nature portfolio

Peer Review File

Direct recognition of an intact foreign protein by an αβ T cell receptor

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the present work, Almeida et al characterized a CD4- CD8- αβ T cell clone (4D4 TCR) in Jα18-/- BALB/c mice that directly binds PE hexamers independently of MHC. In particular, they showed that the 4D4 TCR was able to recognize PE isoforms from Thermo Fisher Scientific (Porphyra tenera) but not from Prozyme (red algae of undisclosed species).
They also estimated a 6:1 TCR:PE stoichiometry (SV-AUC) with six molecules of TCR that bind PE heterodimer within the hexamer with an affinity of 2.2 μM by ITC and 2.7 μM by SPR.
They also solved the structure of the 4D4 TCR bound to PE using X-ray crystallography and evidenced that the epitope of PE recognized by 4D4 TCR harboured an MHC-like conformation.
Finally, by generating 4D4 TCR retrogenic mice, they found that the majority of 4D4 TCR were expressed on T cells in peripheral organs, thus suggesting the thymic maturation and selection of this 4D4 TCR capable to recognize PE in the periphery.
Despite no specific flaws in the methods, data analysis, interpretation and conclusions can be found, the work is neither relevant nor significant.
For instance, it describes a further rare example of an αβ TCR derived from a mouse model that directly recognizes a molecule independently of MHC. The development and biological relevance of such rare event in a physiological immune context is not demonstrated and PE is not a common natural antigen.

Reviewer #2 (Remarks to the Author):

The manuscript by Almeida et al. tried to address there exist αβTCR T cells which can recognize antigen molecules in an MHC-independent manner. They provided evidence that 4D4 αβTCR T cells can recognize foreign phycoerythrin (PE) protein in an antibody-like manner. This study was interesting; however, the gap lied in the lack of mechanistic study and some key controls as well as the writing was some rough.

1. Figure 1c

a. for SAV-BV421: the dot plot blue to red was 25148 versus 387; however, the figure

showed similar. This was problematic.

b. The references should not appear in the legend.

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3. Figure 7:

a. In the text, the authors used GFPhigh, but in the figure the author used GFP+.

b. Figure 7a Rg mouse #2: the thymus dot plot seemed to be modified. Did the same voltage used for the analysis?

c. Could the T cells from 4D4 TCR retrogenic mice be activated by PE in vivo and in vitro? Are these T cells functional?

d. The result showed that most 4D4 TCR T cells were CD4 and CD8 double negative. Do they differentiate to CD8$\alpha\alpha$+ T cells in the mucous membrane, considering in which double negative T cells can develop to the CD8+ T cells.

e. The authors should clarify that 4D4 TCR T cells is not artificial using wild-type mice.

4. A shortcoming for this study is the enigma on how PE activate 4D4 TCR T cells.

Reviewer #3 (Remarks to the Author):

Adaptive immunity bifurcates into two important arms, namely humoral immunity that is exemplified by the secretion of antibodies, and cellular immunity that is mediated by T cells. Compared to the general notion that antibody directly binds to intact antigens, the antigen specificity of T cell receptors, particularly $\alpha\beta$TCRs, is restricted to peptide-bound major Histocompatibility complex (pMHC). MHC restriction is thus the fundamental of T cell immunology. Recent mechanistic studies on T cell development and thymic selection in engineered Lck mutant mice and mice deficient in MHC and coreceptors CD4/CD8

demonstrated that TCRs could recognize intact self-proteins and then be selected in thymus. These pioneer studies revealed that TCRs might not be encoded to react with MHC molecules and they possessed the potentials of recognizing MHC-independent antigens, which deepened our current understandings of thymic selection.

In current manuscript, the authors characterized an accidentally identified αβTCR (4D4 TCR) from screening of CD1d restricted NKT cells. 4D4 TCR recognizes a foreign antigen, R-phycoerythrin(PE) and could be activated by plate bound PE. The X-ray crystallography illustrated the molecular details that MHC-independent recognition of PE by 4D4 TCR resembles antibody-antigen interaction with a major contribution of CDR3 loops from VDJ recombination. In addition, 4D4 TCR could be selected in thymus and emigrate to the peripheral.

Overall, the cellular and structural characterization of 4D4 TCRs are well carried out, showing this TCR can respond to a native antigen PE. However, the evidence for 4D4 being MHC-independent rather than CD1 dependent is not sufficient.

1. The in vivo evidence supporting 4D4 T cells recognizing non-MHC ligands came from their retrogenic mice experiment showing mature 4D4 T cells are mostly CD4- CD8-. However, it's not surprise that these T cells come out CD4- as they are originally from NKT cells. Since PE is unlikely a physiological ligand, it is possible that the TCR recognizes an unknown ceramide presenting CD1d in vivo but crossreactive to PE. The so called "MHC-independent" TCR do not include CD1d-restricted invariant chain bearing NKT receptor, which are naturally MHC independent. To rule out the possibility of 4D4 being another NKT TCR, the authors need to show the GFP+TCRb+ but not GFP-TCRb+ cells proliferate on CD1d-/- APC.

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Point by point responses

Reviewer #1

***1.1**: 'no specific flaws in the methods, data analysis, interpretation and conclusions can be found.'*

Thank you.

***1.2**: 'the work is neither relevant nor significant.'*

We are grateful that the editor over-ruled this comment from reviewer #1. To reiterate, we 1) describe a novel αβTCR recognition mechanism in the absence of antigen presenting molecules and 2) reveal a new antigen for αβ T cells.

Reviewer #2

We thank the reviewer for stating that '*This study was interesting*'. However the reviewer made a number of salient points and mentioned *'..the lack of mechanistic study and some key controls'*. We have now performed additional experiments to address these issues (see below).

2.1 Figure 1c, point a: ***'For SAV-BV421: the dot plot blue to red was 25148 versus 387; however, the figure showed similar. This was problematic.'***

We apologize for the confusion. The blue dots referred to the NKT TCR-transduced cell line which bound the CD1d-tetramer conjugated with SAV-BV421 (bottom plot) with a mean fluorescent intensity (MFI) of 25,148. However, the 4D4 TCR-transduced cell line, shown in red, did not bind the CD1d-SAV-BV21 tetramer, an MFI of 387. By comparison, in the above plot these NKT (blue) and 4D4 (red) cell lines bound CD1d-tetramers conjugated with SAV-PE, with comparable MFIs of 19,738 and 13,094 respectively. This has now been clarified within the results section, we revised the figure panel and clarified the figure legend. Specifically, we now state:

Results section, page 4, line 201-204: "Both 4D4 and A10B8.2 TCR$^+$ cell lines stained with PE-labelled CD1d-α-GalCer tetramers (MFIs of 19738 and 13094, respectively, **Figure 1C** top) however, whilst the A10B8.2 NKT TCR$^+$ cell line was also stained by BV421 labelled CD1d-α-GalCer tetramers (MFI 25148, **Figure 1C** bottom), the 4D4 TCR$^+$ cell line was not (MFI 387, **Figure 1C** bottom).

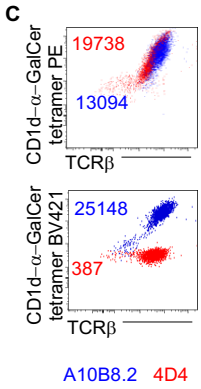


Figure 1C. α-GalCer-loaded CD1d was tetramerized using SAV-PE (top plot) or SAV-BV421 (bottom plot) and assessed for staining of the BW58 lines expressing the 4D4 TCR (red) or control A10B8.2 TCR (blue) by flow cytometry.

2.1 Figure 1c, point b: ***'The references should not appear in the legend.'***

The references have now been removed from the legend.

2.2 Figure 2, point a*: 'Only IL-2 detection was used to evaluate the T cell activation. This was too simple and not suitable. Some activation marks such as CD44, CD25 and CD69 should be measured.'*

As requested, we have now expanded our activation marker measurements to include: CD44, CD25 & CD69 upregulation. We have also measured TCR/CD3 surface downregulation following SAV-PE mediated activation of both a 4D4 TCR^+ BW58 cell line, as well as a 1C5H (γδ) TCR^+ Jurkat cell line. Importantly, these results are consistent with our IL-2 activation data. Mitogenic control stimulation using anti-CD3 supports that the Jurkat cell lines are incapable of upregulating CD25. Notably, only when there was a strong CD69 increase, such as in response to SAV-PE, did we observe TCR/CD3 downmodulation, and a slight CD44 and CD25 upregulation in the 4D4 TCR^+ cell line but not in the control lines. Using these expanded activation markers, we could not detect activation by NAV-PE in the 4D4 TCR^+ cell line, which binds weakly to 4D4 (**Figure 1E**) but did induce IL-2 production (**Figure 2A**). However, NAV-PE was able to activate the 1C5H line, as measured by CD69 upregulation. This is consistent with our original use of IL-2 production as a readout of activation for BW58 cell lines. Indeed, we (and others) have shown that the IL-2 readout for BW58 cell lines is more sensitive than CD69 upregulation (Almeida *et al.* Nat. Comms. 2019).
These new data have been included in the revised manuscript as **Figure 2C, Supplementary Figure 2A**, and described within the results section. The manuscript now reads:

Results section, page 7, line 198-210: "We also investigated whether PE-ligation could modulate the surface-expression of CD69, CD44, CD25 activation markers (as well as CD3/TCR), using the 4D4 TCR^+ cell line (**Figure 2C**) as well as the 1C5H TCR^+ Jurkat line (**Figure S2**). Mitogenic control stimulation using plate-bound anti-CD3 supports that Jurkat cell lines are not capable of upregulating CD25. Notably, only when there was a strong CD69 increase (in response to SAV-PE – which bound strongly to 4D4 (**Figures 1D and E**), or to control anti-CD3 stimulation), did we observe TCR/CD3 downmodulation, and slight CD44 upregulation (as well as CD25 in the 4D4 TCR^+ cell line). We detected CD69 upregulation in response to plate bound SAV-PE and SAV-PECy7 conjugates, in the 4D4 and 1C5H TCR^+ lines, but not in the control NKT TCR^+ lines (VII68 and 9C2 [9]), nor in response to other SAV-controls and non-fused SAV (**Figure 2C**). Notably, NAV-PE stimulation, which binds weakly to 4D4 (**Figure 1E**) and elicits IL-2 production (**Figure 2A**), did not lead to CD69 upregulation in the 4D4 TCR^+ cell line, despite doing so in the 1C5H.TCR.Jurkat line (**Figure S2A**), which in turn did not respond to the non-fused NAV-control. Akin to the 4D4 line, 1C5H also failed to respond to heat-inactivated SAV-PE.."

