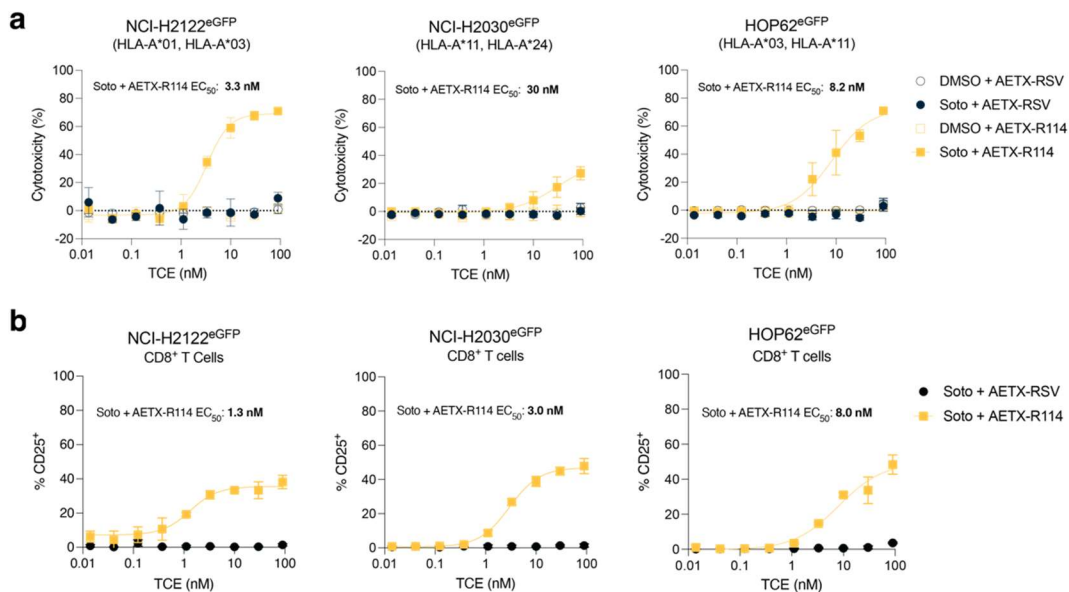
allele/mutation and were resistant to sotorasib. (Note that, e.g., NCI-H358 cells are highly sensitive to sotorasib).

To directly explore the relationship between potency and HLA allele in a constant cell system, we engineered the NCI-H2122 cells by knocking out HLA-A*03 and then re-introducing A*03, A*11, or A*68. These data (**Fig. 4b**) highlight that AETX-R114 achieves comparable potency across alleles, despite their different inherent stabilities and the antibody's different affinity profile for each allele. We submit that this is the best way to draw conclusions about the impact of AETX-R114's efficacy across different HLA alleles.

3- All the functional assays are performed with expanded T cells, what is observed with unactivated (freshly isolated from PBMC) T cells?

Response: To address this point, we performed the cytotoxicity assay using PBMCs against NCI-H2122s, NCI-H2030s, HOP62s with a 5:1 E:T ratio and assessed tumor cell death using the Incucyte and CD8+ T cell activation by flow cytometry at 72 h. These new data are reported in **Supplementary Fig. 10a,b**, along with **Supplementary Fig. 10c** in response to Reviewers #1 and #2. The following text was added to the Results of the revised manuscript:

*“AETX-R114 also elicited cytotoxicity and T cell activation using PBMCs as effector cells. Importantly, there was no evidence of fratricide observed against HLA-matched or unmatched donor cells, providing further evidence of TCE specificity (**Supplementary Fig. 10**).”*



a, IncuCyte analysis of AETX-R114-induced cytotoxicity against cell lines endogenously expressing KRAS^{G12C} in the presence of sotorasib (soto) or vehicle (DMSO). Cell lines were plated at a 5:1 E:T ratio of PBMCs (donor ID RG3564) to target cells and cytotoxicity was assessed at 72 h. **b**, Flow cytometry analysis of CD8+ T cell activation in cytotoxicity assays performed as in Fig. 4a with eGFP-expressing cell lines and a dose response of TCEs at 72 h. All dose response plots show mean $\pm$ standard deviation of 3 technical replicates per datapoint and are representative of 3 independent experiments.

4- What is the rationale for mixing tumor and T cells at time of implantation. What is the long-term survival of AETX-R114 treated mice (Fig 4f)?

