

Flt3L-mediated tumor cDC1 expansion enhances immunotherapy by priming stem-like CD8+ T cells in lymph nodes

Corresponding Author: Professor Paul Beavis

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Author comments:

In this work, the authors add detail to our understanding of how Flt3L potentiates anti-tumour immunity, showing that Flt3L overexpression or treatment promotes the differentiation of CD62L+SLAMF6+ stem-like precursor exhausted T cells (TPEX) in both tumors and draining lymph nodes. This effect is proposed to be dependent upon CCR7-mediated trafficking of dendritic cells from the tumor to draining lymph nodes. Significantly, the authors provide mechanistic evidence that lymph nodes serve as a critical reservoir for these stem-like T cells, as blocking lymph node egress using FTY-720 abolishes the Flt3L-mediated increase in tumor-infiltrating TPEX cells. Furthermore, they demonstrate that combining Flt3L treatment with anti-CTLA-4 checkpoint blockade leads to enhanced therapeutic efficacy, characterised by broader TCR repertoire diversity and the emergence of a unique IL-21R-expressing CD8+ T cell subset that shares clonal relationships with both effector and stem-like T cell populations. Together, these findings provide a refined understanding of mechanisms by which Flt3L treatment may enhance anti-tumour immunity, by enhancing antigen presentation to a reservoir of stem-like T cells within draining lymph nodes.

I have a few comments:

1. Data arise from tumour Flt3L overexpression which poorly reflects the role of pathophysiological levels of Flt3L expressed by tumours – previous work for instance has shown non-linear effects with deletion of tumour-endogenous Flt3L and overexpression resulting in paradoxically similar changes in certain tumour models when Flt3L is either overexpressed or deleted in tumours (<https://pmc.ncbi.nlm.nih.gov/articles/PMC10772324/>). It may be helpful to examine the effect of Flt3L knockout in tumour cells on the frequency of stem-like CD8+ T cells in tumours, and on the anti-tumour response induced by anti-CTLA4 and anti-PD-L1

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3. It would be useful to look at whether the frequency of DC expressing tumour antigen-derived peptide:MHC complexes are increased within dLN upon Flt3L administration using antibodies against specific peptide-MHC (eg using the commercially available fluorochrome-conjugated 25-D1.16 mAb which recognises OVA257-264 peptide bound to H-2Kb and which has been used in such studies of DC: <https://www.nature.com/articles/s41467-021-22535-z>).

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abolish the effect of Flt3L?

5. Fig 6a – Flt3L administration synergised more potently with anti-CTLA4 than with anti-PD-1 although the proposed mechanism involving cDC1 presentation of tumour antigens in the draining lymph nodes would presumably enable greater expansion T_{pex} cells in dLN, which are an important progenitor cell from the effective anti-PD-1 response. Some murine Anti-CTLA4 clones can function to deplete Treg cells. Is anti-CTLA4 in this case functioning as a Treg-depleting therapy, as Treg depletion has previously been shown to synergise with Flt3L overexpression (<https://pmc.ncbi.nlm.nih.gov/articles/PMC10772324/>). What is the frequency of Treg cells in dLN and tumours of treated animals?

6. Is the broadening of the TCR repertoire induced by combination CTLA-4 and Flt3L treatment blocked upon FTY720 treatment?

Reviewer #2

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In this manuscript by Lai, Chan, Armitage et al., the authors dissect the effects of flt3L overexpression in various mouse syngeneic tumor models. They show that enhanced tumor control of flt3 expressing tumors is associated with increased DC, NK and T cell infiltration into tumors, including stem-like CD8 T cells. They also use Ova overexpression to study adoptively transferred tumor specific CD8 T cells to show similar skewing towards a stem-like phenotype in flt3 expressing tumors. They use FTY-720 and CCR7 KO BM chimeras to support the hypothesis that DCs trafficking to LN are required for the generation of these stem-like tumor infiltrating CD8 T cells. They also showed that anti-CTLA4 further improved tumor control of flt3L expressing tumors and was associated with increased tumor specific and diverse clonally expanded T cell responses in dLN and tumors. Overall, this is a carefully executed and described high quality study. My general concern in terms of the impact of this paper for this journal is that the novelty seems to come mostly from some mechanistic dissection (Importance of dLN) for the effect of Flt3 expanded DCs on the induction of stem-like CD8 T cells responses. There is very little attempt to relate these findings to human studies including those involving the combined use of Flt3L and checkpoint blockade.