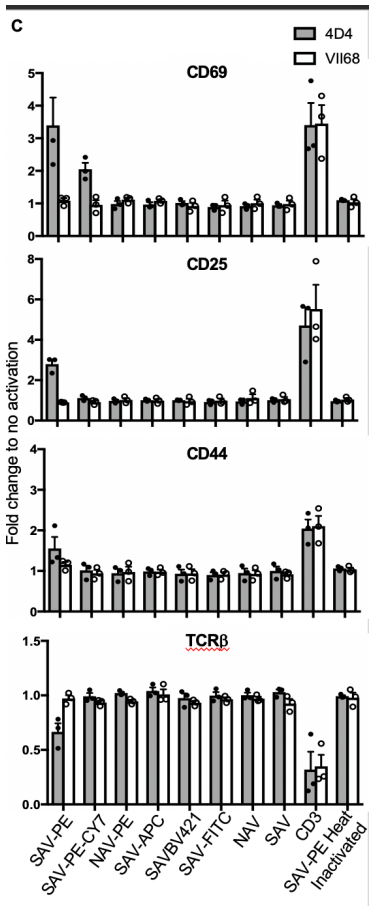


Figure 2C. After 16h of stimulation with plate bound SAV-PE SAV-BV421 or anti-CD3, cells were stained for CD44, CD69 CD25 or TCRβ surface expression. Mean Fluorescence Intensity fold variation relative to no activation +/- SEM for each marker across 3 independent repeat experiments, each represented by the individual dots. Error bars represent SEM.

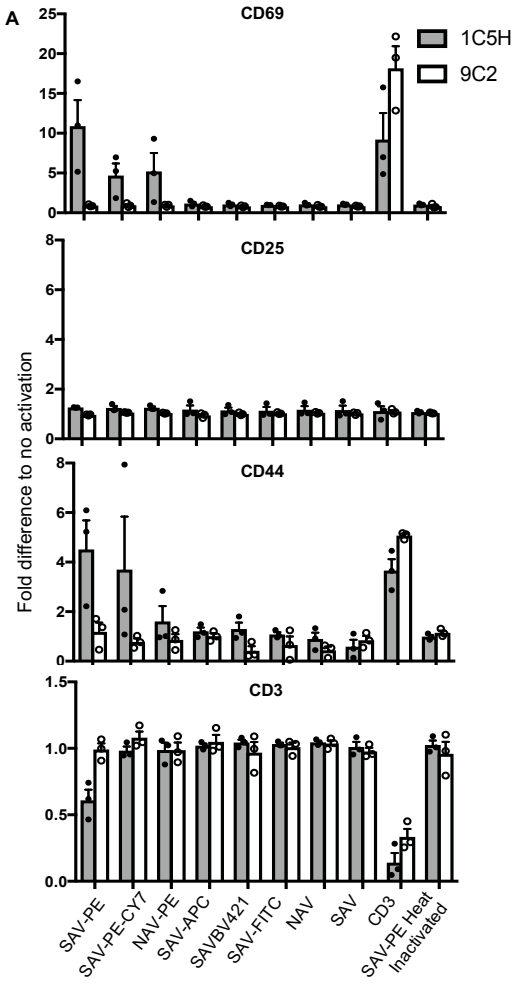


Figure S2. PE activates cells expressing the 1C5H TCR A. Various forms of SAV- or NAV-conjugated PE (from different suppliers) at 50 µg/mL - were assessed for their ability to activate the Jurkat cell line expressing the human γδ 1C5H TCR (PE-reactive) or 9C2 TCR (CD1d-reative). Non-conjugated SAV and NAV were included as controls. Anti-CD3 (10 µg/mL) was included as a positive control for stimulation. After 16h cells were stained for CD44, CD69 CD25 or CD3 surface expression. Graphs show mean fluorescence intensity fold variation to no activation +/- SEM for each marker across 3 independent experiments, each represented by individual dots.

2.2. Figure 2, point b: *'Figure 2a: sole SAV and NAV without fusion should be tested as controls.'*

Thank you for this suggestion. We did trial other SAV-fluorochrome conjugated forms, which failed to elicit a response and so we did not include these in the IL-2 detection assays (**Figure 2**). We have now clarified this within the revised manuscript. We did include SAV and NAV without fusion as controls in the activation experiments (shown above **Reviewer #2, 2.2 Figure. 2, point a**), and have described these controls within the Results section. The manuscript now reads:

Results section, page 6, line 180-183: "Consistent with the PE staining assays (**Figures 1D and E**)**,** plate-bound non-conjugated PE from Prozyme, SAV-conjugated PE or PE-Cy7 from BD, and NAV-PE from Molecular Probes elicited IL-2 production by the 4D4 TCR^+ cell line but not by the VII68 control NKT cell line (**Figure 2A**), whilst other SAV-fluorochrome conjugates failed to elicit a response."

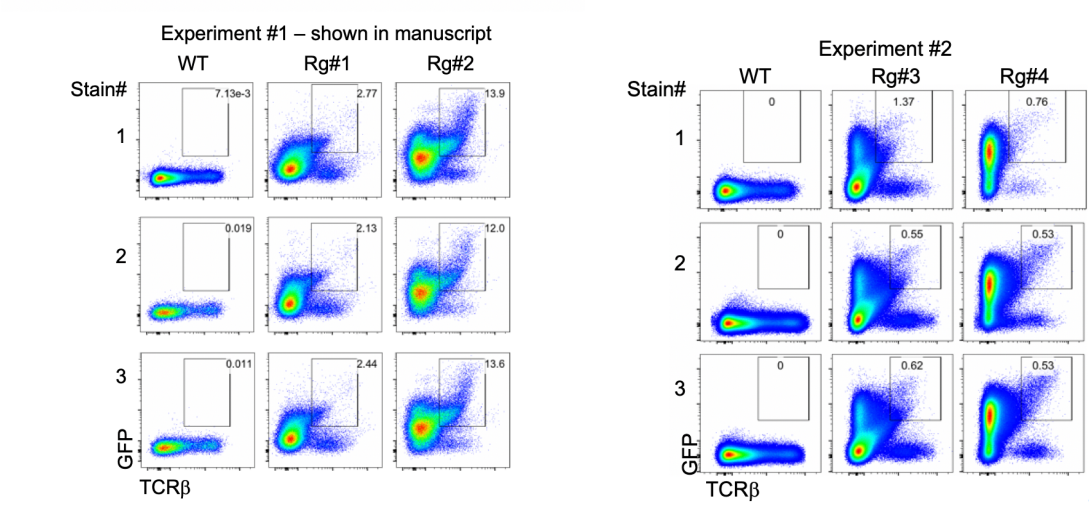
Results section, page 7, line 204-209: "We detected CD69 upregulation in response to plate bound SAV-PE and SAV-PECy7 conjugates, in the 4D4 and 1C5H TCR$^+$ lines, but not in the control NKT TCR$^+$ lines (VII68 and 9C2 [9]), nor in response to other SAV-controls and non-fused SAV (**Figure 2C**). Notably, NAV-PE stimulation, which binds weakly to 4D4 (**Figure 1E**) and elicits IL-2 production (**Figure 2A**), did not lead to CD69 upregulation in the 4D4 TCR$^+$ cell line, despite doing so in the 1C5H.TCR.Jurkat line (**Figure S2A**), which in turn did not respond to the non-fused NAV-control."

2.3. Figure 7, point a*: 'In the text, the authors used GFPhigh, but in the figure the author used GFP+.'*

Thank you for pointing this out, we have now amended the text to be GFP$^+$ throughout.

2.3. Figure 7, point b: ***'Rg mouse #2: the thymus dot plot seemed to be modified. Did the same voltage used for the analysis?'***

We thank the reviewer for highlighting the different GFP intensities for the thymus plots from retrogenic (Rg) mouse #1 and #2. We re-analysed the experiment and confirmed that all of the samples were acquired within a single experiment, and used the same voltages for each parameter, including the 530/30 blue channel that detects GFP. Notably, the differences in GFP intensity between individual retrogenic thymi were observed across two independent experiments with separate staining with three different cocktails. In full transparency we have included these data as a panel below, although this was not included within the revised manuscript.



Rebuttal figure 1. Flow cytometry analysis of thymus of wild type (WT) C57BL/6 or 4D4 retrogenic mice (Rg). Representative plots of GFP and TCRβ expression pre-gated on 7AAD$^-$ B220$^-$ cells across three independent stains from two independent experiments. *Note that in all cases TCRβ-AF700, with the exception of stains #2-#3 from Experiment #2, where TCRβ-BV786 was used.

These are derived from the system used to generate the mice and reflect the intensity of GFP+ in the thymus. We revised **Figure 7A** with plot scales that better highlight this and have clarified this within the Results section of the revised manuscript, which now reads:

Results section, page 11, lines 353-356: “T cell accumulation in the thymus, spleen, lungs, liver and lymph nodes of the C57BL/6 wild type (WT) recipients could be detected as early as 10 weeks after bone marrow transfer from *Rag1*$^{-/-}$ retrogenic donors (**Figure 7A),** with GFP signals that varied in intensity and proportion.”