Response: There is established precedent for mixing immune cells and tumor cells at the time of implantation in tumor prevention models, utilized in IND-enabling studies by both Immunocore (Kimmtrak[®] (tebentafusp), see US8519100B2 or similar method in [reference 40](#), Liddy et al., Nat Med, 2012) and Amgen (Imdeltra[®] (tarlatamab), see [reference 36](#), Giffin et al., Clin Cancer Res, 2021). The duration of these studies lasts between 15-29 days (see references above), consistent with our reported results.

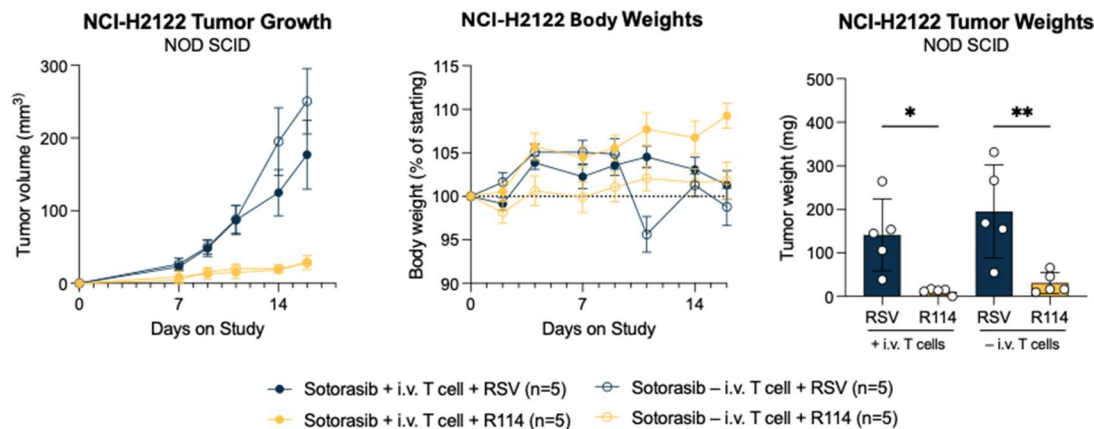
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Unfortunately, immunodeficient mouse models are not compatible with long-term survival studies because T cells are unable to persist. Syngeneic mouse models that incorporate a chimeric HLA molecule offer an attractive solution but are a significant undertaking to engineer and require an alternate mouse surrogate CD3 effector arm. Nevertheless, they have become a focus for our preclinical investigations, which we will report in due time.

The model has numerous deficiencies as it requires multiple T cell + engager injections to observed efficacy. Have the authors consider using huCD34 reconstituted NSG-FLT3 mice?

Response: We appreciate the reviewer's comment and agree the weekly peripheral T cell refreshments are a limitation. To test whether this was necessary for efficacy, we ran a study recapitulating the design in **Fig. 4**, with or without T cell refreshments and found that AETX-R114 was as efficacious in the absence of T cell refreshments. As the bulk of the *in vivo* data we have generated utilized weekly T cell refreshments (including new data in response to Reviewers #1 and #2 showing reproducibility across donors), we have elected not to include this result in the manuscript. We appreciate the reviewer's suggestion to streamline our *in vivo* prevention model protocol and to strengthen the resulting data.



We appreciate the reviewer's suggestion to utilize huCD34 mice for testing our TCEs in a therapeutic context. While we acknowledge the translational value of this model, its substantially higher cost—approximately tenfold that of NOD-SCID mice—makes it cost-prohibitive within the scope of this study. However, we view this as a promising approach and intend to pursue it in future IND-enabling preclinical investigations. To acknowledge this, we have added the following text to the Discussion of the revised manuscript:

“Future in vivo studies using more physiologically relevant models, such as humanized or syngeneic systems, will be important to refine the evaluation of therapeutic efficacy and translational potential of AETX-R114.”

5- For the characterization of R302 and AETX-R302, the authors limit their analysis to cells pulsed with supra concentration of peptide. In the absence of evidence that R302 (and AETX-R302) recognizes processed and presented hap-KRAS G12C/HLA complexes, the relevance of this data is questioned. Although, a peptide derived from mutKRAS G12 has been reported to bind HLA-A*02:01, to date there is no evidence that such peptide is naturally processed and presented. The study will greatly benefit if functional characterization of AETX-R302 is conducted using KRASG12C/A2 cancer cell lines.

