

Convergence, plasticity, and tissue residence of regulatory T cell response via TCR repertoire prism

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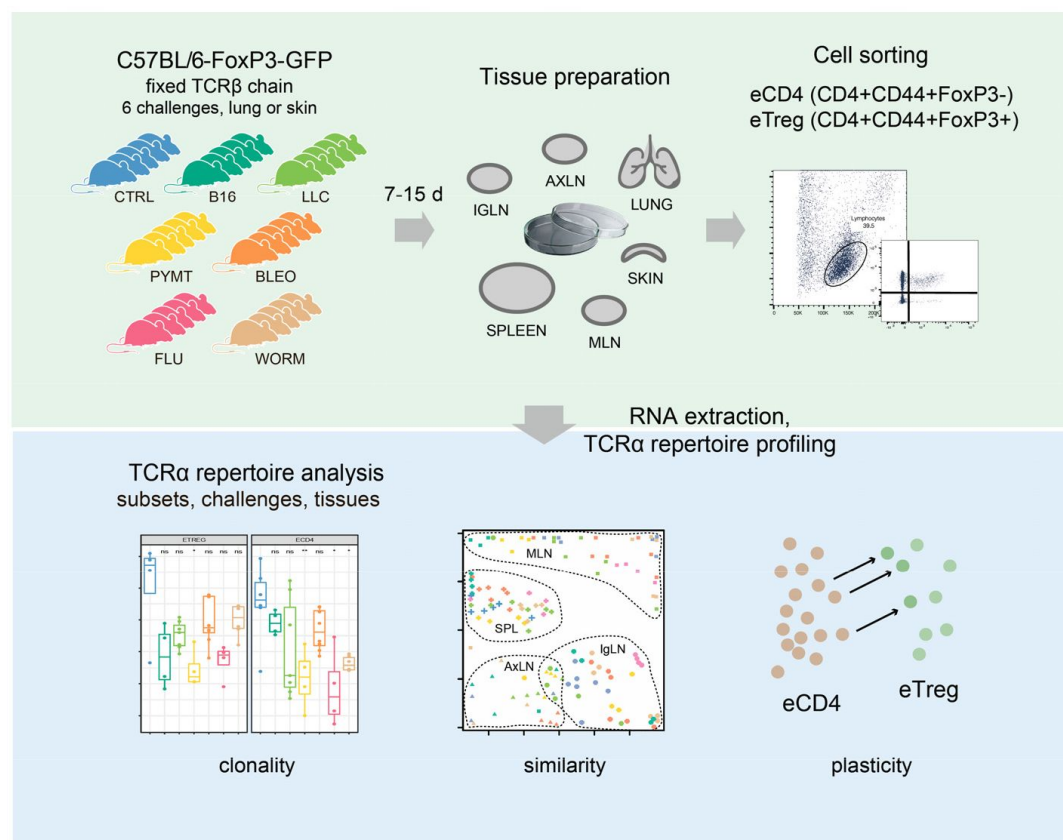
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Abstract

Summary

Suppressive function of regulatory T cells (Treg) is dependent on signaling of their antigen receptors triggered by cognate self, dietary or microbial antigens in the form of peptide-MHC class II complexes. However, it remains largely unknown whether distinct or shared repertoires of Treg TCRs are mobilized in response to different challenges in the same tissue or the same challenge in different tissues. Here we used a fixed TCR β chain FoxP3-GFP mouse model to analyze conventional (eCD4) and regulatory (eTreg) effector TCR α repertoires in response to six distinct antigenic challenges to the lung and skin. This model showed highly “digital” repertoire behavior, allowing for easy-to-track challenge-specific TCR α CDR3 clusters. For both studied subsets, we observed challenge-specific clonal expansion yielding homologous TCR α clusters within and across animals and exposure sites, which were also reflected in the draining lymph nodes but not systemically. Some clusters were shared across cancer challenges, suggesting response to common tumor-associated antigens. For most challenges, eCD4 and eTreg clonal response did not overlap, indicating the distinct origin of the two cell subsets. At the same time, we observed such overlap at the sites of certain tumor challenges. The overlaps included dominant responding TCR α motif and characteristic iNKT TCR α , suggesting the tumor-induced eCD4-eTreg plasticity. Additionally, our TCR α repertoire analysis demonstrated that distinct antigenic specificities are characteristic for eTreg cells residing in particular lymphatic tissues, regardless of the challenge, revealing the homing-specific, antigen-specific resident Treg populations. Altogether, our study highlights both challenge-specific and tissue-specific responses of Treg cells associated with distinct clonal expansions.

Graphical abstract



eLife assessment

This manuscript presents a **valuable** approach to exploring CD4⁺ T-cell response in mice across stimuli and tissues through the analysis of their T-cell receptor repertoires. The authors use a transgenic mouse model with reduced diversity of the T-cell receptor repertoire to elicit more consistent T-cell responses across individuals, demonstrating challenge-specific and tissue-specific responses of regulatory T-cells. The evidence for the authors' conclusions is **solid**, and the work will be of interest to immunologists studying T cell responses.

Introduction

Regulatory T cells (Treg), which constitute 5-10% of peripheral CD4⁺ T cells are indispensable for the maintenance of immunological self-tolerance^{1,2} and regulation of ongoing immune responses to microbiota³⁻⁵ and foreign⁶⁻⁹ antigens, contributing to tissue and metabolic homeostasis and regeneration². Treg suppress immune responses through various mechanisms, but primarily do so via antigen-specific¹⁰ interaction with professional and non-professional antigen-presenting cells¹¹. There is considerable data suggesting that Treg employ higher-affinity T cell receptor (TCRs) and CTLA4 to compete with conventional CD4⁺ T cells in an antigen-specific manner¹².

Most Treg cells are produced by the thymus as a functionally distinct FoxP3⁺ T cell subset. Thymic Treg are generated based on positive selection against self-peptide/MHCII (pMHCII) complexes in the thymic medulla, and multiple other immune cell types may further contribute to this process, including medullary thymic epithelial cells (mTECs), resident and immigrating dendritic cells, macrophages, and B cells^{2,13-15}.

Here we investigated the TCR landscape of conventional (eCD4) and regulatory (eTreg) effector CD4⁺ T cells in lungs, spleen, skin, and three lymph node locations after response to six distinct antigenic challenges to the lungs or skin (see **Graphical abstract**). We used FoxP3-GFP mice with a fixed TCRβ context¹⁶ and analyzed the TCRα repertoire composition for eCD4 and eTreg cells.

The adaptive immune system in this mouse model relies entirely on TCRα diversity to generate antigen-specific responses. On one hand, the relatively limited TCRα repertoire¹⁶ results in highly convergent responses that are easily traceable across groups of mice facing the same challenge. The selection of the same or highly similar TCRα sequences against each antigen leads to the formation of clusters of homologous TCRα CDR3s within and between mice. On the other hand, this model allows for the tracking of all challenge-specific or tissue-specific features of the TCR repertoire using only one chain¹⁶. Overall, compared to conventional mice, this model provides a more powerful means of monitoring convergent TCR responses. At the same time, the model generally mimics the natural behavior of the full-fledged TCRαβ T cell repertoire.

Both for eCD4 and eTreg subpopulations, our analysis of the TCRα repertoire on the fixed TCRβ background revealed a highly focused response which was distinct for each of the antigenic challenges. The response was clonotypically shared between individual mice and between different tissues and formed clear convergent¹⁷ TCRα CDR3 sequence motifs responding to the challenges.

In conditions with such a highly convergent TCR response involving multiple independently-primed T cell clones with similar TCRα CDR3 sequences, one could expect a similar repertoire of clonotypes to dominate within the eCD4 and eTreg subpopulations, given the assumption that both subsets may start from the same pool of antigen-inexperienced naïve T cells. However, we demonstrate that the TCR landscape of eCD4 and eTreg responses was distinct, suggesting that these effector cell types originate from distinct T cell subsets.

The exceptions were the antigenic challenges with Lewis lung carcinoma (LLC) and MMTV-PyMT-derived tumor cells (PYMT), where we observed increased overlap between the eCD4 and eTreg repertoires at the site of challenge, but not systemically. A dominant eCD4 TCRα motif was also present in corresponding eTreg subsets in the LLC and PYMT challenge.

Similarly, the characteristic innate natural killer T cell (iNKT) TCRα variants (that were abundant within eCD4 subsets upon challenge with LLC, bleomycin, and the helminth *Nippostrongylus brasiliensis*) were represented in the corresponding eTreg subset in the LLC challenge experiments but not with the bleomycin and helminth antigens. We attribute these observations to the transient plasticity of effector CD4⁺ T cells in the context of an immunosuppressive tumor burden.

Furthermore, we reveal tissue-specific eTreg TCRα CDR3 motifs that were always present in specific tissue locations irrespectively of the applied antigenic challenge. This observation highlights the existence of the diverse homing-specific, antigen-specific resident Treg populations.

Altogether, our results show the highly antigen-specific and distinct eCD4 and eTreg responses to different challenges, supporting the initial thymic programming of Treg cells responding to newly incoming challenges, and suggest that the fixed TCRβ chain, FoxP3-GFP mouse offers a valuable “digital” model of TCR response that benefits from highly convergent, easy-to-track TCRα CDR3 motifs.