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Minor:

Ref 11 and 33 do not have journal names.

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Reviewer #3

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The present study intended to define how to enhance stem-like T cell responses in tumor immunotherapy via the use of Flt3L-expanded conventional type I dendritic cells 1 (cDC1). cDC1 are known (1) to efficiently cross present tumor antigens to activate CD8+ T cell responses, (2) to promote T_{PEX} maintenance and activation, and (3) to enhance the number of Tcf1+SLAMF6+ CD8+ T cells within tumors. Profiling of Flt3L overexpressing versus control 24JK tumors via single cell

RNA sequencing revealed the presence of significantly enriched DC populations characterized by high expression of Xcr1 (cluster 8), Cd209 (cluster 4), or Ccr7 and Il12 (cluster 7) and it correlated with an increase in the proportion of CD8+ T cells with a stem-like/memory phenotype. Analysis of Flt3L overexpressing or control AT-3 OVA and MC38 tumors yielded similar results and showed increased proportion of CD8+CD44+ T cells expressing surface markers characterizing TPEX cells endowed with superior stem-like potential. Convergent results were also obtained using AT-3 OVA tumor-bearing mice intraperitoneally injected with a single dose of recombinant Flt3L-Fc prior to analysis 8-9 days later. Using XCR1-DTR mice, the authors formally established a correlation between the presence of specifically reduced numbers of tumor cDC1 and of CCR7+PD-L1+ DC numbers and a reduction of the stem-like CD8+ T cell subset. Using a model involving adoptive transfer of congenic OT-I T cells into Flt3L-Fc preconditioned mice, the authors showed that Flt3L-pre-conditioning led to a significant increase in the proportion of CD62L+SLAMF6+ OT-I T cells inside tumors which showed reduced expression of genes associated with exhaustion. Analysis of the lineage-specific gene regulatory networks of the stem-like/memory CD8+ T cells induced by Flt3L identified several transcription factors whose expression was specifically increased in these cells relative to other CD8+ subsets and the authors specifically focused on Myb. Achieving CRISPR/Cas9 mediated knockout of Myb in OT-I cells and transferred them into mice bearing AT-3 OVA tumors, the lack of Myb led to a significant reduction in the proportion of CD62L+SLAMF6+CD8+ T cells. The authors assessed next whether the LN draining the Flt3L-preconditioned tumors were the source of the CD62L+SLAMF6+ T cells. Administration of FTY-720 suggested that the tumor DLN were necessary for the priming of the CD62L+SLAMF6+ CD8+ T cells found within the Flt3L-expressing tumors. Using a model permitting to delete CCR7 specifically from myeloid cells led to a reduction in the number of CD45.2+ DCs in the dLN of mice bearing Flt3L-expressing tumors and in turn in the proportion of CD62L+SLAMF6+CD8+ T cells in the tumor. Using the AT-3 OVA model and performing bulk TCR sequencing on the tumor DLN following anti-CTLA-4 treatment, the authors suggested that such treatment broadens the anti-tumor immune response and correlated with a novel clonally expanded CD8+ subset of effector T cells denoted as cluster T13. Cluster T13 exhibited features of both stem-like/memory populations (Foxo1, CD28, Runx2) and effector-like populations (Zeb1, Ctla4 and Pdcd1) and it exhibited reduced expression of gene signatures associated with exhaustion. Cells belonging to cluster T13 cells expressed high levels of IL-21R, a finding in line with recent reports indicating the importance of IL-21 in the therapeutic effect of CTLA-4 blockade.

In conclusion, this thorough and convincing study provides a series of novel insights into the mechanism by which Flt3L promotes the differentiation of TPEX and improves responses to immunotherapy combining Flt3L and anti-CTLA-4.