Response: We acknowledge the reviewer's comment on the relevance of AETX-R302's binding to diva-p5/A*02. We began the campaign for AETX-R302 to push the boundaries of cross-HLA recognition, exploring how haptenated peptides might enable cross-supertype recognition in a way that conventional antigens cannot. We see AETX-R302 as a biophysical proof-of-concept rather than biological proof-of-

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concept that pan-HLA cross reactivity of p*MHC peptides is possible and use **Fig. 5** as an opportunity supported by Cryo-EM to dive into the structural basis for such cross-HLA recognition. We have included a sentence stating this point. “

“Therefore, at this stage R302 represents a biophysical proof-of-concept of cross-HLA supertype recognition.”

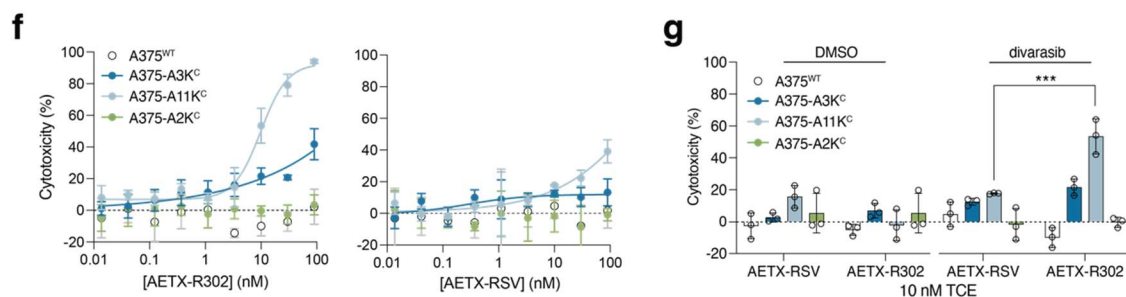
As a result, the peptide pulsed data serve as supporting evidence that the antibody we discovered can bind all 3 p*MHC complexes, not that they are necessarily all biologically actionable. To this end, we openly acknowledge in the discussion of this manuscript numerous caveats of the A*02 KRAS^{G12C} peptide as a biological target:

*“For instance, while KRAS^{G12X} mutant epitopes (e.g. KLVVVGAXGV) on HLA-A*02 have demonstrated immunogenic potential, they exhibit low affinity and poor stability, evading MS detection and rendering TCRm antibodies with nanomolar affinities ineffective in A02/KRAS^{G12X} mutant patients^{1–7}...”*

In our experience, non-affinity matured antibodies against KRAS^{G12C} hapten peptides do not show activity in endogenous HLA matched/KRAS mutant cell lines above background. However, to address the reviewer’s suggestion we have added an additional dataset to **Supplementary Fig. 9**, evaluating the cytotoxicity of AETX-R302 in a panel of A375 cell lines engineered to overexpress the HLA target allele and KRAS^{G12C} treated with either DMSO or divarasilb. Consistent with our hypothesis, AETX-R302 only shows significant activity above background at 10 nM TCE in HLA-A*11:01 expressing cells, which present diva-p7 with the highest endogenous stability compared to HLA-A*03 and HLA-A*02 (lowest stability). While these data do not confirm diva-p7/A*02 presentation, we have been conscious not to claim its existence, but rather to hypothesize that affinity maturation of AETX-R302 or other antibodies targeting low-affinity p/p*MHCs may stabilize these antigens to unlock their potential. While full *in vitro* and *in vivo* evaluation of AETX-R302 is beyond the scope of this study, the structural and biochemical insights presented here offer valuable guidance for AETX-R302’s continued development.

We have added the following text to the Results of the revised manuscript, along with a new figure panel:

*“AETX-R302 mediated potent and selective T cell killing of A375 cells pulsed with divarasilb-conjugated peptides across HLA-A*02, A*03, and A*11 alleles, confirming recognition of the hapten-peptide target across supertypes (Fig. 5b, Supplementary Fig. 14e). However, in drug-treated cells endogenously presenting the hapten peptide, cytotoxicity was observed only in HLA-A*11-expressing cells despite AETX-R302 having comparable affinity for all 3 alleles consistent with diva-p7 exhibiting the highest stability on A*11 (Fig. 1h, Supplementary Fig. 14f,g) These findings support the hypothesis that affinity maturation is necessary to enable effective targeting of less stable hapten-p*MHCs to fully leverage the potential of cross-HLA recognition.”*



f, In situ cytotoxicity analysis of AETX-R302 or AETX-RSV-induced T cell cytotoxicity against A375^{WT} cells or A375 cells engineered to express KRAS^{G12C} and the HLA allele of interest in the presence of 400 nM divarasilb with a 10:1 E:T ratio for 48 h. **g**, Average cytotoxicity across cell lines treated with 10 nM TCE and 400 nM divarasilb (data from f) or DMSO. ***p < 0.0001, Šidák’s multiple comparison’s test. Plots in f and g show n=3 technical replicates per condition and error bars indicate standard deviation.

