

CD137 agonists in mice suppress germinal center responses by increasing the immunosuppressive activity of follicular regulatory T-cells

Ana Martínez-Riaño, Raquel Cuesta, Dayanna Salinas, Nekane Soria, Raluca Alexandru, Paula Molero-Glez, David Ruiz-Guillamon, Arantza Azpilikueta, Carlos Luri-Rey, Álvaro Lopez-Janeiro, Carlos E de Andrea, María S. Aymerich, Pedro Berraondo, Alvaro Teixeira, and Ignacio Melero

Corresponding author(s): Ignacio Melero (imelero@unav.es), Ana Martínez-Riaño (ana.martinezriano@bio-gipuzkoa.eus)

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Melero,

Thank you again for submitting your manuscript EMBOJ-2026-123462 for consideration by The EMBO Journal, and for your patience during peer review. Your manuscript has now been seen by three experts in the field, and we have received the complete set of their comments, which you can find appended below.

As you will see, the referees recognize that this is a compelling, novel, and interesting study addressing a relevant topic in immunology. While they are all rather supportive, they also identify several limitations and raise a number of concerns, while providing detailed and reasonable suggestions in their reports for strengthening the manuscript further and increasing its significance and impact on the field.

In light of this input, I would like to invite you to submit a thoroughly revised version of your manuscript taking the referees' suggestions on board, along with a detailed point-by-point response addressing all referees' comments. Please note that it is The EMBO Journal policy to allow only a single round of major revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Given the nature of the raised concerns, I think that it would be useful to discuss your revision plan already during the initial phase of your revision. You are very welcome to share with me a draft point-by-point response/revision plan explaining if there are any points you do not agree with or cannot address. We could then arrange a video call to discuss the details further, if necessary.

We generally allow three months as standard revision time (12 May, 2026). As a matter of policy, competing manuscripts published during this period will not negatively impact our assessment of the conceptual advance presented by your study. However, we request that you contact us as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we will be able to grant an extension.

Please let me know if you have any questions or comments that you would like to discuss with me. Thank you for the opportunity to consider your work for publication in The EMBO Journal. I look forward to your revision.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor, The EMBO Journal
i.papaioannou@embojournal.org

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- A Word file of the manuscript text (including legends of main Figures, EV Figures and Tables). Please make sure that changes are highlighted (or "tracked") to be clearly visible.

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2. Please note that no statistics should be calculated and shown in Figures if $n=2$. Please also note that each p value should be reported as an exact value.

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"The RNA-seq datasets produced in this study are available in the following database:
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*** The Data Availability Section is restricted to new primary data that are part of this study. In case you have no data that require deposition in a public database, please state so instead of referring to the database: "Our study includes no data deposited in public repositories." under the heading "Data availability". ***

4. The materials and methods need to be described in the manuscript using our structured methods format, which is now required for all research articles. According to this format, the Methods section includes a single "Reagents and Tools Table" - listing key reagents, experimental models, software and relevant equipment including their sources and relevant identifiers- followed by a "Methods and Protocols" section describing the methods. Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guide: <https://www.embopress.org/page/journal/14602075/authorguide#structuredmethods>. When submitting your revised manuscript, please do not include the Reagents and Tools Table in the Methods section of the manuscript but instead upload it as a separate file choosing the file type "Reagent Table".

5. Please check that the title and the abstract of the manuscript are brief, yet explicit, even to non-specialists. The length of the title should not exceed 100 characters, and the abstract should be a single paragraph not exceeding 175 words.

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14. We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a cover.