Results

TCR α repertoire sequencing

Six distinct challenges including influenza virus, *N. brasiliensis*, bleomycin-induced injury, LLC, PYMT, and B16 melanoma were applied to the lung and skin of *Foxp3^{gfp} Tcr α ^{-/-}* mice bearing the DO11.10 TCR β transgene¹⁸ (3–7 mice per group, see **Supplementary Table 1** for details on each tissue and challenge experiment). After incubation, mice were sacrificed and tissue from the lungs, spleen, skin, and three types of lymph nodes (lung-draining mediastinal, MLN, axillary, AXLN, and intraglandular, IGLN) were isolated and digested to generate single-cell suspensions. eCD4 and eTreg CD4⁺ cells were sorted based on FoxP3-GFP signal. RNA-based unique molecular identifier (UMI)-labeled TCR α cDNA libraries were obtained using a previously-reported technique^{19,20} with minor modifications, and then analyzed using MIGEC²¹ and MiXCR²² software. See **Graphical abstract** for the experimental scheme. Altogether, we sequenced 524 TCR α cDNA libraries, yielding $13,605 \pm 14,948$ UMI-labeled TCR α cDNA molecules and $3,392 \pm 3,076$ TCR α CDR3 clonotypes per eTreg sample, and $37,412 \pm 33,129$ UMI-labeled TCR α cDNA molecules and $5,518 \pm 4,601$ TCR α CDR3 clonotypes per eCD4 sample (see **Supplementary Table 2** for details on each cloneset).

Lungs are exposed to a myriad of different insults during their lifetime and are constantly in need of a T cell response to resolve inflammatory insults. Furthermore, Treg with an effector phenotype accumulate in the inflamed lung²³ but it remains poorly understood whether there is a common antigenic denominator driving TCR specificities in the tissue or whether there are selective TCR subsets expanded upon a particular inflammatory insult. Therefore, in the following analyses, we mainly focused on TCR repertoires obtained from T cells infiltrating the lung tissue, for which we have also obtained the largest collection of samples, with several more specific cross-tissue analyses showing the similarity of systemic T cell responses.

Clonality of eCD4 and eTreg repertoires in the lung

First, we explored how clonal the eCD4 and eTreg response is in lungs following distinct antigenic challenges. TCR α repertoire diversity was assessed using several widely used metrics, including observed diversity (number of distinct clonotypes), normalized Shannon Wiener index (repertoire evenness and the extent of clonal expansion) and Chao 1 (estimated lower bound of total diversity based on relative representation of small clonotypes). For normalization, all of these metrics were obtained from datasets that had been down-sampled to 1,000 randomly-chosen, UMI-labeled TCR α cDNAs¹⁹. We observed a prominent clonal response to each of the challenges compared to the control group (**Fig. 1a–c**). The decrease in diversity metrics was comparable for the lung-infiltrating eCD4 and eTreg cells. This indicates that the amplitude and focused nature of the effector and regulatory T cell response in lungs is generally comparable.

Responding eCD4 and eTreg repertoires are distinct

Next, we analyzed all-versus-all pairwise overlap of the amino acid TCR α CDR3 repertoires of lung-infiltrating eTreg and eCD4 cells. We used F2 similarity metrics in the VDJtools software²⁴, which employs a clonotype-wise sum of geometric mean frequencies that takes into account the relative size of shared clonotypes. As such, F2 metrics generally enable comparison of pairs of TCR repertoires with regard to the relative share occupied by common clonotypes. This analysis showed that the eTreg and eCD4 repertoires are highly distinct across distinct challenges (**Fig. 1d,e**).

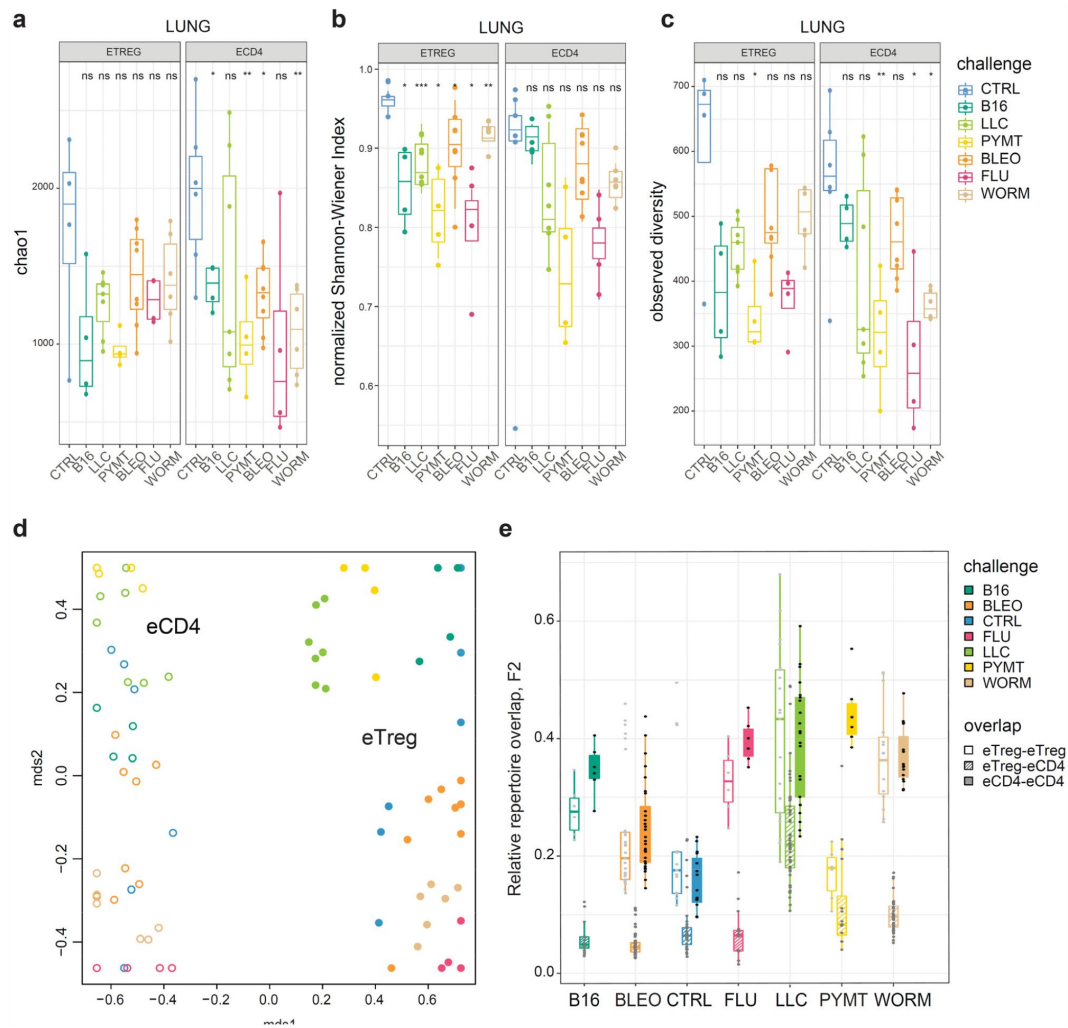


Figure 1.

eTreg and eCD4 subsets.

a-c. Clonality and diversity of lung eTreg and eCD4 subsets in response to distinct antigenic challenges. We calculated the (a) Chao1 estimator, (b) normalized Shannon-Wiener index, and (c) observed diversity for each TCRα repertoire obtained from each CD4⁺ T-cell subset from each animal ($3 < n < 7$) with different antigenic challenges. P-values shown as: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, based on parametric t-test for each group versus control. **d.** Relative overlap between amino acid-defined lung TCRα CDR3 repertoires, visualized as a VDJtools MDS plot. Euclidean distance between points reflects the distance between repertoires. Data were normalized to the top 1,000 most frequent clonotypes and weighted by clonotype frequency (F2 metric in VDJtools). Clonotypes were matched on the basis of identical TRAV gene segments and identical TCRα CDR3 sequences. The closer two circles are, the higher the overall frequency of shared clonotypes. Node colors correspond to the challenge antigen. Filled circles are lung eTreg, open circles are lung eCD4 cells. **e.** The same F2 metrics as in (d), shown separately for each challenge and for eTreg-eTreg, eTreg-eCD4, and eCD4-eCD4 repertoire overlap. For both (d) and (e), mice were analyzed in an all-versus-all fashion, irrespective of whether eTreg and eCD4 subsets were obtained from the same or distinct mice.

eTreg response is convergent and differs for distinct challenges

We next zoomed in on the eTreg repertoire in order to investigate how focused their antigen-specific response is in the lungs. Repertoire overlap analysis showed that eTreg repertoires were highly similar across mice for each of the challenges, and differed between challenges (Fig. 2a, b). Cluster sequence analysis further revealed common dominant TCRα CDR3 motifs in response to each of the challenges (Fig. 2a, Supplementary Figs. 1, 2, Supplementary Tables 4, 5). It should be noted that the eTreg repertoires in the three different cancer challenges (B16, LLC, and PYMT) clustered together and shared some CDR3 motifs, suggesting response to shared tumor-associated antigens. In LLC and PYMT, but not in other challenges, some of the characteristic CDR3 motifs were shared with the eCD4 subset.

eTreg repertoire upon lung challenge is reflected in the draining lymph node

It has previously been shown in a mouse model that the antigen-specific Treg response to influenza in the lungs is also reflected in the lung-draining mediastinal lymph node (MLN)²⁵. Our data revealed this at the repertoire level, showing that the same TCR clonotypes and CDR3 motifs distinguish antigen-specific eTreg response to each of the different lung challenges in the lung and MLN (Fig. 2b, Supplementary Figs. 1, 2, Supplementary Tables 4, 5). It should be noted that each challenge produces its own specific response, wherein eTreg TCRα CDR3 repertoires from the lung and MLN are located side by side. At the same time, the lung and MLN eTreg repertoires of control mice obtained in the absence of any antigenic challenges were distinct, confirming that repertoire similarity is dictated by corresponding antigenic challenges (Fig. 2b). In contrast, the eTreg repertoires in distant AXLN and IGLN lymph nodes and the spleen were less similar to that observed in the lungs, and clustered separately (Fig. 2c–f). Altogether, these results demonstrate the selective tissue localization of the antigen-focused Treg response.