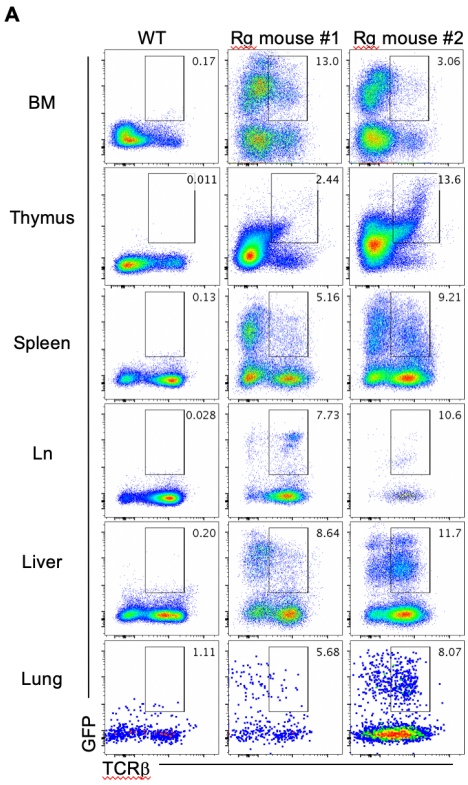


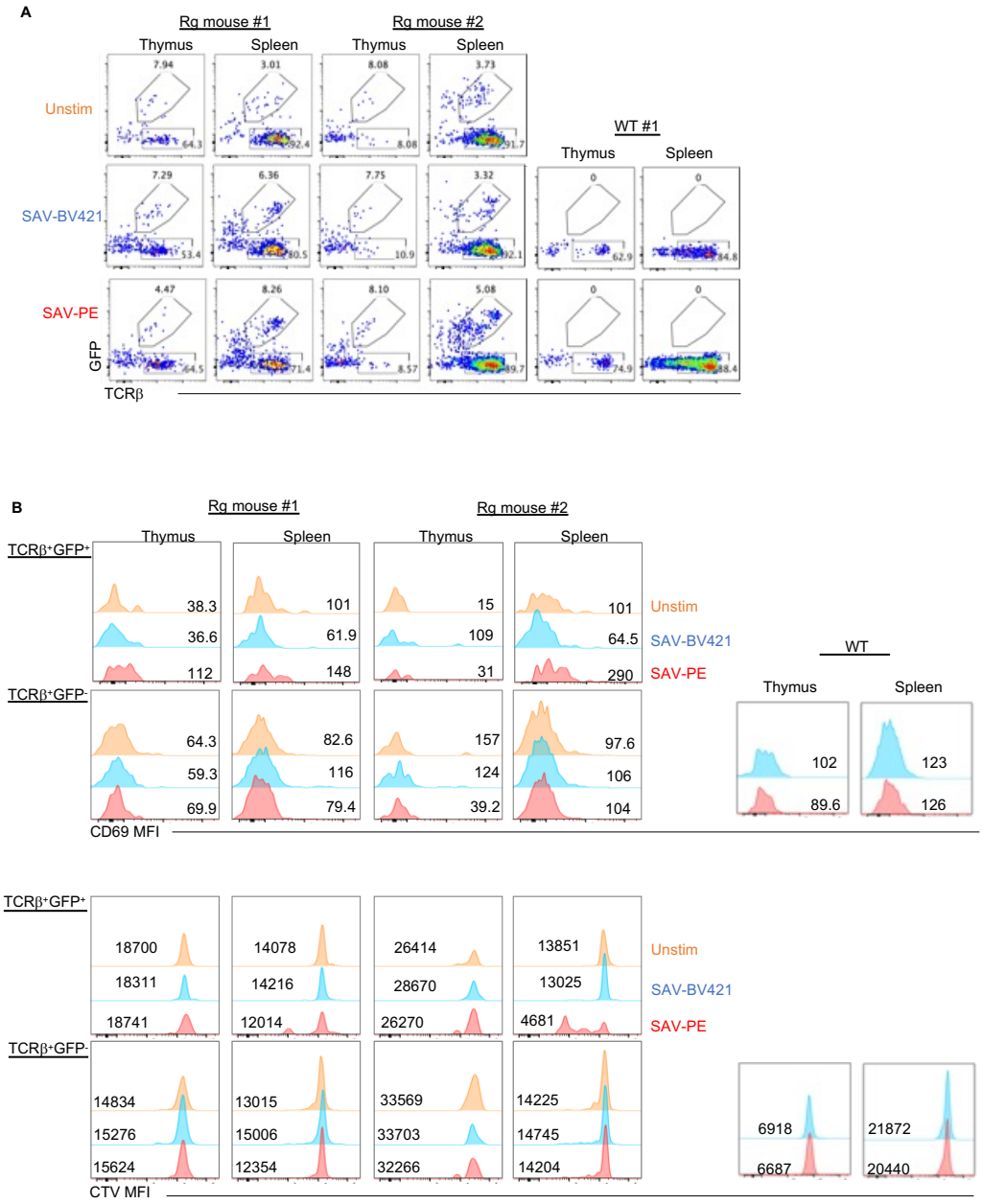
Figure 7. 4D4 TCR^{+} T cells develop *in vivo* in a retrogenic mouse model A. Flow cytometry analysis of bone marrow (BM), thymus, spleen, lymph node (LN), liver, lung of wild type (WT) C57BL/6 or 4D4 retrogenic mice (Rg). Representative plots of GFP and TCRβ expression on 7AAD^{-} B220^{-} cells.

2.3. Figure 7, point c: *‘Could the T cells from 4D4 TCR retrogenic mice be activated by PE in vivo and in vitro? Are these T cells functional?’*

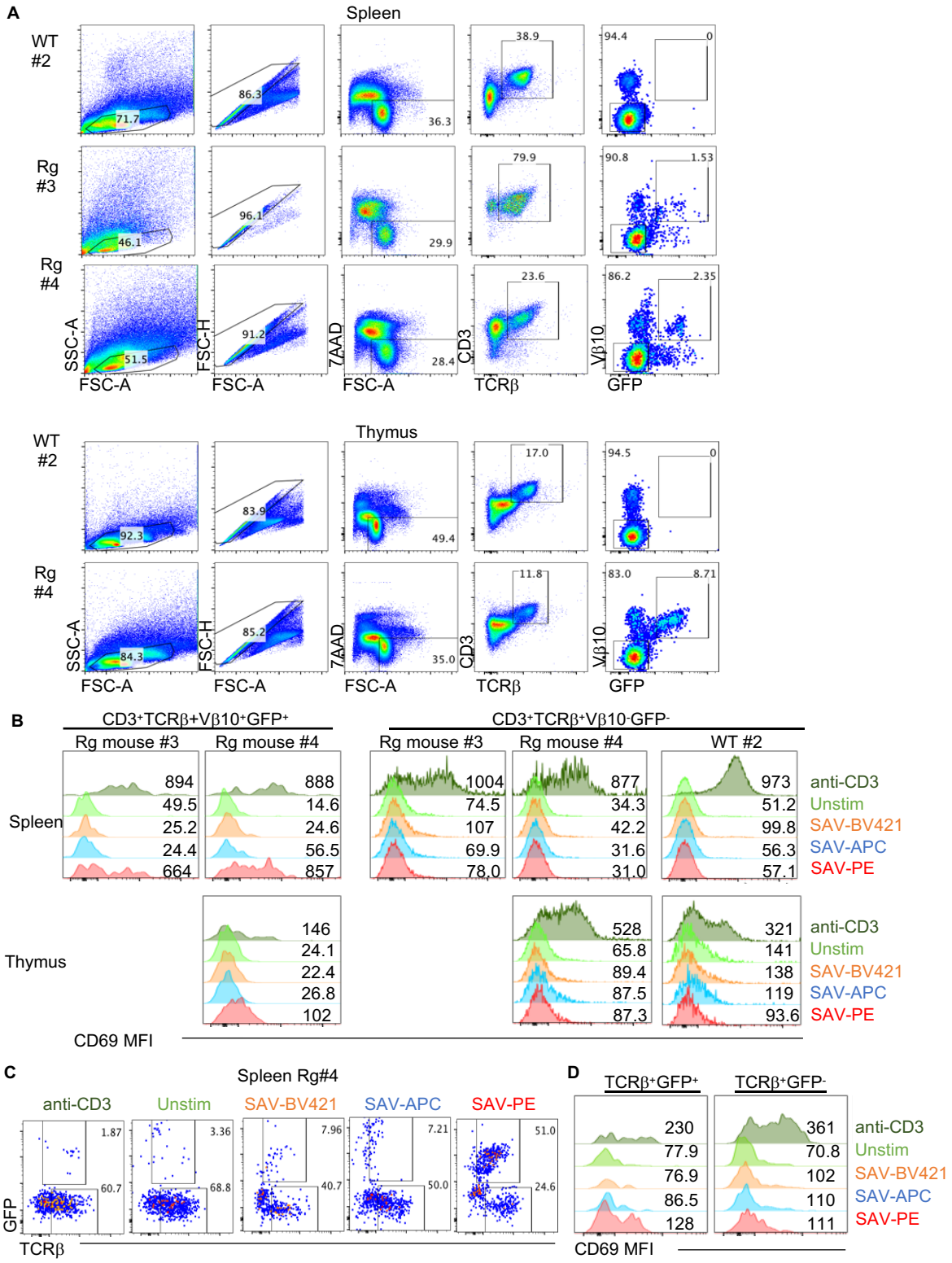
Thank you for raising this important point. We had originally performed *in vitro* activation assays, with our results suggesting that 4D4 TCR retrogenic cells can be activated by PE. However, we did not include these data in the original submission as three distinct approaches across the 4 retrogenic mice were used rather than a unifying approach. Nevertheless, each approach supported the conclusion that these cells are functional (see details below). We have included these data below but shall not include them in the revised manuscript due to the different approaches used. We include the data in the knowledge that the rebuttal will be published alongside the manuscript, readers can still evaluate this data with respect to T cell functionality of the 4D4 TCR retrogenic mice.

Our additional retrogenic data are outlined below. We investigated if 4D4 retrogenic cells could respond to PE stimulation using three separate approaches (**Rebuttal Figure 2 and 3**). Firstly, we assessed whether plate bound SAV-PE could stimulate 4D4 retrogenic cells after three days in culture. Despite the low numbers of 4D4 cells (TCRβ^{+}GFP^{+}) detected amongst retrogenic thymocytes (**Rebuttal Figure 2A**), those derived from the spleen of retrogenic mouse #2 (and #1,

to a lower extent) displayed an increase in CD69 MFI, accompanied by a decrease in CTV MFI in response to SAV-PE but not to SAV-BV421. This was in contrast to control $TCR\beta^{+}GFP^{-}$ cells from the retrogenic mice or from WT mice, which did not respond to either stimulation (**Rebuttal Figure 2B**). This suggested that 4D4 cells were able to proliferate and be activated in response to PE. As a separate approach, the effects of SAV-PE-stimulation were assessed after an overnight stimulation. This revealed clear populations of 4D4 retrogenic cells as $GFP^{+}V\beta10^{+}$ amongst splenocytes of mice #3 and #4, and thymocytes of mouse #4 – to a lower extent, - (**Rebuttal Figure 3A**). Importantly 4D4 cells showed increased CD69 MFI in response to SAV-PE stimulation, as well as to control anti-CD3 stimulation, but not to SAV-BV421 or SAV-APC. On the contrary, $GFP^{-}V\beta10^{-}$ T cells from the retrogenic mice or from WT mice only responded to anti-CD3 stimulation (**Rebuttal Figure 3B**). Notably, splenocytes from retrogenic mouse #4 left in culture for 5 days were enriched for 4D4 cells (identified as $TCR\beta^{+}$ GFP^{+}) in the well coated with SAV-PE, when compared to wells coated with anti-CD3, SAV-BV421 or SAV-APC or unstimulated (**Rebuttal Figure 3C)**. This further supports expansion or survival of these cells in response to the PE-antigen. 4D4 retrogenic cells ($TCR\beta^{+}$ GFP^{+}) from mouse #4 at day 5 also showed increased CD69 MFI in response to SAV-PE and anti-CD3, but not to other SAV-conjugates. In contrast GFP^{-} T lymphocytes in the same well ($TCR\beta^{+}$ GFP^{-}) only responded to anti-CD3 control stimulation (**Rebuttal Figure 3D**).