Referee #1:

Review of EMBOJ-2026-123462, "Costimulation of follicular regulatory T cells with CD137 (4-1BB) agonists disrupts B-cell responses"

- general summary and opinion about the principal significance of the study, its questions and findings

In this manuscript the authors attempt to address the mechanistic basis for how anti-CD137 agonists can disrupt humoral immunity. To address this, they employ various models of immunisation (such as OVA) and study the impact of CD137 agonism on the multiple cell types potentially involved. They show that agonistic anti-CD137 antibodies target activated CD8⁺ T cells, Tregs, follicular regulatory T cells (Tfrs), and FDCs and that CD137 agonism impairs GC reactions upon vaccination, impairing antibody production. They also show that anti-CD137 antibodies suppress ectopic B-cell responses in TLS of tumors. They go on to show that these effects are dependent upon Foxp3⁺ cells, with anti-CD137 treatment increasing both the abundance and immunosuppressive activity of Tregs and Tfrs in their vaccination model.

The data are novel, interesting and address a longstanding immunological phenomenon. The results are in general clear, well described and presented with an easily to follow narrative.

My comments below i hope will serve to improve the paper

One potential limitation that all of the observed effects are demonstrated with a single antibody clone (3H3, a potent and known agonist). Therefore the data could potentially reflect an antibody clone-specific phenomenon. Did the authors evaluate other agonistic CD137 mAb?

Importantly the authors already discuss whether the observed effects relate to normal physiology or due to drug-mediated

pharmacology.

Similarly, previously the authors have elegantly shown that another CD137 antibody, 1D8 is to some extent toxic in mice, evoking hyper-inflammatory responses (especially liver inflammation) that are dose limited. To counter this the authors previously designed a masked/prodrug 1D8 molecule, leading to activation in only the right (in that case, tumor) location (Etxeberria et al *pnas* 2021). Did the authors attempt to exploit the same molecule in this study (in the TLS work). That would certainly increase the translational impact of the study - potentially providing a ready-made solution to any toxicity issues (which have so far limited CD137 agonists such as urelumab).

I could not readily discern from the figure legends how many times each experiment had been performed. In Figure 1, 4 data points presumably indicate 4 mice, but how many times was the experiment performed? Figure 2 shows 3 datapoints. Figure 4 by comparison shows >10 datapoints. Is this from >1 experiment? This (numbers of experiments as well as mice) should be detailed for all figures to evaluate reproducibility.

Of direct relevance, do the authors expect that they saw only limited defective plasmablast generation/ differentiation in anti-CD137-treated mice due to the relatively short time-frame of the study? Or was it simply that they had insufficient numbers of animals to conclude this (n=4)? See comment above

Data in some other figures lack routine statistical significance at $p < 0.05$ due to small numbers.

- specific major concerns essential to be addressed to support the conclusions

Do the authors expect that they saw only limited defective plasmablast generation/ differentiation in anti-CD137-treated mice due to the relatively short time-frame of the study? Or was it simply that they had insufficient numbers of animals to conclude this (n=4)? See comment above

Perhaps the plots have been mixed in Figure 4A - the text reads "Fourteen days post-immunization, anti-CD137-treated mice displayed Tfh frequencies comparable to those of non-immunized controls and approximately two-fold decrease as compared with isotype-treated mice (Fig. 4A, Fig. EV3B)" and yet the %'s indicated on the representative plots show that the TFH increase with anti-CD137.

Did the authors evaluate the efficacy of the depleting/blocking antibodies used in Figure 5? i.e. assess controls as evidence that the CD8, IFNG and IL-12 mAbs had their desired effects? Particularly after immunisation this might be important.

- minor concerns that should be addressed

In Figure 2A was statistical analysis performed? This would complement the visual presentation

To appreciate better the data displayed in figure 2C, some raw flow cytometry plots comparing ex-vivo staining (day 0) versus day 1 might be helpful.

Some minor typos - e.g. line 402 "To explore further the possible effects on Tregs induced by the anti-CD137 agonism, Foxp3-GFP hCD2-RFP were" (missing the word mice or cells)

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

Possibly the white diagonal line for 3C is missing on the isotype magnification? Also the white box to show which area is being expanded

The IgD legend in figure 3C might be better placed to improve visualisation

Referee #2:

In this manuscript the authors assess how agonistic a4-1BB alters humoral immunity. The authors show that a4-1BB limits GC B cells, while simultaneously expanding Treg and Tfr cells, an effect that is not dependent on CD8s, IFNg or IL-12. Furthermore, RNAseq and functional experiments demonstrate that Treg from a4-1BB may be more activated with higher suppressive capacity. Lastly, the authors show that the decrease in B cell responses with a4-1BB can also occur in tumor derived TLS and antibody drug conjugates. Overall, this is a fairly compelling study on how a4-1BB controls humoral immunity. However, some claims are not substantiated with data presented and some data could be strengthened to enhance significance of the work.