Repertoire focus is the same for the lung and skin tumor localization

The eTreg TCR repertoire in lungs (upon lung tumor challenge) and in skin (upon corresponding tumor challenge in skin) was also highly similar (Fig. 3), demonstrating that the antigen-specific character of the Treg response dominates over the tissue location of the challenge. TCR repertoires from eCD4 T cells in the lungs and MLN in response to distinct lung challenges, as well as repertoires of skin eCD4 T cells in response to various skin challenges also clustered together (Fig. 3), with a magnitude of repertoire convergence that was at the same level as for eTreg cells (Fig. 1e).

eCD4 to eTreg conversion is only observed in two cancer challenges

As can be seen in Figure 1e, TCRα CDR3 eTreg and eCD4 amino acid repertoires in the lungs were more similar in the context of the LLC tumor challenge compared to other challenges, and this could reflect induced plasticity of the eCD4 subset. In order to assess possible eCD4-to-eTreg clonal conversion due to natural helper T cell plasticity, we analyzed eTreg versus eCD4 repertoire overlap at the level of CDR3a nucleotide clonotypes for paired eCD4-eTreg samples obtained from the same mice. Estimated eCD4-to-eTreg conversion was strongest in skin and in lung following LLC and PYMT challenge, but was low or absent in the aftermath of other antigenic challenges, including B16 tumor (Fig. 4a, c). The effect was probably local and transient, and was not observed at systemic level in the spleen (Fig. 4b, d). CDR3a cluster #408 was the major contributor to the observed conversion (Fig. 4e).

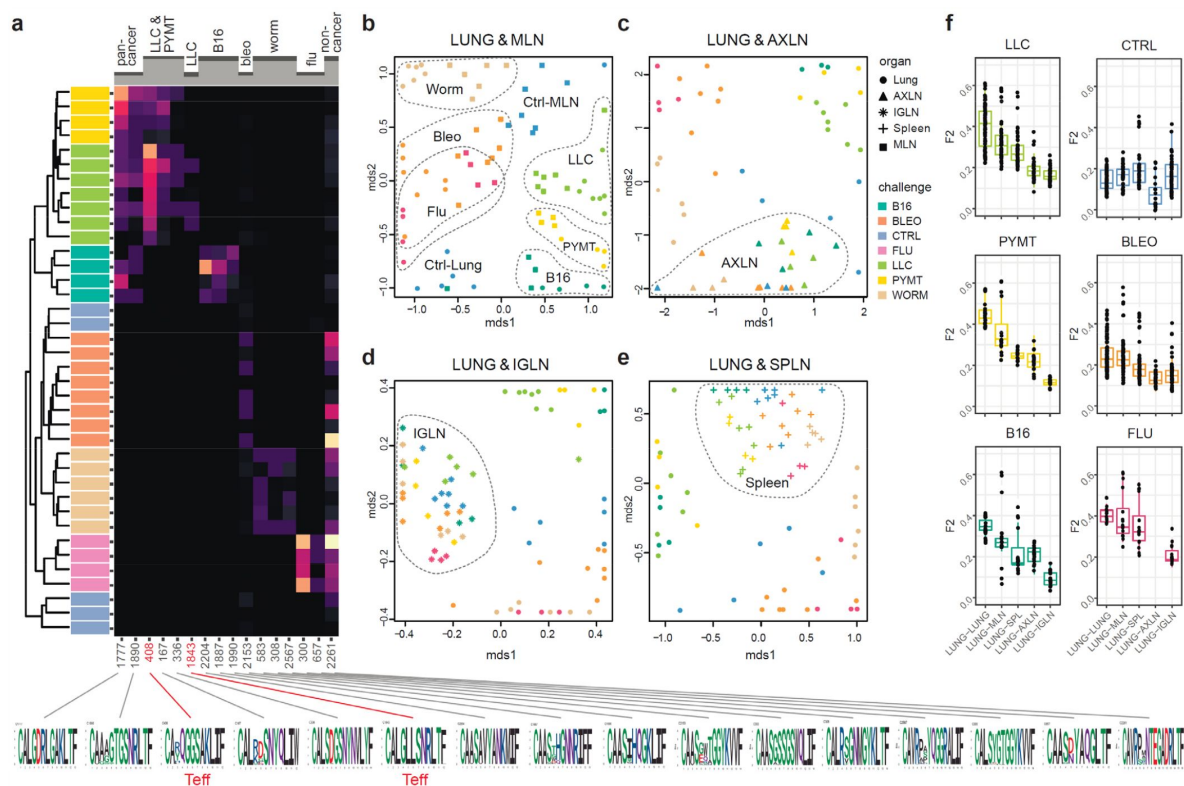


Figure 2.

eTreg TCR repertoire in various tissues following antigen challenge in the lung.

Relative overlap between amino acid-defined eTreg TCRα CDR3 repertoires visualized using VDJtools. Data were normalized to the top 1,000 most frequent clonotypes and weighted by clonotype frequency (F2 metric). Clonotypes were matched on the basis of identical TRAV gene segments and identical TCRα CDR3 sequences. **(a)** Dendrogram of Lung eTreg TCRα CDR3 repertoires, where branch length shows the distance between repertoires. Heat map at right shows the distribution of selected TCRα CDR3 clusters associated with specific challenges. Corresponding TCRα CDR3 logos are shown at bottom; clusters also observed in corresponding eCD4 samples are indicated in red. **(b–e)** MDS plots comparing repertoires between pairs of tissues, where the Euclidean distance between points reflects the distance between repertoires. Overlap is shown for **(b)** lung versus MLN, **(c)** lung versus AXLN, **(d)** lung versus IGLN, and **(e)** lung versus spleen. The closer two samples are, the higher the overall frequency of shared clonotypes. Node colors correspond to different challenges. Mice were analyzed in an all-versus-all fashion, irrespective of whether tissues were obtained from the same or distinct mice. Circles: lung. Squares: MLN. Triangles: AXLN. Snowflakes: IGLN. Crosses: spleen. **(f)** Graphs show eTreg F2 repertoire overlap between lung tissue from different animals, and between lungs and other tissues of all animals.

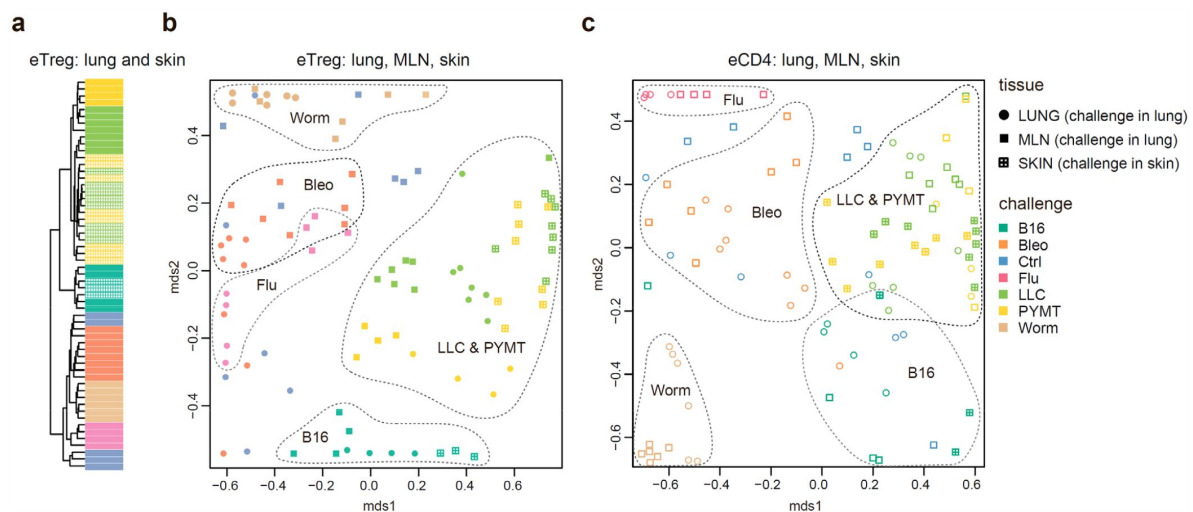


Figure 3.

Convergence of eTreg and eCD4 TCR response in distinct tissues.

(a,b) Relative overlap between amino acid-defined lung and MLN (upon lung challenge) and skin (upon skin challenge) eTreg TCRα CDR3 repertoires as visualized using VDJtools. Data were normalized to the top 1,000 most frequent clonotypes and weighted by clonotype frequency (F2 metric in VDJtools). Clonotypes were matched on the basis of identical TRAV gene segments and identical TCRα CDR3 sequences. (a) Dendrogram branch length shows the distance between repertoires. (b) Euclidean distance between points on the MDS plot reflects the distance between repertoires. The closer two samples are, the higher the overall frequency of shared clonotypes. Mice were analyzed in an all-versus-all fashion, irrespective of whether tissues were obtained from the same or distinct mice. Node colors correspond to different challenges. Circles: lung. Crossed squares: skin. (c) Relative overlap between amino acid-defined lung and MLN (upon lung challenge) and skin (upon skin challenge) eCD4 TCRα CDR3 repertoires.