Rebuttal Figure 2 4D4 retrogenic cells respond to PE – Experiment #1. Splenocytes and thymocytes from two retrogenic mice (Rg #1 and #2) or a control C57BL/6 wild type (WT) mouse were labelled with cell trace violet (CTV) and cultured in wells containing plate-bound SAV-PE or SAV-BV421. After 3 days cells were analysed by flow cytometry for CD69 upregulation and CTV dilution. **A.** Plots show GFP and TCRβ expression on 7AAD$^-$ B220$^-$ cells. Numbers indicate percentage of gated cells. **B.** Histograms show Mean Fluorescence Intensity (MFI) values for CD69 or CTV amongst 4D4 retrogenic GFP$^+$ TCRβ$^+$ and control GFP$^-$ TCRβ$^+$ cells. Data is representative of one experiment.



Rebuttal Figure 3. 4D4 retrogenic cells respond to PE – Experiment #2. Splenocytes and thymocytes from retrogenic (Rg) mouse #3 and #4 or a control C57BL/6 wild type (WT) mouse were cultured in wells containing plate-bound SAV-PE, SAV-BV421, SAV-APC. Unstimulated cells or cells co-cultured with anti-CD3 mAb were included as controls. After 24 h, cells were analysed by flow cytometry for CD69 upregulation. **A.** Plots show representative gating strategies for identification of 4D4 retrogenic T cells (TCRβ$^+$ CD3$^+$ Vβ10$^+$ GFP$^+$). Numbers indicate the

percentage of gated cells. **B.** Histograms show CD69 surface expression (numbers indicate the mean fluorescent intensity (MFI)) amongst 4D4 retrogenic or control Vβ10$^-$ GFP$^-$ T cells. As a separate and complementary approach, splenocytes from retrogenic mouse #4 were kept in culture and analysed at day 5. **C.** Plots show GFP and TCRβ expression on 7AAD$^-$ B220$^-$ cells. Numbers indicate percentage of gated cells. **D.** Histograms show CD69 surface expression and numbers indicate MFI amongst 4D4 retrogenic cells TCRβ^+ GFP$^+$ cells or control TCRβ^+ GFP$^-$ T cells. Data from each approach represent one experiment.

Furthermore, our studies in this setting were impacted by the COVID-19 pandemic, and the work on the retrogenic mice were essentially halted for 2 years due to prioritisation of COVID-19 work over non-essential work at our institutions. To reinitiate these retrogenic studies would minimally require an additional 6 months of work and as such we feel that more extensive follow up studies on the 4D4 TCR retrogenic mice would unnecessarily delay publication of this study. Taking into consideration the reviewer's point we revised the discussion sections. The revised manuscript now reads:

Discussion section, page 13, 420-421: 'Future studies in the 4D4 TCR retrogenic mice will establish the functionality of these PE-reactive T cells in vitro and in vivo'

2.3. Figure 7, point d: ***'The result showed that most 4D4 TCR T cells were CD4 and CD8 double negative. Do they differentiate to CD8$\alpha\alpha$+ T cells in the mucous membrane, considering in which double negative T cells can develop to the CD8+ T cells.'***

We have looked at 4D4 cells in the lung tissue and, at least in this mucosal site, they retain the CD4 and CD8 double negative profile (**Figure 7A**). We revised the discussion sections. The revised manuscript now reads:

Results section, page 11, lines 359-360: "GFP$^+$ cells in the periphery, including the lung mucosa, were similarly DN in contrast to their GFP$^-$TCRβ^+ counterparts."

2.3. Figure 7, point e: ***'The authors should clarify that 4D4 TCR T cells is not artificial using wild-type mice.'***

Thank you for the prompt. To clarify, the 4D4 clone was derived from natural TCR-gene rearrangement in a *Traj18*$^{-/-}$ mouse, and thus the TCR is not artificial. This contrasts with the TCRs derived in the QuadKO all MHC-deficient (β*2m*$^{-/-}$*H-2Ab*$^{-/-}$) and coreceptor-deficient (*CD4*$^{-/-}$*CD8*$^{-/-}$), or Lck deficient mice, whereby normal events associated to thymic selection were bypassed (Van Laethem, Immunity 2007, Tikhonova, Immunity 2012, Van Laethem, Front Immunol 2020). Although the 4D4 T cell clone appeared to be an infrequent clonotype and other clones were not identified in other mice during our attempts to sort-enrich/expand SAV-PE$^+$ cells. This could reflect that these cells are very rare and hard to detect and/or isolate. Nonetheless, PE-reactive human $\gamma\delta$ T cells also exist, as shown by us (**Figure S1**) and others (Zeng, Immunity 2012). We have highlighted this point in the discussion. The revised manuscript now reads:

Discussion section, page 12, line 382-385: "However, such T cell clones, unlike 4D4, were derived from mouse models whereby normal events associated to thymic selection were bypassed, involving "QuadKO" all MHC-deficient (β*2m*$^{-/-}$*H-2Ab*$^{-/-}$) and coreceptor-deficient (*CD4*$^{-/-}$*CD8*$^{-/-}$), or Lck deficient mice."

2.4: ***'A shortcoming for this study is the enigma on how PE activate 4D4 TCR T cells.'***

We thank the reviewer for the opportunity to clarify this point. There are many soluble molecules that can activate the immune system through unconventional and enigmatic pathways, for example TRAIL (Hanada, Blood 2011). Our results (**Figure 2A**) demonstrate that immobilized but not soluble PE molecules elicited 4D4 TCR^+ T cell activation, suggesting that crosslinking of the 4D4 TCR was important. In a physiological setting, this may be facilitated by antigen presenting cells expressing receptors that can immobilize PE, such as FcγRI receptors (Bell and Gray, JEM 2003, Takizawa, J Immunol Methods 1993; van Vugt, Blood 1996) or B cells via their B cell receptors (Pape, Science 2011; Wu, Immunology 1991, Cassatela, J Biol Chem 1991) may present PE to PE-reactive T cells. To investigate this, we co-cultured 4D4 TCR^+ and 1C5H TCR^+ (the PE-reactive γδ TCR) cell lines with SAV-PE in the presence of K562 cells or human monocyte-derived dendritic cells. We assessed CD69 upregulation and TCR down-modulation. Whereas activation of the 4D4 clone by soluble SAV-PE was not detected in any of the tested systems, the 1C5H clone responded to soluble SAV-PE, even in the absence of antigen-presenting cells. These results suggest that the 1C5H TCR^+ Jurkat cell line can immobilise and cross-present PE antigens even in the absence of antigen-presenting cells. These data are now incorporated within **Figure S2B**, we also revised the results and discussion sections appropriately. The revised manuscript now reads:

Results section, page 7, lines 211-218: "Antigen-presenting cells can express receptors that can bind PE, such as FcγRI receptors [39, 40, 41] or B cell receptors [34, 35, 42]. To investigate if soluble PE was immobilised and presented by antigen-presenting cells, we co-cultured 4D4 TCR^+ cells or 1C5H.TCR.Jurkat lines with K562 or human monocyte derived DCs in the presence of SAV-PE, SAV-BV421 or anti-CD3 and assessed CD69 upregulation and TCR downmodulation. Whereas activation of the 4D4 clone by soluble SAV-PE was not detected in any of the tested systems, the 1C5H line upregulated CD69 in response to soluble SAV-PE, even in the absence of other antigen-presenting cells (**Figure S2B**). These results suggest that the 1C5H.TCR.Jurkat line can immobilise and present PE antigens.

Discussion section, page 13, lines 427-431: "Our results suggest PE molecules need to be immobilised to be able to crosslink the TCRs expressed by 4D4 cells and elicit their activation. In a physiological setting, this mechanism could likely be accomplished by cells expressing receptors that can interact stably with PE, such as FcγRI receptors [39, 40, 41], B cell receptors [34, 35, 42] or even γδ TCRs [26]."

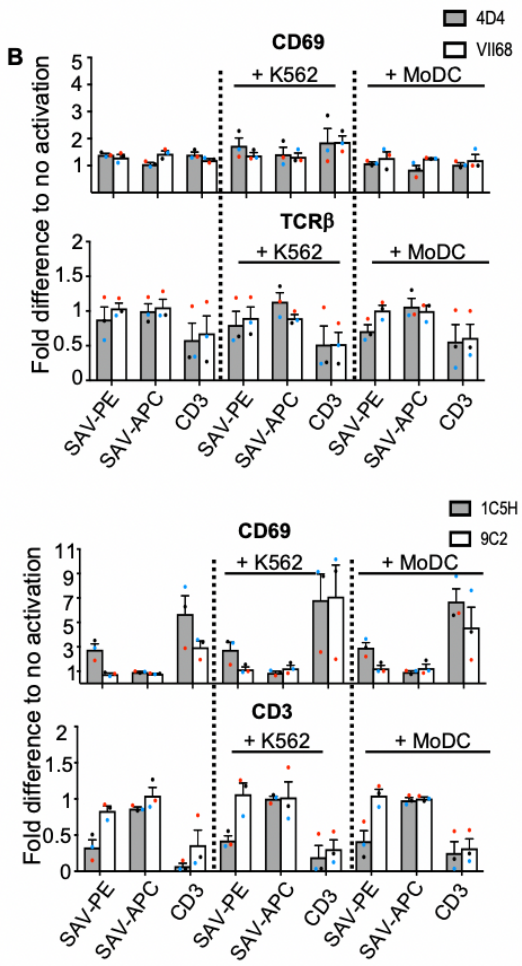


Figure S2. PE activates cells expressing the 1C5H. B. Soluble SAV- PE was assessed for its ability to activate the 4D4 TCR$^+$ BW58 cell line (**left**) or the 1C5H.TCR.Jurkat line (**right**) in co-cultures with K562 or CD1a$^+$ MoDCs lines. SAV-BV421, anti-CD3 (10 µg/mL) mAb and the CD1d-reactive TCRs (VII68 TCR$^+$ BW58 cell line or 9C2.TCR.Jurkat) were included as controls. Graphs show CD69 and TCRβ/CD3 mean fluorescence intensity fold difference to no activation +/- SEM after 16h across 3 independent experiments, each represented by individual colour-coded dots.