Major points

1. This entire study was performed with the 3H3 a4-1BB clone. Key results (decrease in GC B, etc.) should be confirmed with a second a4-1BB agonistic antibody clone for confirmation.
2. A central claim (see title) is that a4-1BB affects Tfr cells both by expansion and suppressive function, but neither of these are

shown directly. Fig. 4c should show Tfr % of total Tregs, not of Tfh. This will help determine whether a4-1BB mostly affects Treg expansion and increased Tfr are due to increased precursors to Tfr, or whether Tfr differentiation is affected. Additional timepoints in Fig. 4 should also be included. Moreover, suppression assays in EV4e should be performed with sorted Tfr/Tfh/B in context of immunization to confirm whether Tfr cells have altered suppressive function.

3. Data in Fig. 6c-d indicate that a4-1bb still limits GC responses in the absence of Treg. There are no statistical increases between WT aCD137 and DTR aCD137, despite the authors claims. This should be strengthened.

4. OVA specific IgG should be assessed in serum in Figure 1 to substantiate the claim that a4-1BB enhances antigen specific responses.

5. FDC usually do not expand that much after immunization, so results in Fig. 3a are surprising. This is especially a concern since total FDC do not seem to be different by IF in Fig. 3c. Gating of FDC per condition should be shown for Fig. 3a.

6. The plots in Fig. 4a show increase in Tfh, but do not match up with the bar graphs.

7. Engraftment in Fig. 6a should be shown by flow cytometry. It is surprising that sufficient B and Treg can engraft in an intact host with total transfer.

8. Most Tfr references are over 15 years old, and newer references have further clarified Tfr differentiation and programming and should be cited (doi: 10.1038/s41590-025-02373-7).

Minor points

9. EV2c has multiple gates so it is unclear what is carried forward. Likewise, EV2d has CD4 on both axes.

10. Ref #40 shows that some Tfh can upregulate FoxP3 during GC contraction, however it is not clear if these are actually Tfr cells.

11. Line 337. It is suggested that Tfr suppress via PD-1, but PD-1 actually inhibits Tfr suppression (doi: 10.1038/ni.2496)

Referee #3:

The manuscript by Martinez-Riano and colleagues describes the impact of a CD137 (4-1BB) on the Tfh/Tfr cell balance, leading to disrupted humoral responses and GC/TLS formation. The phenotype that is observed with agonist CD137 antibodies is striking in its magnitude. Furthermore, the cellular circuits underlying the observed phenotype could be dissected using robust mouse models. Therefore, the conclusions are strongly supported by the experimental data, and the findings have clear clinical implications. There are some minor issues that could be addressed to improve the manuscript.

1. The magnitude of CD137 expression on Tfr and Treg cells represented in the histograms from Fig 2A is surprisingly low. It raises the question of whether the expression depends on an activation stage of these cells, which would suggest that the agonist antibody was acting primarily on Treg/Tfr cells engaged in the ongoing immune response rather than targeting all Treg/Tfr cells (with implications for biodistribution and off-target effects). The manuscript would benefit from information on the CD137 expression in Tfr/Treg at different stages of activation.

2. The immunosuppressive score, in Fig 6E and the companion text, may require further discussion. The expression of "characteristic" Treg-suppressive markers, most notably CD25 (IL2RA gene), changes significantly as Tfr cells mature (see Kumar Sci Immunol 2021). As such, it may constitute a better approach to treat Treg and Tfr "suppressive" signatures differently.