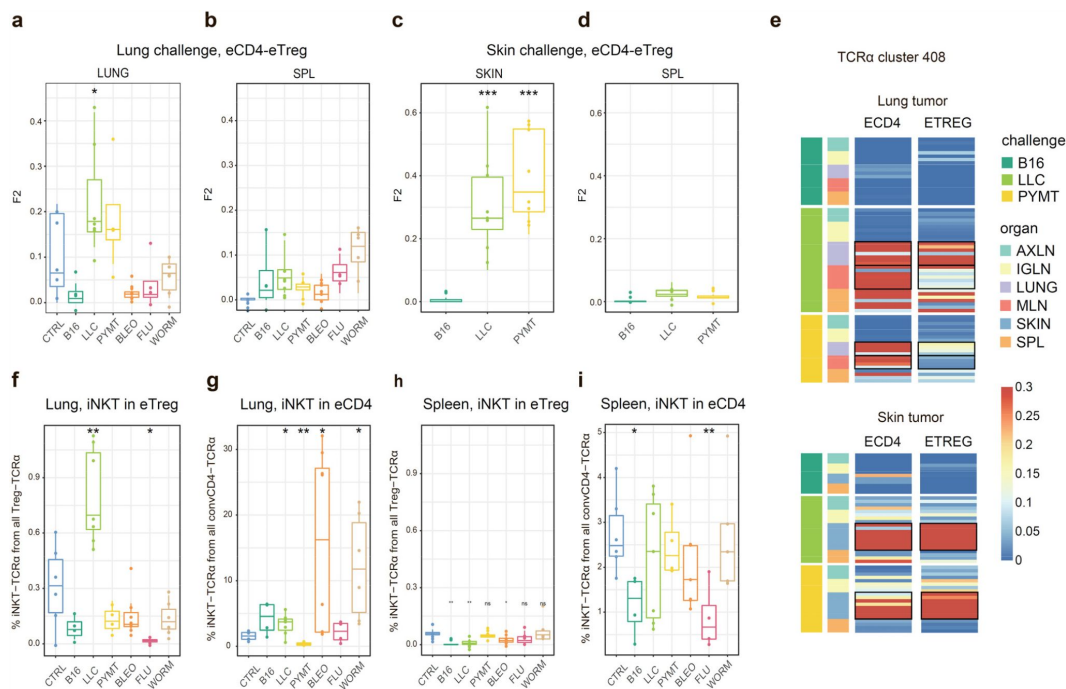


Figure 4.

Estimated eCD4 to eTreg conversion.

(a-d) Relative overlap between eTreg and eCD4 nucleotide-defined TCRα CDR3 repertoires upon lung (a, b) and skin (c, d) challenges. Overlaps are shown for the same organ (a, c) and spleen (c, d). Data were normalized to the 100 most frequent clonotypes and overlap was weighted by clonotype frequency (F2 metric). Clonotypes were matched on the basis of identical TRAV and TRAJ gene segments and identical TCRα CDR3 nucleotide sequences. Overlap was analyzed separately for each challenge for eTreg and eCD4 subsets obtained from the same mice. The closer two samples are, the higher the F2 metric, which reflects the overall frequency of shared clonotypes. e. Distribution of TCR cluster 408 in eTreg and eCD4 repertoires upon tumor challenge in lung and skin. (f) Treg-iNKT proportion of eTreg cells in lung upon lung challenge. (g) iNKT proportion of eCD4 cells in the lung upon lung challenge. (h) Treg-iNKT proportion of eTreg cells in spleen upon lung challenge. (i) iNKT proportion of eCD4 cells in the spleen upon lung challenge. P-values shown as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ based on parametric t-test for each group versus control.

Foxp3⁺ Treg-iNKT cells do not naturally arise during development in the thymus, but can be induced and acquire suppressive functions in the periphery under particular pathophysiological conditions^{26,27}. We specifically observed an increased proportion of Treg-iNKT cells (defined as classic TRAV11-CVVGDRGSALGRLHF-TRAJ18 TCRα) among all eTreg cells upon LLC challenge in lungs (**Fig. 4f**). This was not due to increased presence of eCD4 iNKT cells (**Fig. 4g**), suggesting induced iNKT conversion upon LLC lung challenge. The conversion, which was similar to the general conversion shown in **Figure 4a–d**, was local and was not observed systemically (*e.g.*, in the spleen; **Fig. 4h**).

Local lymphatic tissue-resident eTreg and eCD4 cells

Global TCRα CDR3 cluster analysis revealed that characteristic eTreg TCR motifs were present in distinct lymphatic tissues, including spleen and thymus, irrespective of the applied challenge (**Supplementary Figs. 1,2**, **Supplementary Tables 4,5**). To better illustrate this phenomenon, we performed MDS analysis of TCRα CDR3 repertoires for distinct lymphatic tissues, excluding the lungs due to their otherwise dominant response to the current challenge. This analysis demonstrated close proximity of eTreg repertoires obtained from the same lymphatic tissues upon all lung challenges and across all animals (**Fig. 5a, b**). These results indicate that distinct antigenic specificities are generally characteristic for eTreg cells that preferentially reside in particular lymphatic niches. Notably, the convergence of lymphatic tissue-resident TCR repertoires was less prominent for the eCD4 T cells (**Fig. 5c, d**).

Discussion

We observed a prominent local clonal response to different antigenic challenges within the lung. Remarkably, this effect was comparable for both lung-infiltrating eCD4 and eTreg cells, suggesting similar amplitude for the effector and regulatory response (**Fig. 1a–c**). The TCR repertoires of the eTreg and eCD4 subsets were highly distinct across antigenic challenges (**Fig. 1d, e**). At the same time, eTreg repertoires were highly similar across mice for each challenge (**Fig. 2a, b**), and common TCRα CDR3 motifs dominated each response (**Supplementary Fig. 1**). That being said, the eTreg repertoires were similar for the three different cancer challenges, suggesting a response to shared tumor-associated antigens. Antigen-specific Treg response in lungs was reflected in the lung-draining MLN, but not in distant tissues including the AXLN, IGLN, and spleen (**Fig. 2b**, **Supplementary Fig. 1**), showing the local character of the antigen-specific regulatory response.

For the LLC and PYMT tumors—but not other antigenic challenges—the overall repertoires and dominant CDR3 motifs were shared between the two subsets, suggesting induction of eCD4-eTreg plasticity. Further analysis showed that eCD4 to eTreg conversion was strongest in the skin and lung following LLC and PYMT tumor challenge, and was observed in the site of challenge but not systemically (**Fig. 4a–e**). The presence of an increased proportion of Treg-iNKT cells among the total pool of eTreg cells in the lung upon LLC challenge provided further support for the notion of tumor-induced plasticity (**Fig. 4f–h**). This observation is of particular interest in the context of recent reports on innate-like T cell anti-tumor response²⁸.

The local eTreg response to a given tumor type was clonally very similar upon lung and skin challenge, showing that the antigen-specific character of the eTreg response dominates over the tissue location of the initial challenge (**Fig. 3**).

Strikingly, we observed commonality between eTreg repertoires obtained from the same lymphoid tissue in different mice irrespectively of the antigenic challenge (**Fig. 5a,b**). This observation indicates that distinct antigenic specificities are characteristic for Treg cells with distinct tissue residence.

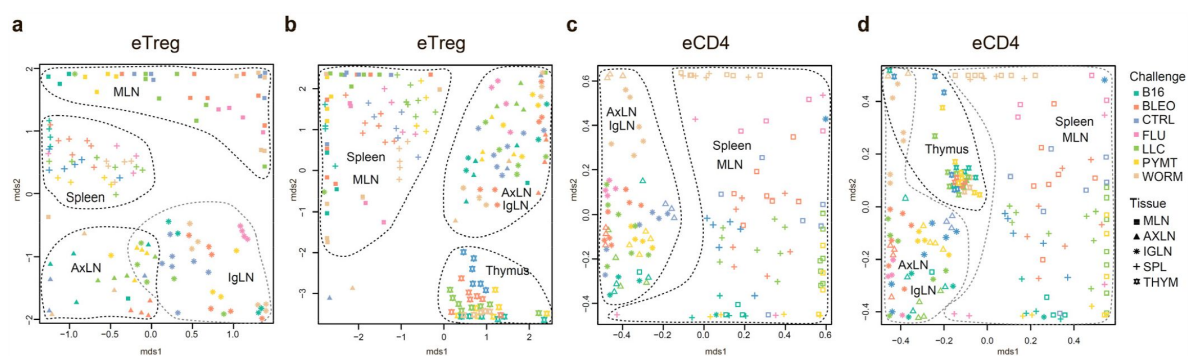


Figure 5.

Lymphatic tissue-resident eTreg and eCD4 cells.

(a–d) MDS plots showing relative overlap between amino acid-defined eTreg TCRα CDR3 repertoires as visualized using VDJtools. Repertoire overlaps are shown for (a) eTreg in MLN, spleen, AxLN, and IGLN; (b) eTreg in MLN, spleen, AxLN, IGLN, and thymus; (c) eCD4 in MLN, spleen, AxLN, and IGLN; and (d) eCD4 in MLN, spleen, AxLN, IGLN, and thymus. Data were normalized to the top 1,000 most frequent clonotypes and weighted by clonotype frequency (F2 metric). Clonotypes were matched on the basis of identical TRAV gene segments and identical TCRα CDR3 sequences. Euclidean distance between points reflects the distance between repertoires; the closer two samples are, the higher is the overall frequency of shared clonotypes. Mice were analyzed in an all-versus-all fashion, irrespective of whether the tissues were obtained from the same or distinct mice. Node colors correspond to the different challenges. Squares: MLN. Triangles: AxLN. Snowflakes: IGLN. Crosses: spleen. Stars: thymus.

The eCD4 subset generally exhibited similar patterns to the eTregs, although the extent of convergence among eTreg repertoires was higher in most scenarios compared to the eCD4 subset, indicating the highly antigen-specific nature of the Treg response.