In addition to the above revisions, we have also performed TCR-CD3 clustering assays using single molecule imaging. Here we showed that the 4D4 TCR is capable of clustering and proximal signaling. We have included these results into the revised manuscript as **Figure 2D-F, Figure S3** and we also revised the results section appropriately. The revised manuscript now reads:

Results section page 7, lines 219-226: "Next, we investigated ligand-induced TCR cluster formation using single-molecule imaging of antibodies against CD3ε (**Figure 2D, S3 and Table S1**) and phosphorylated CD3ζ (pCD3ζ) (**Figure 2E and S3**). We exposed BW58 cell lines expressing the 4D4 TCR or the control 2C12 type I NKT TCR [32] to supported lipid bilayers containing either ICAM-1 only as an negative control, or ICAM-1, SAV-PE and CD1d-α-GalCer, alongside a positive control of anti-CD3 and anti-CD28 mAb-induced activation. The number and size of TCR clusters increased significantly for both the 4D4 and 2C12 TCRs in response to SAV-PE and CD1d-α-GalCer, respectively, although the TCR localizations per cluster remained unchanged throughout (**Figures 2F and S3**)."

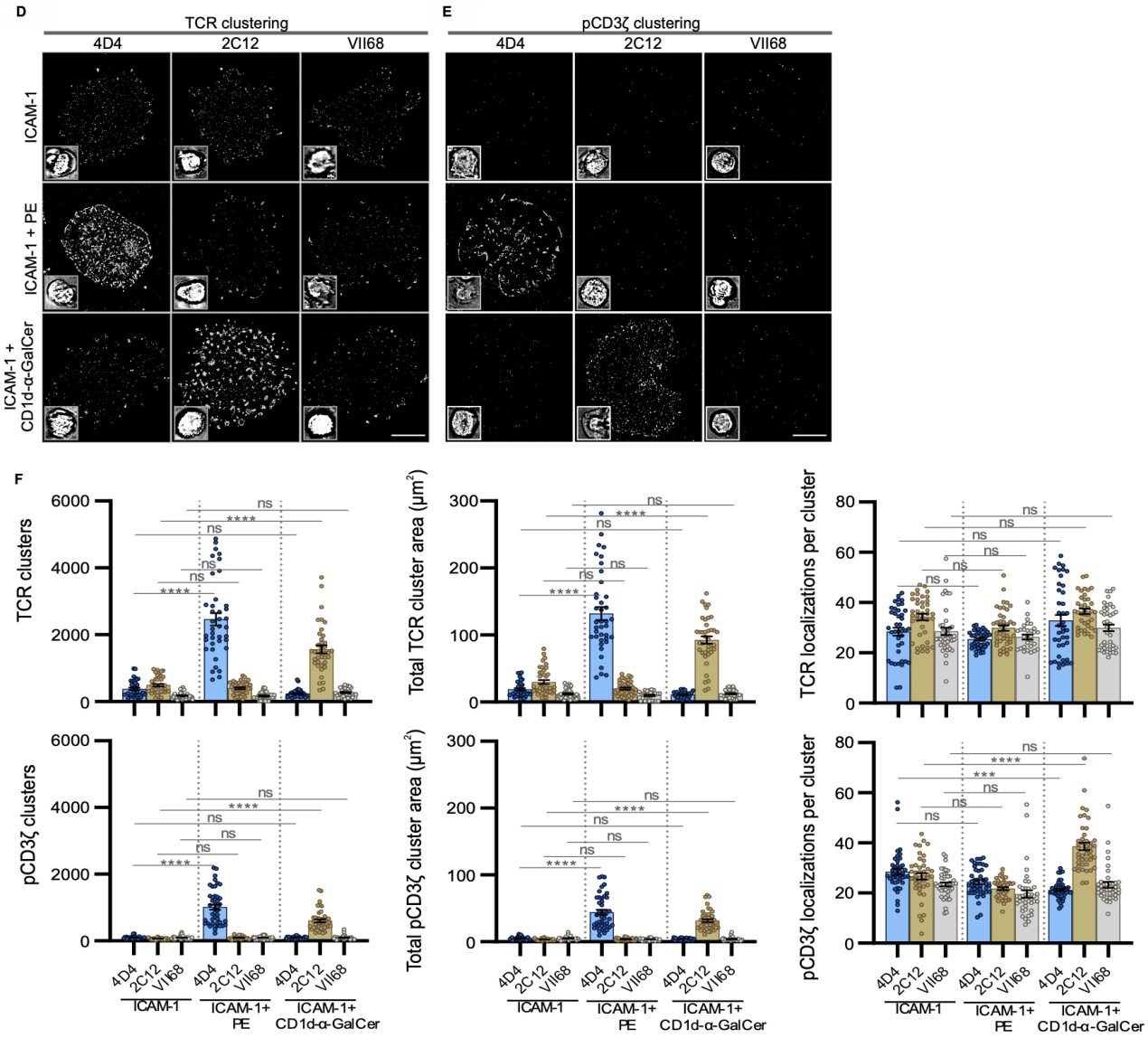


Figure 2. D. Single-molecule images of TCRβ and **E.** pCD3ζ clustering, in BW58 thymoma cells expressing 4D4 or control CD1d-reactive TCRs (2C12 type I NKT and VII68 type II NKT) stimulated on a supported lipid bilayer, decorated with ICAM-1 only, ICAM-1 and SAV-PE or ICAM-1 and CD1d-α-GalCer for 5 min. The inset bright-field images correspond to the single-molecule image, Scale bar= 5 μm. **F.** TCRβ and pCD3ζ clustering was quantified by DBSCAN analysis across $n \geq 40$ thymoma cell measurements which included >3 experimental replicates. Statistical significance reported from comparisons of the stimulated mean with the mean of unstimulated (ICAM only) control using one-way ANOVA; ns, not significant; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$. The exact P values are shown in **Table S1**. Error bars represent the SEM.

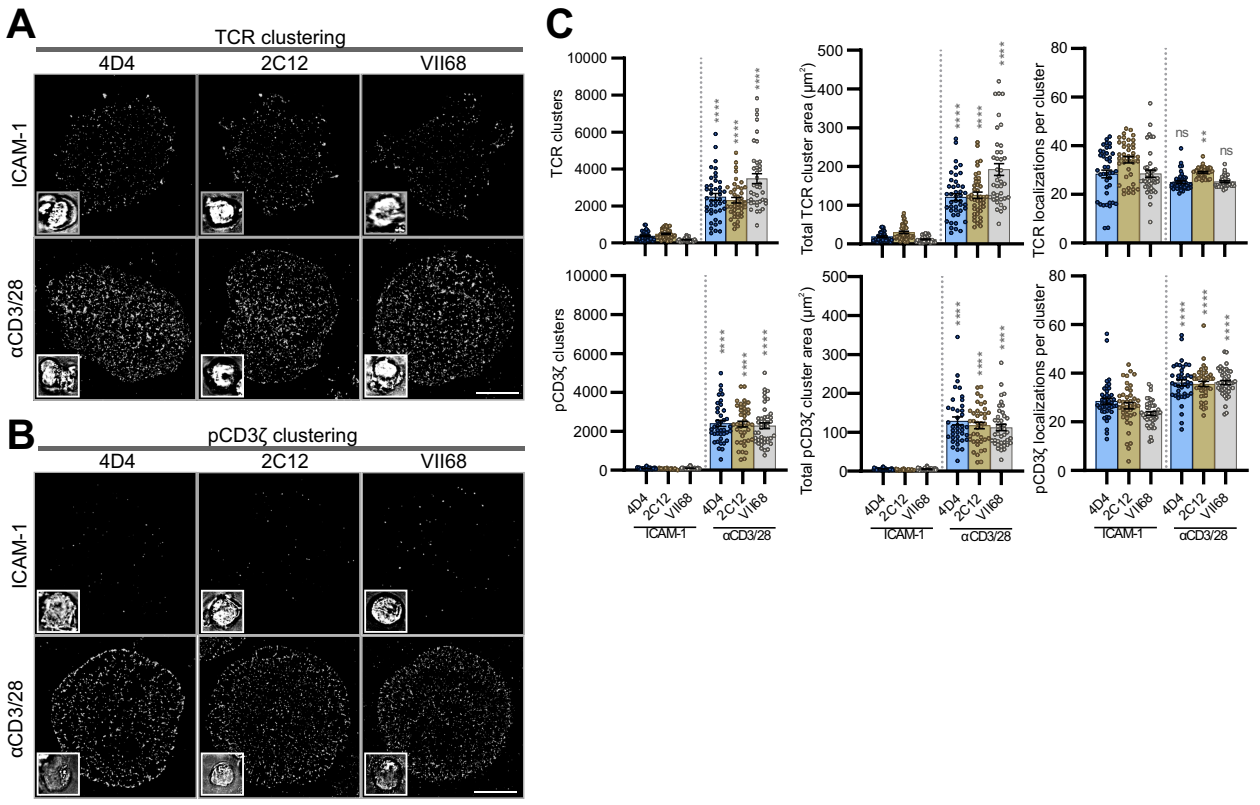


Figure S3. Control TCR and CD3 clustering of TCR-transduced BW58 lines. Single-molecule images of **A** TCRβ and **B.** pCD3ζ clustering in BW58 thymoma cells expressing 4D4, or the 2C12 type I NKT and VII68 type II NKT TCRs stimulated on a supported lipid bilayer, decorated with ICAM-1 only and anti-CD3/CD28 mAbs for 5 min. Scale bar, 5 μm. The inset bright-field images of thymoma cells correspond to each single cell in the single-molecule image. **C.** TCRβ and pCD3ζ clustering was quantified by DBSCAN analysis across $n \geq 40$ thymoma cells which includes at least three independent replicates. Statistical significance reported from multiple comparisons of mean with the mean of unstimulated (ICAM only) control using one-way ANOVA; ns, not significant; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$. The exact P values are shown in **Supplementary Table S1** Error bars represent the SEM.

Reviewer #3

<u>3.1:</u> 'Overall, the cellular and structural characterization of 4D4 TCRs are well carried out, showing this TCR can respond to a native antigen PE. However, the evidence for 4D4 being MHC-independent rather than CD1 dependent is not sufficient.'

We thank the reviewer for this insightful suggestion. We have now clarified this point with further surface plasmon resonance (SPR) experiments. Our data does not show any cross-reactivity and thus does not support the suggestion that the 4D4 TCR could be CD1d or MHC dependent. We have included these results into the revised manuscript as **Figure 3E** and we also revised the results section appropriately, which now reads:

Results section, page9, 278-285: "In a separate SPR experiment we tested the specificity of the 4D4 TCR against murine MHC and MHC-like molecules (**Figure 3E**). Here, the 4D4 TCR specifically bound to PE and showed little or no binding to CD1d-α-GalCer, I-A^b-CLIP, H2-D^b-NP_{366} and MR1-5-OP-RU (**Figure 3E**). The control 2C12 NKT TCR bound specifically to CD1d-α-GalCer and showed no specific binding to other molecules tested. Collectively, these experiments indicated that the soluble 4D4 TCR directly interacts with PE with an affinity that is comparable to that of functional αβTCR-peptide MHC interactions [2], and was specific to PE, with no cross-reactivity to a panel of MHC and MHC-like molecules."