Specific comments

1/ In a former study (10.1080/2162402X.2015.1019198), one of the authors of the present paper stressed the importance of proper tumor cutaneous inoculation to avoid metastatic tumor cells to reach the tumor DLN and in turn impedes proper analysis of the role of the migration of DC from the tumor to the tumor DLN. Have the authors followed the protocol described in Ref. for all the experiments performed in the present paper and more specifically those in paragraph 'Draining lymph nodes are a key site for the emergence of stem-like memory T cells 232 induced by Flt3L'.

1/ Is there a role for CD4+ T cells in the models used in the present study (see 10.1126/sciimmunol.adi9517) ?

Decision Letter:

31st Mar 2025

Dear Dr Beavis,

As you are aware, your Article, "Flt3L-expanded conventional type I dendritic cells formed in the tumor prime "stem-like" T cells in the draining lymph nodes to evoke enhanced responses to immunotherapy" has now been seen by 3 referees. Their comments are copied below

We have also looked over your Author Response to these comments. We are generally satisfied with this revision plan and therefore would be happy to see a revised version.

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* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at <http://www.nature.com/nl/authors/index.html>. Refer also to any guidelines provided in this letter.

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- that control panels for gels and western blots are appropriately described as loading or sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

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Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Sincerely,

Nick Bernard, PhD
Senior Editor
Nature Immunology

Reviewers' Comments:

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It would be helpful to include the data presented in rebuttal figure 3 in the main paper as evidence that Flt3L promotes antigen presentation of Ova model antigen expressed by the tumour in dLNs is useful in interpreting the findings.

With these additions to the manuscript, I believe the study findings are now suitable for publication.

Reviewer #2

(Remarks to the Author)

I appreciate the additional analysis and agree that the revisions have nicely improved the manuscript. My comments have all been adequately addressed.

Decision Letter:

14th Nov 2025

Dear Professor Beavis,

Your Article, "Flt3L-expanded conventional type I dendritic cells formed in the tumor prime "stem-like" T cells in the draining lymph nodes to evoke enhanced responses to immunotherapy" has now been seen again by referees 1 and 2.

The reviewers are happy with the revisions but reviewer 1 wants inclusion of some reviewer-only data in the paper, so although we are prepared to accept the paper I am returning it to you for a short revision to include those data now to ensure that our quality control checks on the figures include all data that will be published.

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

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We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

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Senior Editor
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The authors have gone to substantial lengths to respond to my previous comments, providing new evidence that Flt3L increases trafficking of tumour antigen-expressing cDC1 cells to tumour-draining lymph nodes (New Ext Data 2) and that these cells are presenting peptide antigens (Rebuttal Fig 3), providing better definition of the role of CCR7 in enabling expansion of TPEX cells within tumours upon FLT3L expression (Fig 4e-f), and showing that lymph node egress is required for increased polyclonality of TPEX cells within tumours upon Flt3L expression (Fig 6I).

It would be helpful to include the data presented in rebuttal figure 3 in the main paper as evidence that Flt3L promotes antigen presentation of Ova model antigen expressed by the tumour in dLNs is useful in interpreting the findings.

With these additions to the manuscript, I believe the study findings are now suitable for publication.

Reviewer #2 (Remarks to the Author):

I appreciate the additional analysis and agree that the revisions have nicely improved the manuscript. My comments have all been adequately addressed.

Version 2:

Decision Letter:

Our ref: NI-A39682B

1st Dec 2025

Dear Dr. Beavis,

Thank you for submitting your revised manuscript "Flt3L -expanded conventional type I dendritic cells formed in the tumor prime "stem-like" T cells in the draining lymph nodes to evoke enhanced responses to immunotherapy" (NI-A39682B). It has

now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Immunology, pending minor revisions to comply with our editorial and formatting guidelines.

We will now perform detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

If you had not uploaded a Word file for the current version of the manuscript, we will need one before beginning the editing process; please email that to immunology@us.nature.com at your earliest convenience.

Thank you again for your interest in Nature Immunology Please do not hesitate to contact me if you have any questions.