Altogether, our data demonstrate highly antigen-specific and distinct Teff and Treg responses to different challenges and support the use of the fixed TCR β chain FoxP3-GFP mouse as a “digital” model of TCR response that benefits from access to highly convergent and easy-to-track TCR α CDR3 clusters.

Methods

Mice and challenges

C57BL/6J DO11.10 TCR β transgenic mice (kindly provided by Philippa Marrack) and crossed to C57BL/6J Foxp3^{eGFP} TCR α ^{-/-} mice. Age-matched DO11.10 TCR β Foxp3^{eGFP} TCR α ^{-/-} males were used for experiments. The animals were kept under specific pathogen-free conditions and studied at 6–9 weeks of age.

PyMT cell line was isolated previously from MMTV-PyMT tumor-bearing mice²⁹, Lewis Lung Carcinoma (LLC) cell line was a gift of J. Massague (Memorial Sloan-Kettering Cancer Center) and B16-F10 melanoma cells were a gift from C. Ariyan (Memorial Sloan Kettering Cancer Center). All cell lines were grown in DMEM supplemented with 10% FBS, 1% l-glutamine, 1% penicillin-streptomycin, and 10 mM Hepes. Tumor lines were trypsinized and washed with serum-free DMEM. A dose of 1×10^5 cells in 200 μ L DMEM was injected to each mouse intravenously (i.v) via the tail vein or subcutaneously (s.c) in right flank to induce lung or skin tumors, respectively. Influenza A/Puerto Rico/8/34 (PR8) virus was grown in the cavity of 10-day embryonated chicken eggs, and a dose 100 pfu in 35 μ L PBS was used to challenge mice intranasally (i.n)⁸. *Nippostrongylus brasiliensis* L3 stage larvae were passaged and isolated from the feces of Wistar rats³⁰, 500–700 infective L3 larvae were injected s.c to mice in 500 μ L PBS. Bleomycin for injection was obtained from the pharmacy and each mouse was challenged i.n with a dose of 0.1 U in 35 μ L PBS. Control mice were mock challenged with PBS. Antibodies for magnetic cell isolation and fluorescence activated cell sorting were purchased from Biolegend, ThermoFisher and TONBO BIO. Mouse breeding, challenges and procedures were performed under protocol 08-10-023 approved by the Sloan Kettering Institute (SKI) Institutional Animal Care and Use Committee.

Cell isolation, sorting of T cell subsets and library preparation

To identify and isolate tissue infiltrating T cells and exclude circulating blood lymphocytes, mice from different challenge groups were injected i.v with 0.5 μ g of APC anti-CD45 antibody (clone 30-F11) 3 min before euthanasia³¹. Mice bearing lung and skin tumors were euthanized 2 weeks after challenge and influenza, *Nippostrongylus brasiliensis* and bleomycin experimental mice at day 9 after challenge.

Lungs from all challenge groups were flushed from excess blood via intracardiac injection with 10 mL of PBS, and each lobe was cut into small pieces and digested with Collagenase A (Roche, 1 mg/mL), DNase (30 μ g/mL) in DMEM 2% FBS for 30 min at 37°C in an orbital shaker. Skin tumors were dissected, minced into small pieces and digested with Collagenase A as described above for lung samples. Single cell suspensions were spun and resuspended in sterile EDTA containing FACS buffer (PBS 2% FBS, 1 mM EDTA). Spleens and individual lymph nodes were dissociated with frosted glass slides into single-cell suspensions in FACS buffer. All cell suspensions were filtered with 70 μ m strainers (BD Biosciences) and kept on ice until further use.

Individual cell suspensions were enriched for CD4⁺ T cells using a custom cocktail of biotinylated antibodies (F4/80(clone BM8), anti-mouse I-A/I-E (clone M5/114.15.2), anti-mouse B220(clone RA3-6B2), anti-CD11b (clone M1/70), anti-mouse gamma delta TCR (clone GL3), anti-mouse CD8a (clone 53-6.7), anti-mouse CD11c (clone HL3), anti-mouse Ter119 (clone Ter119), anti-mouse Ly6-G (clone 1A8) at a concentration of 10 ug/mL for each antibody) followed by negative selection with LS columns (Miltenyi). Negative fractions were stained with anti-CD4 (GK1.5, BV605), CD8b (YTS157.7.7, AF700), CD44 (IM7, EF450), CD62L (MEL-14, PE-TexasRed), CD90.2 (clone 30-H12, APC-Cy7), CD45 (clone 30-F11, BV510) and Vb8.2 (clone KJ16-133, PE) antibodies. Effector Treg cells and effector CD4⁺ T cell subsets were sorted using an Aria-II Cell Sorter (BD Biosciences). Briefly, total effector CD4⁺ T cells were gated as Vb8.2⁺ CD4⁺ CD44^{hi} CD62L⁺ CD90⁺ CD45-BV510⁺ CD45 APC-cells. Thereafter, from this gate, conventional effector CD4⁺ T cells as CD44^{hi} CD62L⁺ Foxp3-GFP⁻ cells and effector Treg cells as CD44^{hi} CD62L^{lo} Foxp3-EGFP⁺ were sorted individually into separate tubes, spun, and lysed in buffer RLT plus (Qiagen) and froze at -80°C until further use. RNA purification from individual samples (RNeasy Micro Kit, Qiagen), TCR library preparation from cDNA and Library Next Generation Sequencing has been previously described¹⁸.

TCR repertoire extraction

Raw 150+150 nt sequence data were analyzed using MIGEC software version 1.2.7. UMI sequences were extracted from demultiplexed data using the Checkout utility. Then, the data were assembled using the erroneous UMI filtering option in the Assemble utility. The minimum required number of reads per UMI was set at two for most tasks. In-frame TCRα and TCRβ repertoires were extracted using MiXCR software (version 3.0.13). Normalization, data transformation, in-depth analyses, and statistical calculations were performed using VDJtools software version 1.2.1²⁴. R scripts were used to build figures.

TCRα repertoires diversity analysis

TCRα repertoire diversity was assessed using several widely used metrics, including observed diversity, normalized Shannon Wiener index, and Chao1. For normalization, all diversity metrics were obtained for datasets that had been downsampled to 1,000 randomly-chosen, UMI-labeled TCRα cDNA molecules. Samples with UMI < 700 were excluded from analysis.

TCRα repertoires overlap analysis

Repertoire overlap was analyzed using the weighted F2 (reflecting the proportion of shared T cells between paired repertoires) metric in VDJtools software version 1.2.1. For amino acid overlap metrics calculations, we selected top 1,000 largest clonotypes from each cloneset. Samples with clonotype counts <700 were excluded from analysis. The top N clonotypes were selected as the top N clonotypes after randomly shuffling the sequences and aligning them in descending order. This was done in order to get rid of the alphabetical order for clonotypes with equal counts (e.g. count = 1 or 2).

eCD4-to-eTreg conversion analysis

The top 100 clonotypes were extracted from all samples. F2 (weighted on clonotype size) overlaps in terms of nucleotide CDR3 sequence plus identical V and J were calculated for matched pairs of samples as shown in **Supplementary Table 3**. Samples with clonotype counts <100 were excluded from analysis. The top N clonotypes were selected as the top N clonotypes after randomly shuffling the sequences and aligning them in descending order. This was done in order to get rid of the alphabetical order for clonotypes with equal counts (e.g. count = 1 or 2).

Statistical analysis

Results are shown as mean ± SEM. Statistical analyses were performed on processed datasets in R. Multiple parameter inferences were estimated using parametric t-test.