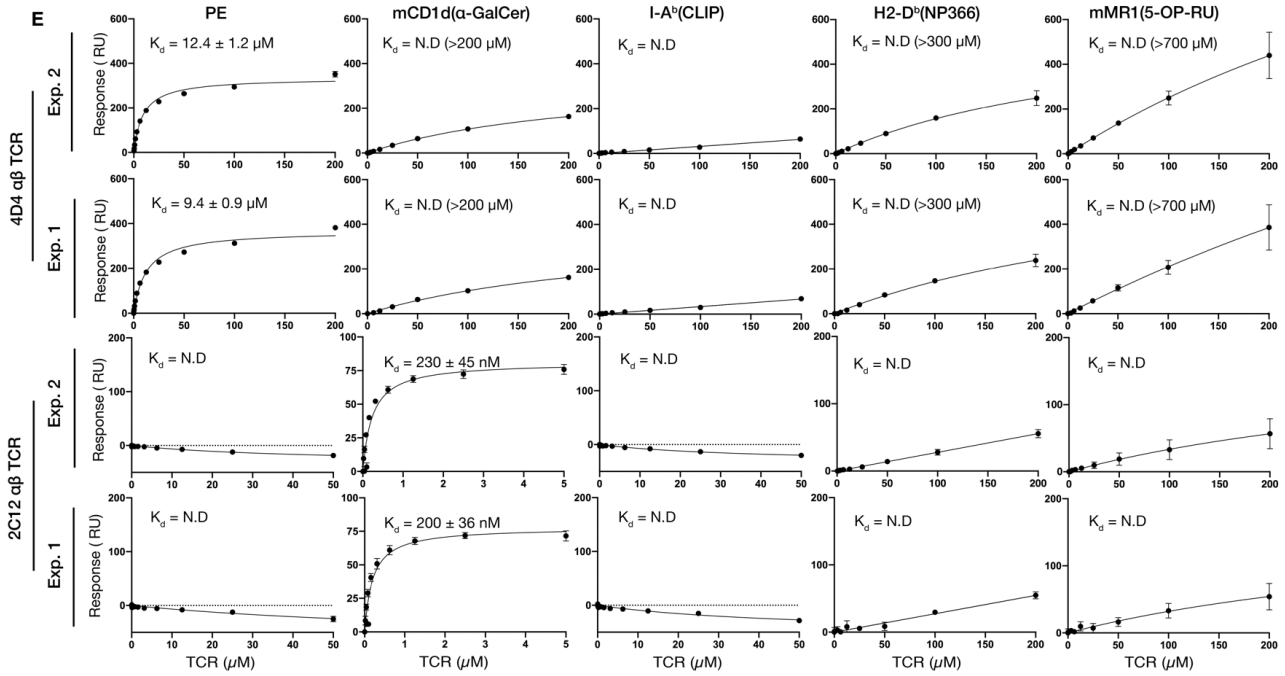


Figure 3. Biophysical analysis of the 4D4 interaction with PE. E. Serial dilutions of the 4D4 TCR and 2C12 type I NKT TCR were passed over a surface plasmon resonance (SPR) chip with immobilised PE, murine (m)CD1d-α-GalCer, I-A^b-CLIP, H2-D^b-NP_{366} and mMR1-5-OP-RU to a surface density of ~1000 response units. SPR derived equilibrium binding curves were conducted twice in independent repeat experiments, as shown. Each experiment was conducted in experimental replicate (n=2, error bars reflect SEM) and used to determine the dissociation constants for each experimental repeat. (K_D). ND - not detected.

<u>3.2:</u> ***'The in vivo evidence supporting 4D4 T cells recognizing non-MHC ligands came from their retrogenic mice experiment showing mature 4D4 T cells are mostly CD4- CD8-. However, it's not surprise that these T cells come out CD4- as they are originally from NKT cells. Since PE is unlikely a physiological ligand, it is possible that the TCR recognizes an unknown ceramide presenting CD1d in vivo but crossreactive to PE. The so called "MHC-independent" TCR do not include CD1d-restricted invariant chain bearing NKT receptor, which are naturally MHC independent. To rule out the possibility of 4D4 being another NKT TCR, the authors need to show the GFP+TCRb+ but not GFP-TCRb+ cells proliferate on CD1d-/- APC.'***

We wish to clarify that we have no evidence that the 4D4 TCR is derived from an NKT clone or is selected through a CD1d-dependent interaction. Whilst we coincidentally isolated this clone alongside CD1d-reactive NKT cells using PE-conjugated CD1d tetramers, our results in Figure 1 indicate that the 4D4 TCR bound only the PE fluorochrome, rather than the CD1d component of the CD1d tetramers. Furthermore, we now provide surface plasmon resonance (SPR) data showing that 4D4 does not bind to CD1d and MHC (see above, **Reviwer #3, 3.1**). For this reason we had not considered the need to conduct experiments addressing this particular alternative to the mechanisms we have formally ruled in. To acknowledge that 4D4 TCR may still bind to MHC or CD1d via dependence on a particular peptide or lipid-based antigen, we have revised the discussion to account for this possibility. The revised manuscript now reads:

Discussion section, page 13, lines 406-408: "Whereas we did not find any evidence suggesting MHC or CD1d-dependence, we cannot exclude that the 4D4 TCR might still bind to MHC or CD1d via dependence on a particular peptide or lipid-based Ag."

3.3: ***'4D4 TCR recognizes PE from Prozyme (red algae of undisclosed species) but not from Thermo Fisher Scientific (Porphyra tenera). A structure-based sequence alignment would reveal some additional information on the epitopes for 4D4 TCR binding.'***

Thank you for this suggestion. Based on the X-ray structure we were able to determine of the Prozyme PE we compared the sequence available for Porphyra tenera, highlighting a modest difference of Ile79 to Val79, respectively. Notably without structural data of the Porphyra tenera PE α-chain these analyses do not account for sidechain movements or shifts due to neighbouring residues. To broaden these analyses across species we estimated the evolutionary conservation of the TCR epitope, namely that across ~150 species the TCR epitope is highly variable. We have now included these analyses within the revised results section and supplementary Figure S7. The revised manuscript now reads:

Results section, page 10-11, lines 325-328: "To provide insight into species-specific reactivity to PE from the 4D4 TCR, we conducted multiple sequence alignments of ~150 bacterial PE α-chains and visualised this in the context of the structure. Mapping the sequence variation and conservation within the PE epitope recognized by the 4D4 TCR clearly showed a low conservation across different bacterial species (**Figure S7 and Table S4**)."

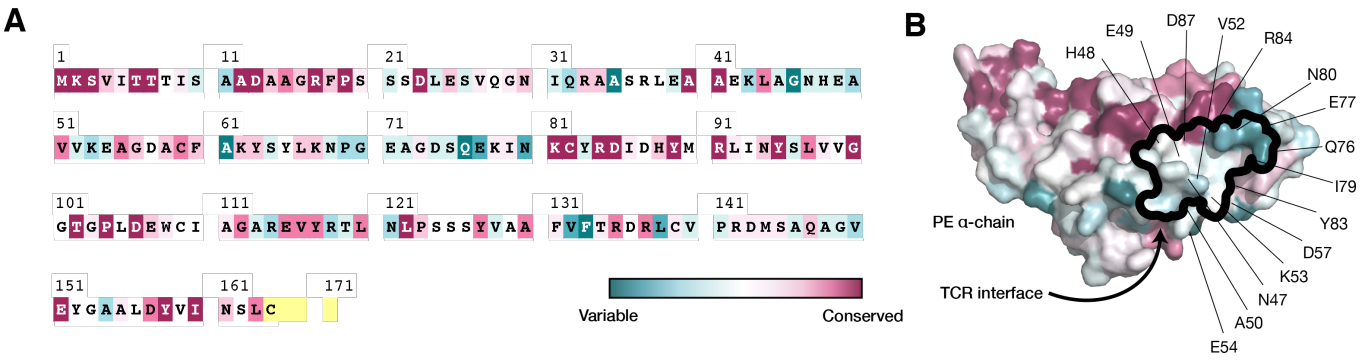


Figure S7. Estimation and visualization of the evolutionary conservation at the PE-TCR epitope. **A.** Colorised representation of a multiple sequence alignments of ~150 bacterial species of PE α-chains (see **Table S4**). **B.** Structural mapping of the sequence conservation with the PE α-chain area recognized by the 4D4 TCR highlighted.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

The reviewer has no other questions.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, the authors provided additional SPR binding data to show 4D4 TCR recognized immobilized PE but not CD1d-α-GalCer, nor MR1-5-OP-RU. These SPR experiments simply confirmed 4D4 does not bind known CD1d-α-GalCer nor MR1 but can not be used to make generalization that the TCR dose not bind CD1d with unknown lipid moiety. In fact, the non-binding of 4D4 TCR to CD1d-α-GalCer was not surprised as the TCR was isolated from a cell population stained negative to CD1d-α-GalCer but positive to CD1d-α-GlcADAG in their 2019 Nat Comm publication. This reviewer suggested to demonstrate 4D4 TCR's independence from CD1d. In their rebuttal, the authors stated that they had no evidence of 4D4 being CD1d-dependent, thus did not find necessary to address the concern experimentally. However, these T cells were largely absent in CD1d-/- mice from their 2019 publication, suggesting they are CD1d-dependent. Since the manuscript describes 4D4 TCR as a functional MHC independent abTCR to recognize intact protein instead of a CD1d-dependent TCR that happens to cross reactive to PE (which is likely not physiologically relevant), it is necessary to show 4D4 proliferate in vivo independent of CD1d. The reviewer does not disagree with the finding that PE can stimulate 4D4, but so can phytohemagglutinin (PHA) and concanavalin A, both stimulate T cells but are not physiological ligands for TCR. The only difference is 4D4 binding involves CDR3. But that in itself is not sufficient to claim PE as a physiological ligand of 4D4. PE may be sufficient to stimulate 4D4 TCR but may not be necessarily a physiological ligand in vivo.

Hence the original comment from this reviewer has not been addressed and needs to be addressed before the manuscript can be accepted for publication.