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Minor Comments:

1-Fig 2d, in Table says AETX-R114 KD A*11 as 160pM, text says 130pM.

Response: Thank you, this has now been corrected in the text to 160 pM.

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ADDITIONAL EDITS IN THE REVISED MANUSCRIPT

The following sentences and figures were added or edited to the revised manuscript for consistency, clarity, and to reflect the most current context.

- “Cryo-EM structural insights reveal that R302 achieves unprecedented cross-reactivity by adopting the same binding mode for both A3 and A2 supertype targets, whereas previously reported cross-reactive binders, which showed significantly different affinities across HLA supertypes, engaged their targets with distinct binding modes³².”
- **Supplementary Figure 17a.** Updated with recently reported datapoint (reference 43).
- **Supplementary Table 1** was updated to include characteristics for all donors used in the revised manuscript.

Donor ID	HLA-A	Ethnicity	Age	Sex
RG3309	A*31:01:02, A*68:01:02	Caucasian	18	Male
RG3594	A*25:02:01, A*25:01:01	Caucasian	31	Male
1110044729	A*30:02:01, A*30:04:01	African American	47	Female
RG1200	A*01:01:01, A*01:01:01	Caucasian	25	Female
RG1532	A*02:01:01, A*24:02:01	Caucasian	39	Male
RG2115	A*02:06:01, A*34:01:01	Caucasian	24	Male
RG3564	A*02:01:01, A*02:01:01	Caucasian	45	Female
CE0009519	A*01:01:01, A*30:01:01	Hispanic	22	Female
RG2195	A*01:01:01, A*03:01:01	Caucasian	28	Male
CE0007153	A*11:01:01, A*32:01:01	Caucasian	39	Male
CE0010030	A*01:01:01, A*02:01:01	Hispanic	48	Male

Supplementary Table 1: Characteristics of donors for *in vitro* and *in vivo* studies.

- **Supplementary Table 2** was added to include cytotoxicity, T cell activation, and cytokine release data across 3 endogenous cell lines for 4-6 independent T cell donors. Each data point is the average of 3 biological replicates, ensuring the validity and reproducibility of the results and conclusions drawn in this study assessing the activity for AETX-R114. Furthermore, Donor ID information was added to all legends for transparency.

Donor ID	Cytotoxicity EC ₅₀ (pM)			T cell activation*			IFN γ release†		
	NCI-H2122	NCI-H2030	HOP-62	NCI-H2122	NCI-H2030	HOP-62	NCI-H2122	NCI-H2030	HOP-62
RG3309	380		2100				90	38	50
RG2115				2.1	8.3	9.4	54	6.2	33
RG3594	320	2500	1900						
110044729	310	2100	1500	10	19	23	26	17	45
RG1200	560	1800	1100	2.6	6.0	1.1			
RG1532	600	1600	1900	13	18	9.9	25	70	39
Average*	430 $\pm$ 130	2000 $\pm$ 430	1700 $\pm$ 480	6.9 $\pm$ 5.4	12.8 $\pm$ 6.6	10.9 $\pm$ 9.1	49 $\pm$ 31	33 $\pm$ 28	42 $\pm$ 7.7

Supplementary Table 2: Comparison of AETX-R114 activity across T cell donors.

*Average values are mean $\pm$ standard deviation of all donors tested.

†Fold change of % CD25⁺ cells with 10 nM AETX-R114 + 300 nM sotorasib treatment over % CD25⁺ cells with 10 nM AETX-RSV + 300 nM sotorasib treatment.

‡Fold change of pg/mL IFN γ with 10 nM AETX-R114 + 300 nM sotorasib treatment over pg/mL IFN γ with 10 nM AETX-RSV + 300 nM sotorasib treatment.