TCRα clusters

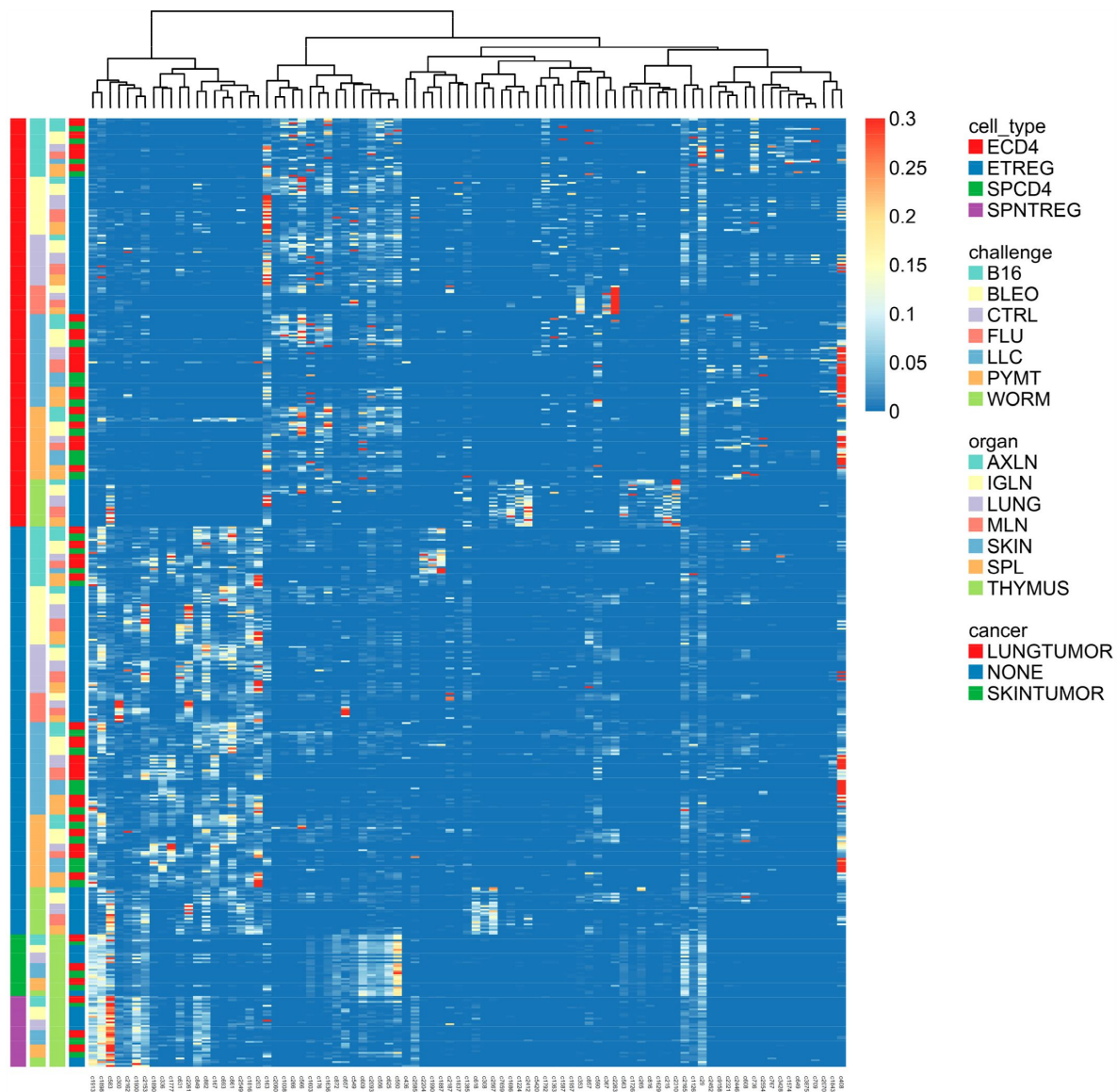
TCRα sequence homology clusters were generated using the TCRNET algorithm³²[↗](#). All TCRα CDR3 sequences were pooled, and unique sequences were used to build a graph. Edges of this graph connected CDR3s that differed by a single amino acid mismatch. CDR3s having more neighbors than expected by chance were selected, and connected components from selected CDR3 sequences were used as clusters. As a baseline, we used random mouse TCRα VDJ rearrangements. Each cluster was assigned a frequency based on the total frequency of T cells encoding corresponding CDR3s within a sample. The CDR3 cluster frequency matrix was then subjected to hierarchical clustering using the ‘aheatmap’ R package with default parameters.

Acknowledgements

Authors are grateful to Alexander Y. Rudensky for his invaluable contribution to the work in all aspects, and to Michael Eisenstein for the helpful edits. Supported by the grant from the Ministry of Science and Higher Education of the Russian Federation 075-15-2019-1789.

Data Availability

All repertoire data used in the manuscript are available on figshare: https://figshare.com/articles/dataset/Convergence_plasticity_and_tissue_residence_of_regulatory_and_effector_T_cell_response/22226155[↗](#)



Supplementary Fig. 1.

TCR group behavior in response to different antigenic challenges.

In this TCR homology cluster expression matrix, each column corresponds to a TCR sequence homology cluster (*i.e.*, group of TCR clonotypes having similar TCR α CDR3 sequences) and each row corresponds to a particular condition (*e.g.*, tissue type, antigenic challenge, etc). The color of each cell highlights the overall (sum of frequency across all clonotypes) expression of each cluster in a given condition. Rows are sorted by cell type, challenge, organ and cancer type as highlighted by the colored bands at left; columns are sorted according to hierarchical clustering of TCR cluster expression.



Supplementary Fig. 2.

All TCR clusters.

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Reviewer #1 (Public Review):

The authors investigate the alpha chain t cell receptor landscape in conventional vs regulatory CD4 T cells. Overall I think it is a very well thought out and executed study with interesting conclusions. Findings are valuable and are supported by convincing evidence. This work will be of interest for immunologists studying T cells.

Strengths:

- One of a kind evidence and dataset.
- State of the art analyses using well accepted in the literature tools.
- Interesting conclusions on the breadth of immune response to challenges across different types of challenges (tumor, viral and parasitic).

<https://doi.org/10.7554/eLife.89382.2.sa1>

Reviewer #3 (Public Review):

This study presents a valuable exploration of CD4+ T cell response in a fixed TCR β chain FoxP3-GFP mouse model across stimuli and tissues through the analysis of their TCR α

repertoires. This is an insightful paper for the community as it suggests several future directions of exploration.

The authors compare Treg and conventional CD4⁺ repertoires by looking at diversity measures and the relative overlap of shared clonotypes to characterize similarity across different tissues and antigen challenges. They find distinct yet convergent responses with occasional plasticity across subsets for some stimuli. The observed lack of a general behavior highlights the need for careful comparison of immune repertoires across cell subsets and tissues. Such comparisons are crucial in order to better understand the heterogeneity of the adaptive immune response. This mouse model demonstrates its utility for this task due to the reduced diversity of the TCR α repertoire and the ability to track a single chain.

The revised manuscript has significantly improved in terms of clarity of explanations and presentations of the results.

<https://doi.org/10.7554/eLife.89382.2.sa0>

Author Response

The following is the authors' response to the original reviews.

eLife assessment

This manuscript presents a valuable approach to exploring CD4⁺ T-cell response in mice across stimuli and tissues through the analysis of their T-cell receptor repertoires. The authors use a transgenic mouse model, in which the possible diversity of the T-cell receptor repertoire is reduced, such that each of a diverse set of immune exposures elicits more detectably consistent T-cell responses across different individuals. However, whereas the proposed experimental system could be utilized to study convergent T-cell responses, the analyses done in this manuscript are incomplete and do not support the claims due to limitations in the statistical analyses and lack of data/code access.

We worked to address the reviewers' concerns below, point-by-point.

All data on immune repertoires are deposited here: https://figshare.com/articles/dataset/Convergence_plasticity_and_tissue_residence_of_regulatory_and_effector_T_cell_response/22226155

We added the Data availability statement to the manuscript.

Public Reviews:

Reviewer #1 (Public Review):

The authors investigate the alpha chain TCR landscape in conventional vs regulatory CD4 T cells. Overall I think it is a very well thought out and executed study with interesting conclusions. The authors have investigated CDR3 alpha repertoires coupled with a transgenic fixed CDR3beta in a mouse system.

Strengths:

- *One of a kind evidence and dataset.*
- *State-of-the-art analyses using tools that are well-accepted in the literature.*

- *Interesting conclusions on the breadth of immune response to challenges across different types of challenges (tumor, viral and parasitic).*

Thank you for the positive view.

Weaknesses:

- *Some conclusions regarding the eCD4->eTreg transition are not so strong using only the data.*

The overlaps between the top-nucleotide clones in both LLC and PYMT challenges are prominently above the average, and this result is reproducible in lungs and skin, so we have no doubts based on these data. Further experiments with different methods, including tracking the clonal fates, should clarify and confirm/correct/disprove our findings.

- *Some formatting issues.*

We are working on the manuscript to correct minor errors and formatting.

Reviewer #2 (Public Review):

This study investigates T-cell repertoire responses in a mouse model with a transgenic beta chain, such that all T-cells in all mice share a fixed beta chain, and repertoire diversity is determined solely by alpha chain rearrangements. Each mouse is exposed to one of a few distinct immune challenges, sacrificed, and T-cells are sampled from multiple tissues. FACS is used to sort CD4 and Treg cell populations from each sample, and TCR repertoire sequencing from UMI-tagged cDNA is done.

Various analyses using repertoire diversity, overlap, and clustering are presented to support several principal findings: 1) TCR repertoires in this fixed beta system have highly distinct clonal compositions for each immune challenge and each cell type, 2) these are highly consistent across mice, so that mice with shared challenges have shared clones, and 3) induction of CD4-to-Treg cell type transitions is challenge-specific.

The beta chain used for this mouse model was previously isolated based on specificity for Ovalbumin. Because the beta chain is essential for determining TCR antigen specificity, and is highly diverse in wildtype mice, I found it surprising that these mice are reported to have robust and consistently focused clonal responses to very diverse immune challenges, for which a fixed OVA-specific beta chain is unlikely to be useful. The authors don't comment on this aspect of their findings, but I would think it is not expected a priori that this would work. If this does work as reported, it is a valuable model system: due to massively reduced diversity, the TCR repertoire response is much more stereotyped across individual samples, and it is much easier to detect challenge-specific TCRs via the statistics of convergent responses.

This was to some extent expected, since these mice live almost normally and have productive adaptive immune responses and protection. In real life, there are frequent TCR-pMHC interactions where the TCR-alpha chain dominates (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5701794/>; <https://pubmed.ncbi.nlm.nih.gov/37047500/>). On the fixed TCR-beta background this mechanics starts working full-fledged, essentially substituting TCR-beta diversity, at the extent of relatively simplified TCRab repertoire and probably higher cross-reactivity.

We agree that this is a valuable model, for sure, and indicated this in the last sentence of our Discussion. Now we are also adding this point to the abstract.

While the data and analyses present interesting signals, they are flawed in several ways that undermine the reported findings. I summarize below what I think are the most substantive data and analysis issues.

(1) There may be systematic inconsistencies in repertoire sampling depth that are not described in the manuscript. Looking at the supplementary tables (and making some plots), I found that the control samples (mice with mock challenge) have consistently much shallower sampling-in terms of both read count and UMI count-compared with the other challenge samples. There is also a strong pattern of lower counts for Treg vs CD4 cell samples within each challenge.