Sincerely,

Nick Bernard, PhD
Senior Editor
Nature Immunology

Version 3:

Decision Letter:

In reply please quote: NI-A39682C

Dear Dr. Beavis,

I am delighted to accept your manuscript entitled "Flt3L-mediated tumor cDC1 expansion enhances immunotherapy by priming stem-like CD8+ T cells in lymph nodes" for publication in an upcoming issue of Nature Immunology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Immunology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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Sincerely,

Nick Bernard, PhD
Senior Editor
Nature Immunology

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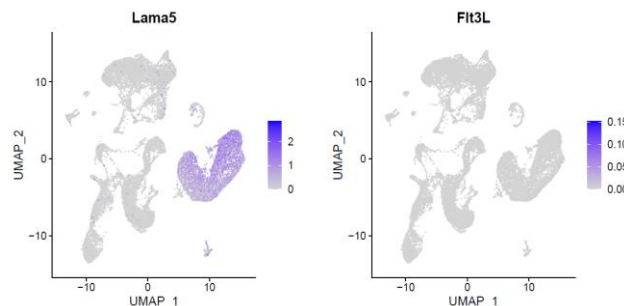
Reviewer 1.

In this work, the authors add detail to our understanding of how Flt3L potentiates anti-tumour immunity, showing that Flt3L overexpression or treatment promotes the differentiation of CD62L+SLAMF6+ stem-like precursor exhausted T cells (TPEX) in both tumors and draining lymph nodes. This effect is proposed to be dependent upon CCR7-mediated trafficking of dendritic cells from the tumor to draining lymph nodes. Significantly, the authors provide mechanistic evidence that lymph nodes serve as a critical reservoir for these stem-like T cells, as blocking lymph node egress using FTY-720 abolishes the Flt3L-mediated increase in tumor-infiltrating TPEX cells. Furthermore, they demonstrate that combining Flt3L treatment with anti-CTLA-4 checkpoint blockade leads to enhanced therapeutic efficacy, characterised by broader TCR repertoire diversity and the emergence of a unique IL-21R-expressing CD8+ T cell subset that shares clonal relationships with both effector and stem-like T cell populations. Together, these findings provide a refined understanding of mechanisms by which Flt3L treatment may enhance anti-tumour immunity, by enhancing antigen presentation to a reservoir of stem-like T cells within draining lymph nodes.

I have a few comments:

1. Data arise from tumour Flt3L overexpression which poorly reflects the role of pathophysiological levels of Flt3L expressed by tumours – previous work for instance has shown non-linear effects with deletion of tumour-endogenous Flt3L and overexpression resulting in paradoxically similar changes in certain tumour models when Flt3L is either overexpressed or deleted in tumours (<https://pmc.ncbi.nlm.nih.gov/articles/PMC10772324/>). It may be helpful to examine the effect of Flt3L knockout in tumour cells on the frequency of stem-like CD8+ T cells in tumours, and on the anti-tumour response induced by anti-CTLA4 and anti-PD-L1.

Response: The reviewer has raised an interesting question with regards to the relationship between the concentration of Flt3L and the impact on TpeX formation. In the article cited by the reviewer, the authors demonstrate that Flt3L knockout mice elicit impaired responses to immune checkpoint blockade. Notably, we wish to clarify that this study does not employ Flt3L knockout tumor cell lines. This is likely because tumor cells are not a major source of Flt3L (**Reviewer only Figure 1**). Indeed, analysis of our scRNA-seq dataset of 24JK tumors (**Figure 1** of our manuscript) confirms that tumor cells did not express detectable levels of Flt3L. Therefore, we believe that performing new experiments with Flt3L KO tumors is not warranted.



Reviewer Only Figure 1- Flt3L is not expressed by 24JK tumor cells.

24JK tumors were analyzed by scRNA-seq (as per **Figure 1** of our manuscript). Tumor cells (as demarcated by the Lama5⁺ population) do not express detectable levels of Flt3L.