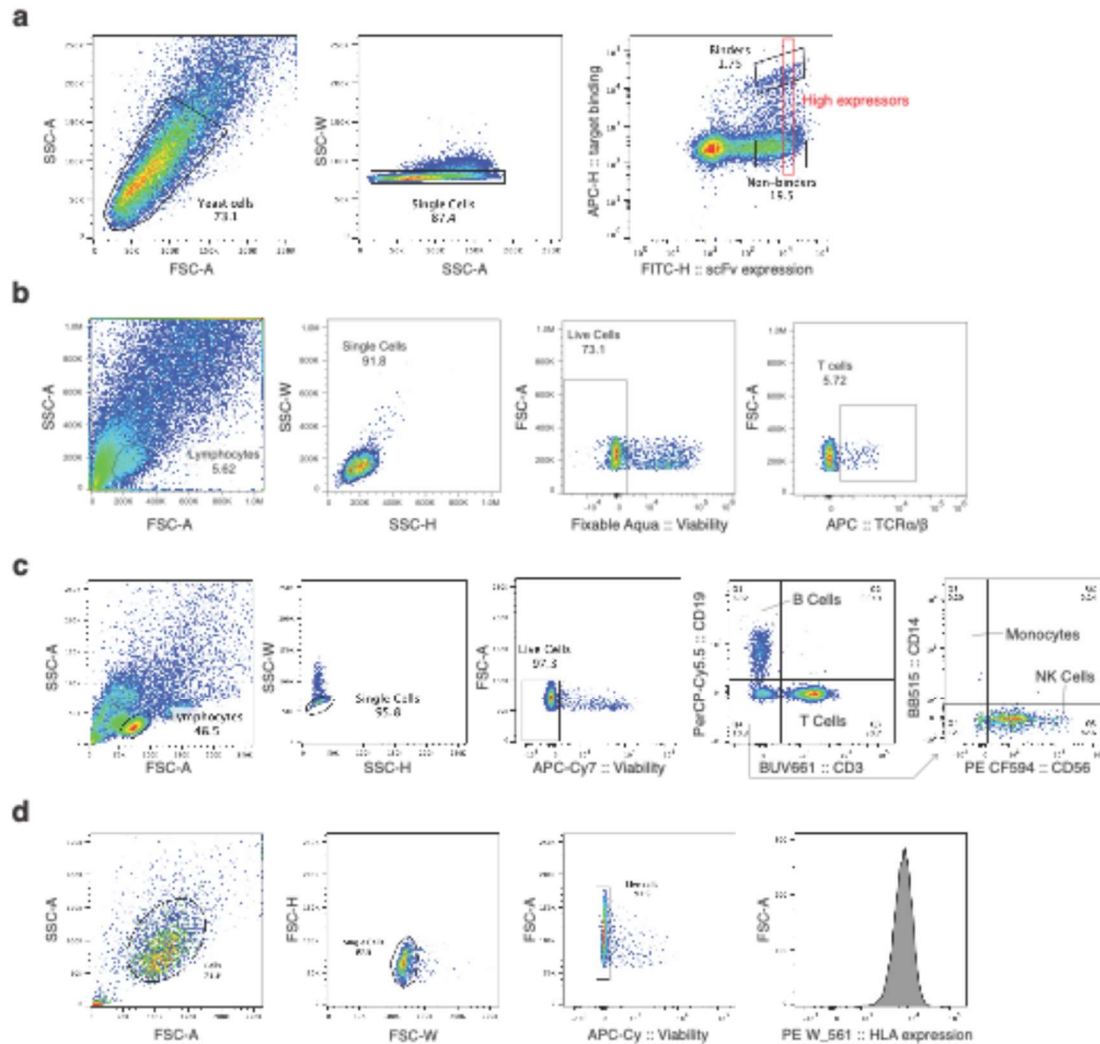
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- Exemplary gating schemes for all flow cytometry-based experiments were included as a new Supplementary Figure (**Supplementary Fig. 19**), shown below and incorporated into the methods section as the following text:

Gating schemes

“Exemplary gating schemes for all flow cytometry-based experiments are depicted in **Supplementary Fig. 19**.”



Supplementary Fig. 19: Example gating schemes for flow cytometry and sorting.

a, Yeast cells scFv expression and target binding. **b**, Live T cells. **c**, Live immune cell proportions in PBMCs. **d**, HLA expression for isogenic cell lines.