The immune response of control mice is less extensive, as it should be. Just like the fact that the number of Tregs in tissues is lower than CD4, this is normal. So this all follows the expectations. But please note that we were very accurate everywhere with appropriate data normalisation, using all our previous extensive experience (<https://pubmed.ncbi.nlm.nih.gov/29080364/>).

In particular (now adding more relevant details to Methods):

For diversity metrics calculations, we randomly sampled an equal number of 1000 UMI from each cloneset. Samples with UMI < 700 were excluded from analysis.

For amino acid overlap metrics calculations, we selected top-1000 largest clonotypes from each cloneset. Samples with clonotype counts < 700 were excluded from analysis.

For nucleotide overlaps metrics calculations (eCD4-eTreg), we selected top-100 clonotypes from each cloneset. Samples with clonotypes < 100 were excluded from analysis.

The top N clonotypes were selected as the top N clonotypes after randomly shuffling the sequences and aligning them in descending order. This was done in order to get rid of the alphabetical order for clonotypes with equal counts (e.g. count = 1 or 2).

Downsampling was carried out using software vDJtools v.1.2.1.

(2) FACS data are not reported. Although the graphical abstract shows a schematic FACS plot, there are no such plots in the manuscript. Related to the issue above, it would be important to know the FACS cell counts for each sample.

Yes, we agree that this is valuable information that should be provided. Unfortunately, this data has not been preserved.

(3) For diversity estimation, UMI-wise downsampling was performed to normalize samples to 1000 random UMIs, but this procedure is not validated (the optimal normalization would require downsampling cells). What is the influence of possible sampling depth discrepancies mentioned above on diversity estimation? All of the Treg control samples have fewer than 1000 total UMIs-doesn't that pose a problem for sampling 1000 random UMIs?

Indeed, I simulated this procedure and found systematic effects on diversity estimates when taking samples of different numbers of cells (each with a simulated UMI count) from the same underlying repertoire, even after normalizing to 1000 random UMIs. I don't think UMI downsampling corrects for cell sampling depth differences in diversity estimation, so it's not clear that the trends in Fig 1A are not artifactual-they would seem to show higher diversity

for control samples, but these are the very same samples with an apparent systematic sampling depth bias.

We evaluated this approach through all our work, and summarised in the ref: <https://pubmed.ncbi.nlm.nih.gov/29080364/>. Altogether, normalising to the same count of randomly sampled UMI seems to be the best approach (although, preferably, the initial sequencing depth should be essentially higher for all samples than the sampling threshold used). Initial sorting of identical numbers of cells and ideally uniform library preparation and sequencing is generally not realistic and does not work in the real world, while UMI downsampling does the same work much better.

(4) The Figures may be inconsistent with the data. I downloaded the Supplementary Table corresponding to Fig 1 and made my own version of panels A-C. This looked quite different from the diversity estimations depicted in the manuscript. The data does not match the scale or trends shown in the manuscript figure.

There was a wrong column for Chao1, now correcting. Also, please note that we only used samples with > 700 UMI. Supplementary Table now corrected accordingly. Also, please note that Figure 1 shows the results for lung samples only.

(5) For the overlap analysis, a different kind of normalization was performed, but also not validated. Instead of sampling 1000 UMIs, the repertoires were reduced to their top 1000 most frequent clones. It is not made clear why a different normalization would be needed here. There are several samples (including all Treg control samples) with only a couple hundred clones. It's also likely that the noted systematic sampling depth differences may drive the separation seen in MDS1 between Treg and CD4 cell types. I also simulated this alternative downsampling procedure and found strong effects on MDS clustering due to sampling effects alone.

That's right, for the overlap analysis (which values are mathematically proportional to the clonotype counts in both compared repertoires, so the difference in the counts causes major biases) the right way to do it is to choose the same number of clonotypes. See Ref. <https://pubmed.ncbi.nlm.nih.gov/29080364/>.

We kept only samples with > 700 for the overlap analyses. Some relatively poor samples are present in all challenges, while MDS1 localization has clear reproducible logic, so we are confident in these results.

It is not made clear how the overlap scores were converted to distances for MDS. It's hard to interpret this without seeing the overlap matrix.

This is a built-in feature in VDJtools software (<https://pubmed.ncbi.nlm.nih.gov/26606115/>). See also here: <https://vdjtools-doc.readthedocs.io/en/master/overlap.html>.

(6) The cluster analysis is superficial, and appears to have been cherry-picked. The clusters reported in the main text have illegibly small logo plots, and no information about V/J gene enrichments. More importantly, as the caption states they were chosen from the columns of a large (and messier-looking) cluster matrix in the supplementary figure based on association with each specific challenge. There's no detail about how this association was calculated, or how it controlled for multiple tests. I don't think it is legitimate to simply display a set of clusters that visually correlate; in a sufficiently wide random matrix you will find columns that seem to correlate with any given pattern across rows.

Particular CDR3 sequences and VJ segments do not mean much for the results of this manuscript. Logos are given just for visual explanation of how the consensus motifs of the clusters look like.

We now add two more Supplementary Tables and a Supplementary Figure with full information about clusters.

We disagree that the Supplementary Figure 1 (representing all the clusters) looks “messy”. Vice versa, it is surprisingly “digital”, showing the clear patterns of responses and homings. This becomes clear if you visually study it for a while. But yes, it is too big to let the reader focus on this or that aspect. That is why we need to select TCR clusters to illustrate this or that aspect discussed in the work, but they were selected from the overall already structured picture.

(7) The findings on differential plasticity and CD4 to Treg conversion are not supported. If CD4 cells are converting to Tregs, we expect more nucleotide-level overlap of clones. This intuition makes sense. But it seems that this section affirms the consequent: variation in nucleotide-level clone overlap is a readout of variation in CD4 to Treg conversion. It is claimed, based on elevated nucleotide-level overlap, that the LLC and PYMT challenges induce conversion more readily than the other challenges. It is not noted in the textual interpretations, but Fig 4 also shows that the control samples had a substantially elevated nucleotide-level overlap. There is no mention of a null hypothesis for what we'd expect if there was no induced conversion going on at all. This is a reduced-diversity mouse model, so convergent recombination is more likely than usual, and the challenges could be expected to differ in the parts of TCR sequence space they induce focus on. They use the top 100 clones for normalization in this case, but don't say why (this is the 3rd distinct normalization procedure).

Your point is absolutely correct: “This is a reduced-diversity mouse model, so convergent recombination is more likely than usual”. Distinct normalisation procedure was required to focus on the most expanded clonotypes to avoid the tail of (presumably cross-reactive) and identical TCRs present in all repertoires in these limited-repertoire mice. So we downsampled as strictly as possible to minimise this background signal of nucleotide overlap, and only this strict downsampling to the top-100 clonotypes allowed us to visualise the difference between the challenges. This is a sort of too complicated explanation that would overload the manuscript. But your comments and our answers will be available to the reader who wants to go into all the details.

The observed (at this strict downsampling) overlaps between the top-nucleotide clones in both LLC and PYMT challenges are prominently above the average, and this result is reproducible in lungs and skin, so we have no doubts in interpretations based on these data. Further experiments with different methods, including tracking the clonal fates, should clarify and confirm/correct/disprove our findings.

Although interpretations of the reported findings are limited due to the issues above, this is an interesting model system in which to explore convergent responses. Follow-up experimental work could validate some of the reported signals, and the data set may also be useful for other specific questions.

Yes, thank you for your really thorough analysis. We fully agree with your conclusion.

Reviewer #3 (Public Review):

Nakonechnaya et al present a valuable and comprehensive exploration of CD4+ T cell response in mice across stimuli and tissues through the analysis of their TCR-alpha

repertoires.

The authors compare repertoires by looking at the relative overlap of shared clonotypes and observe that they sometimes cluster by tissue and sometimes by stimulus. They also compare different CD4+ subsets (conventional and Tregs) and find distinct yet convergent responses with occasional plasticity across subsets for some stimuli.

The observed lack of a general behaviour highlights the need for careful comparison of immune repertoires across cell subsets and tissues in order to better understand their role in the adaptive immune response.

In conclusion, this is an important paper to the community as it suggests several future directions of exploration.

Unfortunately, the lack of code and data availability does not allow the reproducibility of the results.

Thank you for your positive view.

All data on immune repertoires are deposited here: https://figshare.com/articles/dataset/Convergence_plasticity_and_tissue_residence_of_regulatory_and_effector_T_cell_response/22226155

We added the Data availability statement to the manuscript.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

- *In the manuscript at "yielding 13,369 {plus minus} 1,255 UMI-labeled TCRα cDNA molecules and 3233 {plus minus} 310 TCRα CDR3 clonotypes per sample" I'm not sure how can there be fewer unique DNA molecules than clonotypes in each sample.*

That was our mistake for sure, now corrected.

- *In the manuscript at "This indicates that the amplitude and focused nature of the effector and regulatory T cell response in lungs is generally comparable."*

I'm not sure it's possible to conclude that a drop in diversity in all conditions necessarily signals a focused nature. Since at this stage, the nature of the clonotypes was not compared between conditions, it is not possible to claim a focused nature of the response.

We have softened the wording:

"This could indicate that the amplitude and focused nature of the effector and regulatory T cell response in lungs is generally comparable."

- *What are your thoughts on why there is such a large overlap between Treg and Teff in the Lung in control? For some replicates it is almost as much as a post-LLC challenge!*

There is some natural dispersion in the data, which is generally expectable. The overlaps between the top-nucleotide clones in both LLC and PYMT challenges are prominently above

the average, and this result is reproducible in lungs and skin, so we have no doubts based on these data. Further experiments with different methods, including tracking the clonal fates, should clarify and confirm/correct/disprove our findings.