However, the major phenotype of Flt3L deficient mice is a significant reduction in cDC1 and cDC2 populations as highlighted by the article cited by the reviewer (**Reviewer Only Figure 2**- reproduced from Regnier *et al.* 2023, Cell Rep Med). Therefore, we believe that our experiments in XCR1-DTR mice enable us to address the same question in a different way. As shown in **Figure 2i-l** of our current manuscript depletion of XCR1 DCs led to a loss of CD62L⁺ stem-like CD8⁺ T cells and attenuated the ability of Flt3L treatment to expand this population.

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From a therapeutic point of view, whilst we agree with the reviewer that Flt3L overexpressing tumors may not reflect the pathophysiological levels of Flt3L within the tumor microenvironment, we would like to reiterate that we also observed the same effects when Flt3L was systemically administered (e.g. **Figures 2e-g**), which more closely replicates how Flt3L has been applied in the clinic.

[Image redacted-- see caption below for source]

Reviewer Only Figure 2- Flt3L knockout mice exhibit significantly reduced numbers of dendritic cells (reproduced from Regnier et al. 2023, Cell Rep Med).

These data highlight that the major phenotype of Flt3L-deficient mice is loss of dendritic cells, an effect which we have modelled using XCR1-DTR mice.

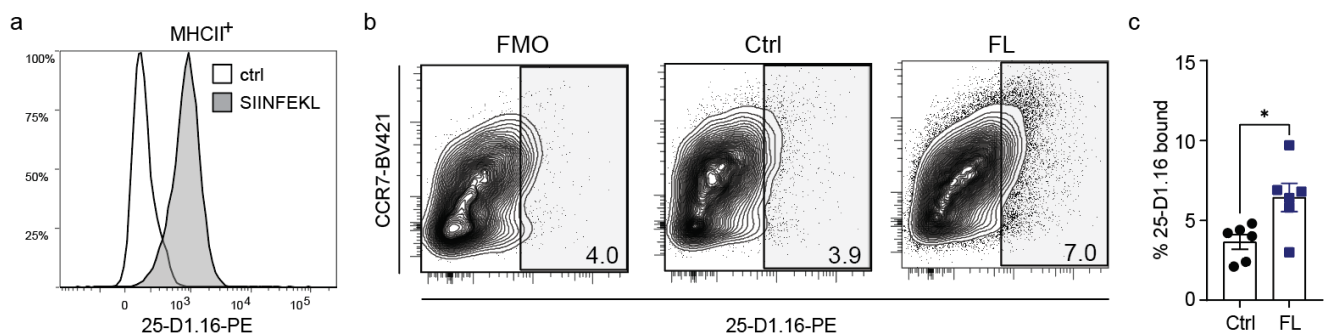
2. The proposed mechanism involves migration of cDC1 from tumours to dLN where they prime CM/Tpex cells to migrate to tumours and supply the ongoing anti-tumour response. A key component of this chain of events which lacks resolution is the drainage of cDC1 from tumours to draining lymph nodes bearing tumour antigens. Could the authors track the migration of cDC1 from tumours to draining lymph nodes by using the Kaede or other optogenetic tracking system, either themselves, or through collaboration with investigators who have this set up for use in tumour models (<https://pmc.ncbi.nlm.nih.gov/articles/PMC9048291/>)? Alternatively, I am not sure whether a tumour-expressed mCherry niche-labelling system can be used (<https://pmc.ncbi.nlm.nih.gov/articles/PMC6797499/>) – can DCs labelled in the tumour be found in the dLN?.

Response: We thank the reviewer for this question. Although it has previously been described that cDC1s are primed for the uptake of tumor antigens and trafficking to the node (PMIDs 27096321, 27424807) we agree it is important to highlight this in the context of our study. To address this, we analyzed the proportion and number of mCherry⁺ DCs in the tumor draining lymph nodes of mice injected with either 24JK mCherry or 24JK mCherry-Flt3L. In line with our hypothesis, the CCR7⁺ DC population elicited the highest proportion of mCherry⁺ cells and the number of these cells were significantly enhanced in the context of Flt3L. This new data is presented in **New Extended Data Fig 2** and referred to in the following text on line 263.