3. Related to the previous point, in addition to the numerical changes of Treg and Tfr cells, the authors could describe whether there are changes in the maturation of those cells, something that can be observed by flow cytometry (see, regarding Tfr maturation, Ribeiro Sci Adv 2025).

In conclusion, this manuscript provides compelling evidence for the impact of agonist anti-CD137 antibodies on humoral responses, dependent on Treg/Tfr cells. It is a major contribution to the research community with clear clinical implications.

Referee #1:

Review of EMBOJ-2026-123462, "Costimulation of follicular regulatory T cells with CD137 (4-1BB) agonists disrupts B-cell responses"

- general summary and opinion about the principal significance of the study, its questions and findings

In this manuscript the authors attempt to address the mechanistic basis for how anti-CD137 agonists can disrupt humoral immunity. To address this, they employ various models of immunisation (such as OVA) and study the impact of CD137 agonism on the multiple cell types potentially involved. They show that agonistic anti-CD137 antibodies target activated CD8⁺ T cells, Tregs, follicular regulatory T cells (Tfrs), and FDCs and that CD137 agonism impairs GC reactions upon vaccination, impairing antibody production. They also show that anti-CD137 antibodies suppress ectopic B-cell responses in TLS of tumors. They go on to show that these effects are dependent upon Foxp3⁺ cells, with anti-CD137 treatment increasing both the abundance and immunosuppressive activity of Tregs and Tfrs in their vaccination model.

The data are novel, interesting and address a longstanding immunological phenomenon. The results are in general clear, well described and presented with an easily to follow narrative.

Response 1:

Thank you for the overall positive appraisal of our manuscript and the detailed review.

My comments below i hope will serve to improve the paper

One potential limitation that all of the observed effects are demonstrated with a single antibody clone (3H3, a potent and known agonist). Therefore the data could potentially reflect an antibody clone-specific phenomenon. Did the authors evaluate other agonistic CD137 mAb? Importantly the authors already discuss whether the observed effects relate to normal physiology or due to drug-mediated pharmacology.

Response 2:

As suggested by the reviewer, we have evaluated the inhibition of GC and IgG1⁺ class-switched responses using the agonistic anti-CD137 clone 1D8 (Fig. EV1E). In addition, we validated the observed reduction in Tfh differentiation and the increase in Treg and Tfr percentages using the 1D8 monoclonal antibody (Fig. EV3G). These results support and generalize the conclusion that the observed effects are associated with pharmacological CD137 activation rather than being specific to the 3H3 antibody.

Similarly, previously the authors have elegantly shown that another CD137 antibody, 1D8 is to some extent toxic in mice, evoking hyper-inflammatory responses (especially liver inflammation) that are dose limited. To counter this the authors previously designed a masked/prodrug 1D8 molecule, leading to activation in only the right (in that case, tumor) location (Etxeberria et al pnas 2021). **Did the authors attempt to exploit the same molecule in this study (in the TLS**

work). That would certainly increase the translational impact of the study - potentially providing a ready-made solution to any toxicity issues (which have so far limited CD137 agonists such as urelumab).

Response 3:

Unfortunately, we were unable to use the masked/prodrug version of the 1D8 molecule in the present study due to an expired contract with Cytomx and lack of interest from the new managers of the company. Nevertheless, we evaluated the impact of the unmodified 1D8 antibody on TLS development by treating KPAR-bearing mice with a single dose of 1D8 (100 µg) (Fig. EV5A). Under these conditions, we observed a reduction in intrapulmonary B cells and GC B cells, again supporting the notion that CD137 agonism impairs TLS-associated humoral responses with a different anti-CD137 antibody.

I could not readily discern from the **figure legends how many times each experiment had been performed**. In Figure 1, 4 data points presumably indicate 4 mice, but how many times was the experiment performed? Figure 2 shows 3 datapoints. Figure 4 by comparison shows >10 datapoints. Is this from >1 experiment? **This (numbers of experiments as well as mice) should be detailed for all figures to evaluate reproducibility.**

Response 4:

We thank the reviewer for this suggestion. We have now added detailed information regarding the number of independent experiments and biological replicates to all figure legends.