Reviewers comments

Reviewer #2 (Remarks to the Author):

The reviewer has no other questions.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, the authors provided additional SPR binding data to show 4D4 TCR recognized immobilized PE but not CD1d-α-GalCer, nor MR1-5-OP-RU. These SPR experiments simply confirmed 4D4 does not bind known CD1d-α-GalCer nor MR1 but can not be used to make generalization that the TCR dose not bind CD1d with unknown lipid moiety. In fact, the non-binding of 4D4 TCR to CD1d-α-GalCer was not surprised as the TCR was isolated from a cell population stained negative to CD1d-α-GalCer but positive to CD1d-α-GlcADAG in their 2019 Nat Comm publication. This reviewer suggested to demonstrate 4D4 TCR's independence from CD1d. In their rebuttal, the authors stated that they had no evidence of 4D4 being CD1d-dependent, thus did not find necessary to address the concern experimentally. However, these T cells were largely absent in CD1d-/- mice from their 2019 publication, suggesting they are CD1d-dependent. Since the manuscript describes 4D4 TCR as a functional MHC independent abTCR to recognize intact protein instead of a CD1d-dependent TCR that happens to cross reactive to PE (which is likely not physiologically relevant), it is necessary to show 4D4 proliferate in vivo independent of CD1d. The reviewer does not disagree with the finding that PE can stimulate 4D4, but so can phytohemagglutinin (PHA) and concanavalin A, both stimulate T cells but are not physiological ligands for TCR. The only difference is 4D4 binding involves CDR3. But that in itself is not sufficient to claim PE as a physiological ligand of 4D4. PE may be sufficient to stimulate 4D4 TCR but may not be necessarily a physiological ligand in vivo.
Hence the original comment from this reviewer has not been addressed and needs to be addressed before the manuscript can be accepted for publication.

Point by point rebuttal

Reviewer #1
No comments to address

Reviewer #2
No comments to address

Reviewer #3
We thank the reviewer for their continued comments on our manuscript and for prompting us to provide more data to substantiate our main claim, namely that the 4D4 TCR can directly recognise PE and does so independently of recognising CD1d.
We wish to draw the reviewer's attention to the fact that while we used CD1d tetramers to isolate CD1d-reactive T cells (Almeida et al., Nat Comms 2019), and also used CD1d tetramers in this study, we subsequently showed that the reactivity of the 4D4 TCR was not attributable to CD1d but to PE itself. This is demonstrated in Figures 1 and Figure 2 of the manuscript.

Moreover, we then refolded the 4D4 TCR, conducted extensive biophysical analyses to show direct interaction of the 4D4 TCR with PE (Figure 3), and then determined the crystal structure of the 4D4 TCR-PE complex (Figures 4 and 5), providing the molecular basis of this interaction. Note that the data in Figures 3-5 are with purified recombinant proteins, where there is no CD1d or MHC present

– thus we formally demonstrate how an αβTCR can directly recognise an antigen independent of an MHC or MHC-I like molecule (such as CD1d).

The reviewer contends that the 4D4 TCR may cross-react with CD1d when bound to a specific antigen, and cite α-GlcADAG as a potential antigen. In Figure 1 of our original submission, we included α-GlcADAG in a CD1d plate bound assay and showed that three CD1d restricted T cell clones responded to this, but 4D4 did not. In the first round of revision we addressed the possibility that 4D4 might be CD1d reactive by more SPR analyses (Figure 2), plus we conducted single molecule analyses (Figure 2) to show that the 4D4 TCR bound to PE and not CD1d. To further address the reviewer's comments regarding their concern over cross-reactivity towards CD1d, we have undertaken two additional series of experiments.

1) We have now conducted an additional series of SPR-based experiments, in addition to that included in the first revision. Namely, we expanded the CD1d panel to include: CD1d-endo, CD1d-α-GalCer and CD1d-α-GlcADAG. We also tested TCRs specific for the loaded mCD1d samples.

This experiment is included as **Supplementary Figure S7** and demonstrates that the 4D4 TCR does not specifically bind mCD1d-endo which is endogenously loaded during production and therefore encompasses a broad repertoire of distinct self-lipids. Our experience dictates that such a very weak response is within the noise of SPR experiments, as also seen with 4D4 TCR interaction with MR1, H-2Db and I-Ab (Figure 3E). Further, we saw no 4D4 TCR mediated reactivity or activation to CD1d-endo across our other experiments. Additionally, the 4D4 TCR did not bind to CD1d-α-GalCer or CD1d-α-GlcADAG. The latter is particularly important because this was the antigen loaded into the CD1d tetramer used to isolate the 4D4 T cell line (Figure 1), further supporting the fact that 4D4 binds to PE, not to CD1d-α-GlcADAG. The control NKT TCRs 2C12 and A11B8.2 confirmed the quality and loading of the CD1d samples, showing CD1d-α-GalCer specificity and the preferentially CD1d-α-GlcADAG reactivity, respectively, but also some binding to CD1d-endo, which is expected for these CD1d-restricted TCRs. The results and supplementary sections were suitably revised as follows.

Results, Page 10, line 300:

'We next conducted SPR with an expanded CD1d panel. Here the 4D4 TCR did not specifically bind to CD1d endogenously loaded with a multitude of distinct self-lipids during production (CD1d-endo). Such a very weak response is within the noise of SPR experiments, as also seen with 4D4 interaction with MR1, H-2Db and I-Ab (Figure 3E). Moreover, we observed no binding to CD1d-α-GalCer or CD1d-α-GlcADAG (**Figure S7**). These SPR-based results are consistent with no 4D4 TCR mediated reactivity or activation to CD1d-endo or loaded with α-GalCer or α-GlcADAG across our other experiments. The control NKT TCRs 2C12 and A11B8.2 [31] confirmed the quality and loading of the CD1d samples, showing CD1d-α-GalCer specificity and the preferentially CD1d-α-GlcADAG reactivity, respectively. Accordingly, this 4D4 TCR does not exhibit notable cross-reactivity to a panel of MHC and MHC-like molecules including CD1d loaded with a range of lipid-based antigens. Collectively, these experiments indicated that the soluble 4D4 TCR directly interacts with PE with an affinity that is comparable to that of functional αβTCR-peptide MHC interactions [2].'

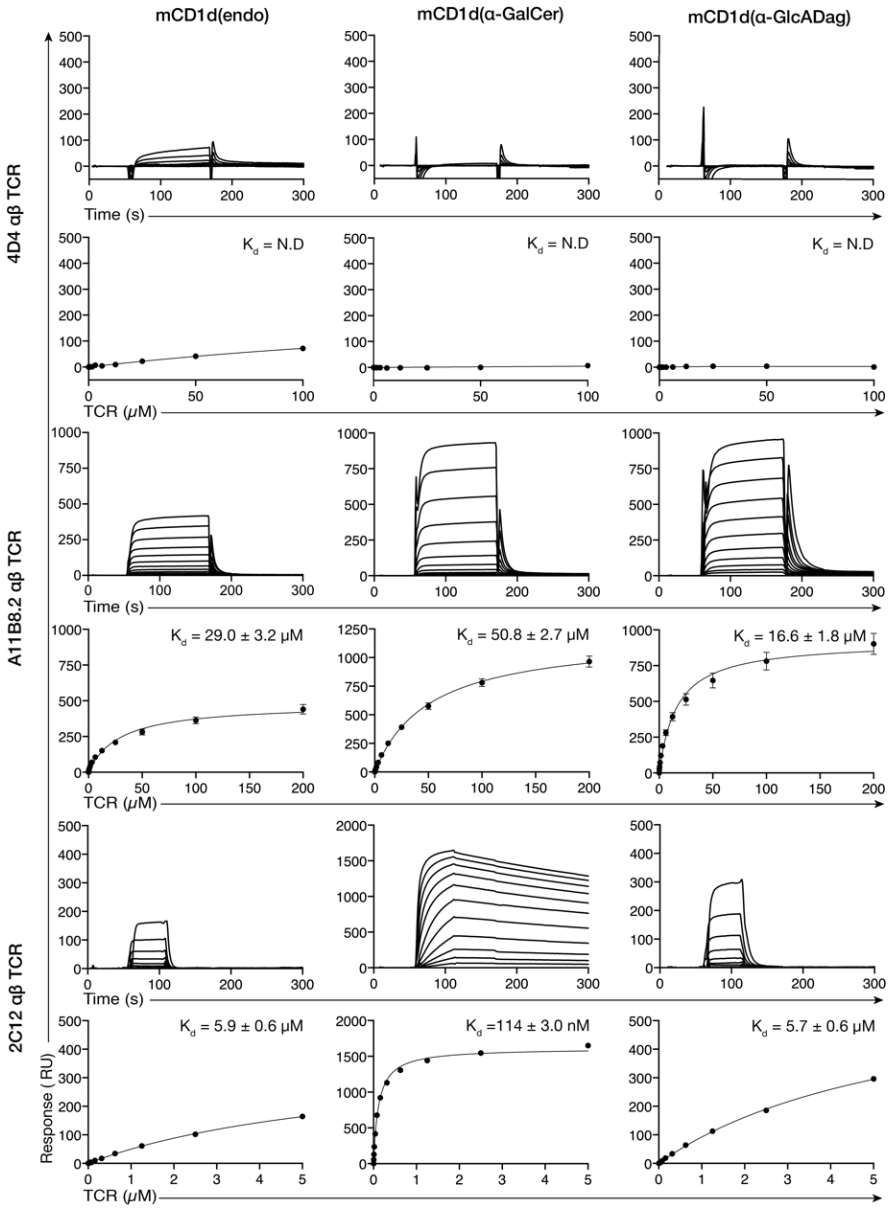


Figure S7. Expanded surface plasmon resonance of the 4D4 TCR and CD1d. Surface plasmon resonance of the 4D4, A11B8.2 and 2C12 TCR binding to immobilised murine CD1d-endo, CD1d-α-GalCer and CD1d-α-GlcADAG. Representative sensorgrams of two-fold dilutions of the 4D4, A11B8.2 and 2C12 TCRs (100, 200 and 5μM respectively). The derived equilibrium binding curves were used to estimate the dissociation constants (K_d) for each experiment (Mean ± SEM derived from duplicates). Data shown are representative of two independent experiments for the 4D4 and 2C12 TCRs, and a single experiment for the A11B8.2 TCR (N.D. = not determined).

2) We have also conducted new activation assays of 4D4 in the presence of bone marrow-derived dendritic cells from C57BL/6 WT mice (expressing CD1d) or $CD1d^{-/-}$ mice (lacking CD1d), including the 2C12 NKT TCR^{+} cell line as a positive control, as well as additional complementary plate-bound activation assays.