- *In the manuscript at "These results indicate that distinct antigenic specificities are generally characteristic for eTreg cells that preferentially reside in particular lymphatic niches" I'm not sure we can conclude this from the Figure. Wouldn't you expect the samples to be grouped by color (the different challenges)? Maybe I'm not understanding the sentence!*

This is a different story, about resident Tregs, irrespective of the challenge.

The whole explanation is here in the text:

“Global CDR3α cluster analysis revealed that characteristic eTreg TCR motifs were present in distinct lymphatic tissues, including spleen and thymus, irrespective of the applied challenge (Supplementary Fig. 1). To better illustrate this phenomenon, we performed MDS analysis of CDR3α repertoires for distinct lymphatic tissues, excluding the lungs due to their otherwise dominant response to the current challenge. This analysis demonstrated close proximity of eTreg repertoires obtained from the same lymphatic tissues upon all lung challenges and across all animals (Fig. 5a, b). These results indicate that distinct antigenic specificities are generally characteristic for eTreg cells that preferentially reside in particular lymphatic niches. Notably, the convergence of lymphatic tissue-resident TCR repertoires was less prominent for the eCD4 T cells (Fig. 5c, d).”

And in the abstract:

“Additionally, our TCRα repertoire analysis demonstrated that distinct antigenic specificities are characteristic for eTreg cells residing in particular lymphatic tissues, regardless of the challenge, revealing the homing-specific, antigen-specific resident Treg populations. ”

- *In the manuscript at " Notably, the convergence of lymphatic tissue-resident TCR repertoires was less prominent for the eCD4 T cells ":*

5b and 5d seem to have the same pattern: Spleen and MLN group together, AxLN and IgLN together and thymus is separate. Do you mean to say that the groups are more diffuse? I feel like the pattern really is the same and it's likely due to some noise in the data...

Yes, we just mean here that eTreg groups are less diffuse - means more convergent.

- *I'm not sold on the eCD4 to eTreg conversion evidence. Why only limit to the top 100 clones? The top 1000 clones were used in previous analyses! Moreover, the authors claim that calculating relative overlap (via F2) of matching CDR3+V+J genes is evidence of a conversion between eCD4 and eTreg. I think to convince myself of a real conversion, I would track the cells between groups, unfortunately, I'm not sure how to track this.. Maybe looking at the thymus population? For example, what is the overlap in the thymus vs. after the challenge? I don't have an answer on how to verify but I feel that this conclusion is a bit on the weaker end.*

Distinct normalisation procedure was required to focus on the most expanded clonotypes to avoid the tail of (presumably cross-reactive) and identical TCRs present in all repertoires in these limited-repertoire mice. So we downsampled as strictly as possible to minimise this

background signal of nucleotide overlap, and only this strict downsampling to the top-100 clonotypes allowed us to visualise the difference between the challenges. This is a sort of too complicated explanation that would overload the manuscript. But your comments and our answers will be available to the reader who wants to go into all the details.

The observed (at this strict downsampling) overlaps between the top-nucleotide clones in both LLC and PYMT challenges are prominently above the average, and this result is reproducible in lungs and skin, so we have no doubts in interpretations based on these data. Further experiments with different methods, including tracking the clonal fates, should clarify and confirm/correct/disprove our findings.

- *There is a nuance in the analysis between Figure 3 and Figure 5 which I think I am not grasping. Both Figures use the same method and the same data but what is different? I think the manuscript would benefit from making this crystal clear. The conclusions will likely be more evident as well!*

As explained in the text and above, on Figure 5 “we performed MDS analysis of CDR3α repertoires for distinct lymphatic tissues, excluding the lungs due to their otherwise dominant response to the current challenge.”

The idea of this mini-chapter of the manuscript is to reveal tissue-resident Tregs, distinct for distinct tissues, resident there in all these mice, irrespectively of the challenge we applied. And they are really there (!).

- *Do the authors plan to share their R scripts?*

All calculations were performed in VDJtools. R was only used to build figures. Corrected this in Methods.

Minor typos and formatting issues to address:

- *Typo in Figure 2a the category should read "worm" instead of "warm"*

Corrected.

- *Figure 2a heatmap is missing a color bar indicating the value ranges*

The detailed information can be found in additional Supplementary materials.

- *Figure 2f is never mentioned in the manuscript!*

Corrected.

- *"eTreg repertoire upon lung challenge is reflected in the draining lymph node" - the word upon is of a lower size*

Corrected.

- *The authors should make the spelling of eTreg uniform across the manuscript (reg in subscript vs just lower case letters. Same goes for CDR3α vs CDR3\alpha)*

Corrected.

- *Figure 4a-d p-values annotations are not shown. Is it because they are not significant?*

Corrected.

- *The spelling of FACS buffer should be uniform (FACs vs FACS, see methods)*

Corrected.

- *In the gating strategy, I would make a uniform annotation for the cluster of differentiation, for example, "CD44 high" vs "CD44^{hi}", pos vs + etc.*

Corrected.

- *Citation for MIGEC software (if available) is missing from methods*

Present in the text so probably sufficient.

Reviewer #2 (Recommendations For The Authors):

I noticed the data was made available via Figshare in the preprint, but there is no data availability statement in the current ms.

We provided Data availability statement.

The methods state that custom scripts were written to perform the various analyses. Those should be made available in a code repository, and linked in the ms.

All calculations were performed in VDJtools. R was only used to build figures. Corrected this in Methods.

The title mentioned "TCR repertoire prism", so I thought "prism" was the name of a new method or software. But then the word "prism" didn't appear anywhere in the ms.

We just mean viewing or understanding something from a different perspective or through a lens that reveals different aspects or nuances.

Figure 1D lacks an x-axis label.

Worked on the figures in general.

Reviewer #3 (Recommendations For The Authors):

- *The paper is very concise, possibly a bit too much. It could use additional explanations to properly affirm its relevance, for example:*

why the choice of fixing the CDR3beta background?

To make repertoire more similar across the mice, and to track all the features of repertoire using only one chain.

to what it is fixed?

As explained in Methods:

“C57BL/6J DO11.10 TCR β transgenic mice (kindly provided by Philippa Marrack) and crossed to C57BL/6J Foxp3eGFP TCR α ^{-/-} mice.”

| *What do you expect to see and not to see in this specific system and why it is important?*

As stated above: we expected repertoire to be more similar across the mice, and it is important to find antigen-specific TCR clusters across mice, and to be able to track all the features of the TCR repertoire using only one chain.

| *Does this system induce more convergent responses? If so, can we extrapolate the results from this system to the full alpha-beta response?*

Such a model, compared to conventional mice, is much more powerful in terms of the ability of monitoring convergent TCR responses. At the same time, it behaves natural, mice live almost normally, so we believe it reflects natural behaviour of the full fledged alpha-beta T cell repertoire.

| *• Is the lack of similarity of other tissues to Lung/MLN due to a lack of a response?*

As indicated in the title of the corresponding mini-chapter: “eTreg repertoire upon lung challenge is reflected in the draining lymph node”. And conclusion of this mini-chapter is that “these results demonstrate the selective tissue localization of the antigen-focused Treg response.”

| *Can you do a dendrogram like 2a for the other tissues to better clarify what is going on there? There is space in the supplementary material.*

We built lots of those, but in such single dimension mostly they are less informative compared to 2D MDS plots.

| *• Figure 5 seems a bit out of place as it looks more related to Figure 2. It could maybe be integrated there, sent to supplementary or become Figure 3?*

This is a different story, about resident Tregs, irrespective of the challenge.

The whole explanation is here in the text:

“Global CDR3 α cluster analysis revealed that characteristic eTreg TCR motifs were present in distinct lymphatic tissues, including spleen and thymus, irrespective of the applied challenge (Supplementary Fig. 1). To better illustrate this phenomenon, we performed MDS analysis of CDR3 α repertoires for distinct lymphatic tissues, excluding the lungs due to their otherwise dominant response to the current challenge. This analysis demonstrated close proximity of eTreg repertoires obtained from the same lymphatic tissues upon all lung challenges and across all animals (Fig. 5a, b). These results indicate that distinct antigenic specificities are generally characteristic for eTreg cells that preferentially reside in particular lymphatic niches. Notably, the convergence of lymphatic tissue-resident TCR repertoires was less prominent for the eCD4 T cells (Fig. 5c, d).”

And in the abstract:

“Additionally, our TCR α repertoire analysis demonstrated that distinct antigenic specificities are characteristic for eTreg cells residing in particular lymphatic tissues, regardless of the

challenge, revealing the homing-specific, antigen-specific resident Treg populations. ”

- *Have you explored more systematically the role of individual variability? If you stratify by individual, do you observe any trend? If not this is also an interesting observation to highlight and discuss.*

This is inside the calculations and figures/ one dot = 1 mice, so this natural variation is there inside.

- *Regarding the MDS plots: why are 2 dimensions the right amount? Maybe with 3, you can see both tissue specificity and stimuli contributions. Can you do a stress vs # dimensions plot to check what should be the right amount of dimensions to more accurately reproduce the distance matrix?*

Tissue specificity and stimuli contribution is hard to distinguish without focussing on appropriate samples, as we did on Fig. 3 and 5. The work is already not that simple as is, and attempting to analyse this in multidimensional space is far beyond our current abilities. But this is an interesting point for future work, thank you.

- *Figure 2: A better resolution is needed in order to properly resolve the logo plots at the bottom.*

Yes, we worked on Figures, and also provide new Supplementary Figure with all the logos.

- *No code or data are made available. There is also a lack of supplementary figures that complement and expand the results presented in the main text.*

We believe that the main text, although succinct, contains lots of information to analyse and conclusions (preliminary) to make. So we do not see it rational to overload it further.