Each dot represents an independent mouse / biological sample.

Of direct relevance, do the authors expect that they saw only limited defective plasmablast generation/ differentiation in anti-CD137-treated mice due to the relatively short time-frame of the study? Or was it simply that they had insufficient numbers of animals to conclude this (n=4)? See comment above
Data in some other figures lack routine statistical significance at $p < 0.05$ due to small numbers.

Response 5:

We analyzed plasmablast generation at later time points following immunization. While at day 10, we only observed a trend toward reduced plasmablast generation, by day 14 we detected a statistically significant decrease between groups (Fig. 1D, EV1 D). Furthermore, reduced plasma cells frequencies were also observed at day 21 in the bone marrow (Fig. 7D). We have now included the day 14 data in the revised manuscript to strengthen this conclusion with statistical significance.

- specific major concerns essential to be addressed to support the conclusions

Perhaps the **plots have been mixed in Figure 4A** - the text reads "Fourteen days post-immunization, anti-CD137-treated mice displayed Tfh frequencies comparable to those of non-immunized controls and approximately two-fold decrease as compared with isotype-treated mice (Fig. 4A, Fig. EV3B)" and yet the %'s indicated on the representative plots show that the TFH increase with anti-CD137.

Response 6:

We apologize for this error and we are immensely grateful for you having spotted it. The representative plots have now been changed accordingly.

Did the authors evaluate the efficacy of the depleting/blocking antibodies used in Figure 5? i.e. assess controls as evidence that the CD8, IFNG and IL-12 mAbs had their desired effects? Particularly after immunisation this might be important.

Response 7:

Although all antibodies used had been commercially purchased, we performed additional in vivo validation experiments as suggested by the reviewer (Fig. EV3J, K). To confirm CD8 depletion efficiency, lymph nodes were analyzed 24 h after treatment (Fig. EV3J). To validate IFN γ and IL-12 neutralization, mice were injected intraperitoneally with recombinant p70 IL-12 (10 μ g), which increase MHC-I expression on dendritic cells in a manner at least partially dependent on IFN γ . MHC-I expression levels on CD11c+ dendritic cells were then used as a functional readout for both stimulation and neutralization efficiency (Fig. EV3K).

- minor concerns that should be addressed

In Figure 2A was statistical analysis performed? This would complement the visual presentation

Response 8:

We have performed a one-way ANOVA test simply to state that there is variability in the level of CD137 expression on the indicated cell types.

To appreciate better the data displayed in figure 2C, some raw flow cytometry plots comparing ex-vivo staining (day 0) versus day 1 might be helpful.

Response 9:

The raw flow cytometry plots are presented in the figure EV2 F comparing the isotype negative control (grey) and the α -CD137 antibody (blue) at 24h.

Some **minor typos** - e.g. line 402 "To explore further the possible effects on Tregs induced by the anti-CD137 agonism, Foxp3-GFP hCD2-RFP were" (missing the word mice or cells)

Response 10:

We have already corrected it.

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

Possibly the white diagonal line for 3C is missing on the isotype magnification? Also the white box to show which area is being expanded

Response 11:

We have corrected it

The IgD legend in figure 3C might be better placed to improve visualisation

Response 12:

We have corrected it

Referee #2:

In this manuscript the authors assess how agonistic a4-1BB alters humoral immunity. The authors show that a4-1BB limits GC B cells, while simultaneously expanding Treg and Tfr cells, an effect that is not dependent on CD8s, IFN γ or IL-12. Furthermore, RNAseq and functional experiments demonstrate that Treg from a4-1BB may be more activated with higher suppressive capacity. Lastly, the authors show that the decrease in B cell responses with a4-1BB can also occur in tumor derived TLS and antibody drug conjugates. Overall, this is a fairly compelling study on how a4-1BB controls humoral immunity. However, some claims are not substantiated with data presented and some data could be strengthened to enhance significance of the work.