This data is included in **Supplementary Figure S3** and again suggests that 4D4 is not activated by CD1d, unlike 2C12 which responds to various CD1d-bound ligands in a CD1d-dependent manner, both in the BMDC co-culture, as well as in response to plate-bound mCD1d. The results and supplementary sections were suitably revised as follows:

Results, Page 7, line 218:

Next, we investigated if 4D4 TCR^+ cells could be activated by mouse dendritic cells expressing CD1d. Here, 4D4 TCR^+ cells, or control type I NKT 2C12 TCR^+ cells, were co-cultured with bone marrow-derived dendritic cells (BMDC) from C57BL/6 WT or $CD1d^{-/-}$ mice **Fig S3A**). IL-2 was detected at high levels in the co-culture supernatant of 2C12 TCR^+ cells with BMDC WT in the presence of α-GalCer and, to a lesser extent, α-GlcADAG, and even in the absence of exogenously added-ligand (**Fig S3B**). Importantly this was prevented by anti-CD1d blocking mAb added at the beginning of culture, or when 2C12 was co-cultured with BMDC $CD1d^{-/-}$, indicating CD1d-dependent activation. In contrast, 4D4 TCR^+ cells were not activated in this context, regardless of the lipid antigen and presence or absence of CD1d. While we also found that neither of the TCR^+ cell lines responded specifically to PE added to BMDC-co-cultures, in comparison to BMDC-co-cultures with no exogenous ligand added, this is in line with our observations in Figure S2 where PE loaded into cellular cocultures did not stimulate 4D4 TCR^+ cells. Using a plate-bound assay, as expected, high levels of IL-2 were detected when 2C12 TCR^+ cells were cultured with plate-immobilised CD1d-α-GalCer and, albeit less so, CD1d-α-GlcADAG and CD1d-endo, which is loaded with an array of self-lipids incorporated into CD1d during its synthesis[39] (**Fig S3C**). This again shows that the type I NKT 2C12 TCR can be activated by a range of CD1d-bound lipids. In contrast, 4D4 was not activated by any of these plate-bound CD1d-lipid antigen complexes but was clearly activated by plate-immobilised PE. Both TCR-transduced lines responded to plate-bound anti-CD3 (**Fig S3C)**. Thus, these data are consistent with the concept that the 4D4 TCR is not CD1d-restricted.

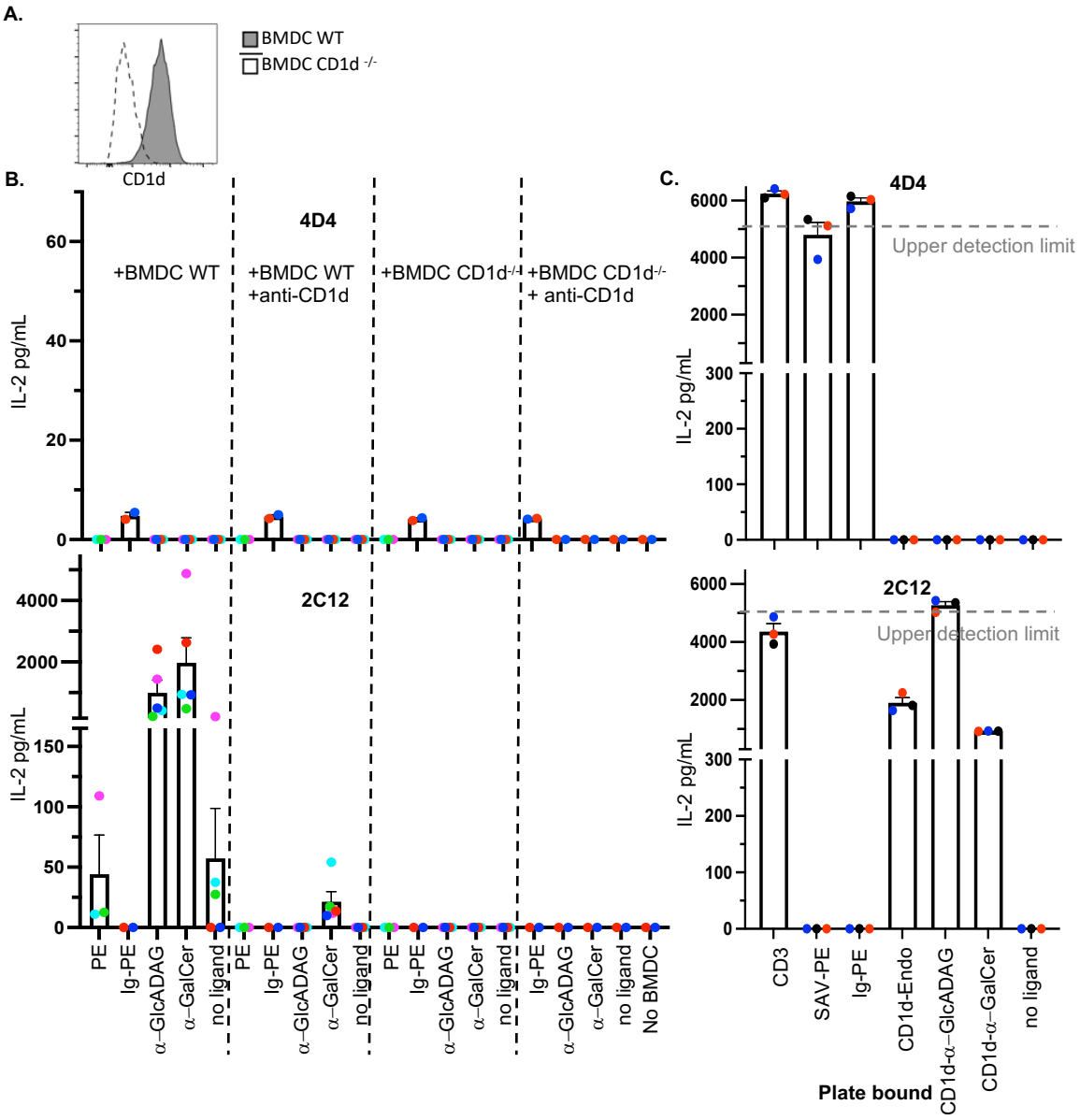


Figure S3. 4D4 is not activated by CD1d. A. Graphs show CD1d expression on C57BL/6 WT or CD1d$^{-/-}$ bone marrow-derived DCs (BMDC), and representative plot showing Ig-PE (anti-PD-L1, CD45, CD80) capture by BMDCs. **B.** 4D4.BW58 or 2C12.BW58 cells were cultured at a 1:1 ratio with BMDCs WT or CD1d$^{-/-}$ in the presence of purified PE (50 µg/mL), Ig-PE (50 µg/mL), α-GlcADAG (30 µg/mL), α-GalCer (400 ng/mL) or in the absence of exogenously added ligands. After 20h, the supernatants harvested for IL-2 quantification by Cytometric Bead Array (CBA). Where indicated anti-CD1d mAb (clone 1B1) was added at the beginning of culture at 10 µg/mL. Data (mean ± SEM) is from n = 5 independent experiments, each represented by a different dot (colour-matched (3 involving purified PE and 2 Ig-PE (red and blue)). **C.** 4D4.BW58 or 2C12.BW58 cells were cultured with plate-immobilised anti-CD3 (2 µg/mL), SAV-PE or Ig-PE (50 µg/mL) or CD1d (25 µg/mL) loaded with endogenous ligands (-Endo), α-GlcADAG or α-GalCer. After 20h, IL-2 secretion was assessed by CBA. Data (mean ± SEM) is from 3 independent experiments, each represented by a different dot (colour-matched to B).

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

In the revised manuscript, the authors provided more Biacore data and stimulation of 4D4 transduced BW58 cells with BMDC from wt or CD1d-/- mice. Rather than activation by both wt and CD1d-/- BMDC, which would confer CD1d independence, the results showed no IL-2 productions under any stimulation conditions. It is a negative result that showed while α-GlcADAG or α-GalCer presenting wt BMDC failed to activate 4D4, CD1d-/- BMDC also failed. In the case of MHC independent TCR for mCD155, the T cells were activated by both wt and MHC-/- APC. The BMDC experiments merely confirmed that α-GlcADAG or α-GalCer are ligands for 2C12 but not 4D4 as 2C12 without added α-GlcADAG or α-GalCer to BMDC also was not activated. As the reviewer mentioned earlier, high affinity soluble ligands are known to activate T cells, including PHA, ConA, antibodies and superantigens that displayed limited TCR specificities. In the current case, the TCR binds PE through CDR region, thus is more specific than, for example, superantigens that recognized based on TCRbeta. An MHC independent physiological ligand should be expressed on the surface of APC and activates T cells in its native form and it simply does not use MHC, nor CD1d. mCD155 is expressed on mouse cells, thus can participate normal T cell function of recognizing cell surface mCD155. PE is not expressed on mouse cell surface, does not constitute as a physiological ligand, but behaves like a 4D4 specific superantigen from cyanobacteria. Given the lack of clear cellular support for 4D4 being CD1d independent and the conceptual misunderstanding for MHC independent physiological ligand, the claim of 4D4 MHC-independency should be removed and PE should be described as a 4D4 specific superantigen ligand. Nonetheless, PE is an interesting superantigen and the 4D4 TCR recognition of it would be interesting as well.

Reviewers comments

Reviewer #2 (Remarks to the Author):

The reviewer has no other questions.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, the authors provided more Biacore data and stimulation of 4D4 transduced BW58 cells with BMDC from wt or CD1d-/- mice. Rather than activation by both wt and CD1d-/- BMDC, which would confer CD1d independence, the results showed no IL-2 productions under any stimulation conditions. It is a negative result that showed while α-GlcADAG or α-GalCer presenting wt BMDC failed to activate 4D4, CD1d-/- BMDC also failed. In the case of MHC independent TCR for mCD155, the T cells were activated by both wt and MHC-/- APC. The BMDC experiments merely confirmed that α-GlcADAG or α-GalCer are ligands for 2C12 but not 4D4 as 2C12 without added α-GlcADAG or α-GalCer to BMDC also was not activated. As the reviewer mentioned earlier, high affinity soluble ligands are known to activate T cells, including PHA, ConA, antibodies and superantigens that displayed limited TCR specificities. In the current case, the TCR binds PE through CDR region, thus is more specific than, for example, superantigens that recognized based on TCRbeta. An MHC independent physiological ligand should be expressed on the surface of APC and activates T cells in its native form and it simply does not use MHC, nor CD1d. mCD155 is expressed on mouse cells, thus can participate normal T cell function of recognizing cell surface mCD155. PE is not expressed on mouse cell surface, does not constitute as a physiological ligand, but behaves like a 4D4 specific superantigen from cyanobacteria. Given the lack of clear cellular support for 4D4 being CD1d independent and the conceptual misunderstanding for MHC independent physiological ligand, the claim of 4D4 MHC-independency should be removed and PE should be described as a 4D4 specific superantigen ligand. Nonetheless, PE is an interesting superantigen and the 4D4 TCR recognition of it would be interesting as well.

We thank the reviewer for recognition that our paper describing 4D4 TCR recognition of PE is interesting. Given their concerns regarding MHC-independent claim, we have modified our title from:

MHC independent αβ T cell receptor recognition of an intact protein

to:

Direct recognition of an intact foreign protein by an αβ T cell receptor