Response 13:

We are very grateful for the encouraging comments and the detailed review of our work.

Major points

1. This entire study was performed with the 3H3 a4-1BB clone. Key results (decrease in GC B, etc.) should be **confirmed with a second a4-1BB agonistic antibody** clone for confirmation.

Response 14:

As suggested, we repeated key experiments using the agonistic anti-CD137 clone 1D8 (see response to reviewer 1). Specifically, we analyzed GC and IgG1⁺ class-switched responses (Fig. EV1E), as well as Tfh, Treg, and Tfr differentiation (Fig. EV3G). In addition, we confirmed the effects of CD137 agonism on TLS biology using the 1D8 clone (Fig. EV5A). Together, these data demonstrate that the observed effects are not restricted to the 3H3 clone.

2. A central claim (see title) is that **a4-1BB affects Tfr cells both by expansion and suppressive function, but neither of these are shown directly**. Fig. 4c should show Tfr % of total Tregs, not of Tfh. This will help determine whether a4-1BB mostly affects Treg expansion and increased Tfr are due to increased precursors to Tfr or whether Tfr differentiation is affected.

Additional timepoints in Fig. 4 should also be included

Moreover, **suppression assays in EV4e should be performed with sorted Tfr/Tfh/B in context of immunization** to confirm whether Tfr cells have altered suppressive function.

Response 15:

We have now reanalyzed the data as suggested and included Tfr frequencies within the total Treg compartment (Fig. 4E). These analyses indicate that the increase in Tfr cells is primarily driven by expansion of the Treg precursor pool rather than preferential differentiation into Tfr cells. This is now presented in results and discussed in the discussion section. We are grateful to the reviewer for having realized this matter.

In addition, we evaluated in vivo proliferation using EdU incorporation and found that anti-CD137 treatment increased proliferation of both Tregs and Tfrs (Fig. 6E), with a comparable ~3-fold increase in both populations.

We also included additional time points (days 4 and 7 post-immunization) analyzing Tfh, Tfr, and Treg frequencies, as well as Tfh/Tfr ratios (Fig. EV3E-F) in addition to day 14 on Figure 4.

Regarding functional suppression assays using sorted Tfr/Tfh/B-cell populations, we attempted multiple experimental attempts; however, the number of viable cells recovered after sorting was insufficient to establish reproducible in vitro assays. We have now acknowledged this technical limitation in the manuscript.

3. Data in Fig. 6c-d indicate that a4-1bb still limits GC responses in the absence of Treg. **There are no statistical increases between WT aCD137 and DTR aCD137**, despite the authors claims. This should be strengthened.

Response 16:

We appreciate this comment. As noted by the reviewer, the initial dataset lacked statistical significance due to variability among animals. We have now added an additional cohort of mice, increased the sample size and thereby reduced variability, which allowed us to reach statistical significance (Figure 6C).

4. OVA specific IgG should be assessed in serum in Figure 1 to substantiate the claim that a4-1BB enhances antigen specific responses.

Response 17:

We performed ELISA analyses as suggested by the reviewer (Fig. 1F). These experiments revealed a significant reduction in OVA-specific IgG1 titers 14 days after immunization in anti-CD137-treated mice, in agreement with reduced B-cell responses.

5. FDC usually do not expand that much after immunization, so results in Fig. 3a are surprising. This is especially a concern **since total FDC do not seem to be different by IF in Fig. 3c. Gating of FDC per condition should be shown for Fig. 3a.**

Response 18:

We have now included the gating strategies used for these analyses (Fig. EV3C). Importantly, previous studies have shown that immunization can induce FDC expansion at magnitudes comparable to those observed here (PMID: 37443283). In addition, we analyzed non-immunized mice treated with isotype or anti-CD137 agonistic antibody and observed no differences in FDC frequencies five days later (Figure 3A). These findings support the idea that the effects of anti-CD137 on FDCs are indirect and driven by the altered immune response rather than direct CD137 targeting of FDCs.

6. The **plots in Fig. 4a** show increase in Tfh, but do not match up with the bar graphs.

Response 19:

Thank you very much for the correction, we apologize for the copy-paste mistake.

7. **Engraftment in Fig. 6a should be shown by flow cytometry.** It is surprising that sufficient B and Treg can engraft in an intact host with total transfer.

Response 20:

We have now included flow cytometric analyses of donor-cell engraftment (CD45.2⁺ cells) in recipient mice (Fig. EV4B). As correctly indicated by the reviewer, engraftment levels were relatively low. Consequently, large numbers of events had to be acquired to obtain reproducible analyses of GC B cells and Treg populations. Unfortunately, Tfr cell numbers remained too low for reliable quantification and those data cannot be included.

8. **Most Tfr references are over 15 years old,** and newer references have further clarified Tfr differentiation and programming and should be cited (doi: 10.1038/s41590-025-02373-7).

Response 21:

We have updated the references as suggested by the reviewer.

Minor points
9. **EV2c has multiple gates so it is unclear what is carried forward.** Likewise, **EV2d has CD4 on both axes.**

Response 22:

Thank you very much for this point, we have clarified the explanations and corrected the gating strategy display.

10. Ref #40 shows that some Tfh can upregulate FoxP3 during GC contraction, however it is not clear if these are actually Tfr cells.

Response 23:

We agree with this limitation of the aluded reference since the immunosuppressive ability of those newly generated Foxp3⁺ Bcl6⁺ cells is not shown. However, their presence correlates with the GC shutdown period and such lymphocytes express the two master regulators that define TFRs. We briefly acknowledge such limitation in discussion.

11. Line 337. It is suggested that Tfr suppress via PD-1, but PD-1 actually inhibits Tfr suppression (doi: 10.1038/ni.2496)

Response 24:

We have modified the text according to the published data and provided the reference.

Referee #3:

The manuscript by Martinez-Riano and colleagues describes the impact of a CD137 (4-1BB) on the Tfh/Tfr cell balance, leading to disrupted humoral responses and GC/TLS formation. The phenotype that is observed with agonist CD137 antibodies is striking in its magnitude. Furthermore, the cellular circuits underlying the observed phenotype could be dissected using robust mouse models. Therefore, the conclusions are strongly supported by the experimental data, and the findings have clear clinical implications. There are some minor issues that could be addressed to improve the manuscript.

1. **The magnitude of CD137 expression on Tfr and Treg cells represented in the histograms from Fig 2A is surprisingly low.** It raises the question of whether the expression depends on an activation stage of these cells, which would suggest that the agonist antibody was acting primarily on Treg/Tfr cells engaged in the ongoing immune response rather than targeting all Treg/Tfr cells (with implications for biodistribution and off-target effects). **The manuscript would benefit from information on the CD137 expression in Tfr/Treg at different stages of activation.**

Response 25:

We appreciate this observation. We have now included an additional independent experiment in Figure 2A and expanded the analysis to days 4 and 7 post-immunization (Fig. EV2E). To facilitate comparisons across populations, CD137 expression is now represented as fold-change over FMO controls. What cannot be addressed is if the level of CD137 expression beyond a detectable threshold has differential functional effects. This is a topic that we are addressing with regard to CD137-based cancer immunotherapy and it is a complex issue.

2. The **immunosuppressive score, in Fig 6E and the companion text, may require further discussion.** The expression of "characteristic" Treg-suppressive markers, most notably CD25 (IL2RA gene), changes significantly as Tfr cells mature (see Kumar Sci Immunol 2021). As such, **it may constitute a better approach to treat Treg and Tfr "suppressive" signatures differently.**

Response 26:

We have expanded the discussion regarding the immunosuppressive score and added the references used to generate the gene signature. We agree with the reviewer that suppressive programs in Tregs and Tfrs may not fully overlap and therefore we now discuss this limitation more explicitly.

3. Related to the previous point, in addition to the numerical changes of Treg and Tfr cells, the **authors could describe whether there are changes in the maturation of those cells**, something that can be observed by flow cytometry (see, regarding Tfr maturation, Ribeiro Sci Adv 2025).

Response 27:

As suggested, we further characterized Treg and Tfr maturation markers (Fig. EV3H-I). We observed that Tfrs from anti-CD137-treated mice displayed lower CD25 surface expression, consistent with a GC-localized phenotype. However, we did not observe differences in double-positive ICOS+PD1+ cells. These discrepancies may reflect species-specific differences or indicate that CD137 agonism does not substantially alter Tfr maturation. In contrast, we observed an increase in highly suppressive PD-1⁺CD25⁺ Treg populations.

In conclusion, this manuscript provides compelling evidence for the impact of agonist anti-CD137 antibodies on humoral responses, dependent on Treg/Tfr cells. It is a major contribution to the research community with clear clinical implications.

Response 28:

We are very grateful for the positive appraisal of our work.

Dear Dr. Melero,

Thank you for the submission of your revised manuscript (EMBOJ-2026-123462R) to The EMBO Journal for our consideration, and for your patience during re-review. Your manuscript and your point-by-point responses to the previous comments of the referees have now been seen by them, and we have received their new reports, which you can find appended below.

I am pleased to say that, as you will see, the referees find the manuscript strengthened and substantially improved, and all previously raised concerns adequately addressed. They now all recommend publication of the manuscript in The EMBO Journal. In light of this expert input, I am pleased to let you know that your manuscript has been accepted in principle for publication in our journal.

Before we can move forward with the next steps towards publication of your article, there are a few changes and corrections from the editorial side we need you to make in a final version of the manuscript:

- Thank you for depositing your RNA-sequencing data to the Gene Expression Omnibus (GEO) database. Please make sure that these data will be publicly available at the time of publication. The reviewer token can now be removed from the Data Availability statement of the manuscript.

- Please also provide in the Data Availability statement an ID and URL to the Source Data deposited in BioStudies. If this is no longer relevant -as we note that you have already uploaded Source Data for your Figures to our online system- please remove the sentence referring to BioStudies from this section.

- Please note that the References format needs to be updated to the style used by The EMBO Journal. In particular, the list of references should be alphabetical (not numerical), and "et al." must be used after the names of the first 10 co-authors of each citation. Please check our author guidelines for more information on our format:
<https://link.springer.com/journal/44318/submission-guidelines#cms-Reference-guidelines>.

- All funding information provided in the Acknowledgements section of the manuscript must also be entered in our online system; currently, the following items are missing from the system:
"the Spanish Research Agency (MICIU/AEI) grant (ID: PID2024-156660OA-100) and the Ramón y Cajal grant (RYC2023-042880-I)".

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Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor, The EMBO Journal
i.papaioannou@embojournal.org

Referee #1:

The authors have addressed my earlier concerns, strengthening the data and conclusions.

Referee #2:

The authors have addressed my concerns.

Referee #3:

The revised version of the manuscript addressed all the issues raised in the initial report. The manuscript is substantially improved and will be of great value to the community.

All minor editorial requests have been addressed by the authors.

In conclusion, this manuscript provides compelling evidence for the impact of agonist anti-CD137 antibodies on humoral responses, dependent on Treg/Tfr cells. It is a major contribution to the research community with clear clinical implications.

Response 28:

We are very grateful for the positive appraisal of our work.

Dear Ignacio,

Congratulations on an excellent manuscript! I am very pleased to inform you that it has been accepted for publication in The EMBO Journal. Thank you for comprehensively addressing the initially raised referees' concerns and our editorial requests for corrections and changes.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

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If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to The EMBO Journal. Working with you has been a pleasure.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Senior Editor, The EMBO Journal - EMBO Reports - Molecular Systems Biology
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](https://doi.org/10.31222/osf.io/9sm4x)). Please follow the journal's guidelines in preparing your

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The data shown in figures should satisfy the following conditions:

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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if $n < 5$, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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