“In line with previous studies, we observed that CCR7⁺ DCs were able to efficiently transport tumor antigens to the tumor-draining lymph nodes (**Extended data Fig 2a-b**)”.

3. It would be useful to look at whether the frequency of DC expressing tumour antigen-derived peptide:MHC complexes are increased within dLN upon Flt3L administration using antibodies against specific peptide-MHC (eg using the commercially available fluorochrome-conjugated 25-D1.16 mAb which recognises OVA257-264 peptide bound to H-2Kb and which has been used in such studies of DC: <https://www.nature.com/articles/s41467-021-22535-z>).

Response: We thank the reviewer for this suggestion. We have evaluated the capacity of 25-D1.16 mAb to detect antigen-presenting DCs in our hands. We first validated that this antibody could detect SIINFEKL presenting APCs *in vitro* through the addition of SIINFEKL to splenocytes, which resulted in a detectable signal (**Reviewer Only Figure 3a**). Having validated the antibody in our hands, we next evaluated its ability to identify antigen presenting APCs *ex vivo* through staining the draining lymph nodes of mice bearing AT3ova or AT3ova-FL tumors. Consistent with our hypothesis, we saw that the staining mediated by 25-D1.16 mAb was significantly increased in mice with Flt3L-expressing tumors (**Reviewer Only Figure 3b-c**). Moreover, staining was predominantly observed in the CCR7⁺ DC population, in line with the notion that DCs were trafficking to the lymph node from the tumor site and presenting antigen. We have currently included this data as a **Reviewer Only Figure** but can include it in the manuscript if requested by the reviewer. The reason we have not included this data at this stage is that the staining mediated by this reagent *ex vivo* is mild and whilst this data supports our conclusions, we believe the mCherry data we refer to above is stronger and sufficiently highlights that CCR7⁺ DCs are adept at presenting tumor antigens in the dLNs.



Reviewer Only figure 3 – Staining on myeloid cells using PE-conjugated 25-D1.16 mAb isolated from a) splenocytes pulsed with SIINFEKL peptide (1 nM) for 48 hours *in vitro* and b-c) draining lymph nodes of AT-3 OVA tumor-bearing mice *ex vivo*. *p<0.05 unpaired t Test.

4. The system in Figure 5 is elegant, and shows a requirement for an intact CCR7 gene in a majority of haematopoietic cells for optimal recruitment of CCR7-proficient Tpex cells in the tumour. Would ablation of CCR7 in this context be sufficient to abolish the effect of Flt3L?

Response: We thank the reviewer for their positive appraisal of this experimental setup. To address the question of whether CCR7 expression on Tpex is required for the expansion of this subset in the tumor we have performed new experiments where CCR7 was deleted on OT-I T cells prior to transfer to mice with control or Flt3L-expressing tumors. This new data indicates that CCR7 deletion abolishes the effect of Flt3L on the expansion of CD62L⁺SLAMF6⁺CD8⁺ cells within the tumor. This new data is shown in new **Figure 4e-f** and is referred to on line 248:

“Similarly, we observed that CRISPR/Cas9-mediated targeting of CCR7 on OT-I T cells prior to transfer to tumor bearing mice ablated the ability of Flt3L to enhance the proportion of CD62L⁺SLAMF6⁺ CD8⁺ T cells (**Fig 4e-f**)”.

5. Fig 6a – Flt3L administration synergised more potently with anti-CTLA4 than with anti-PD-1 although the proposed mechanism involving cDC1 presentation of tumour antigens in the draining lymph nodes would

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presumably enable greater expansion T_{pex} cells in dLN, which are an important progenitor cell from the effective anti-PD-1 response. Some murine Anti-CTLA4 clones can function to deplete Treg cells. Is anti-CTLA4 in this case functioning as a Treg-depleting therapy, as Treg depletion has previously been shown to synergise with Flt3L overexpression (<https://pmc.ncbi.nlm.nih.gov/articles/PMC10772324/>). What is the frequency of Treg cells in dLN and tumours of treated animals?

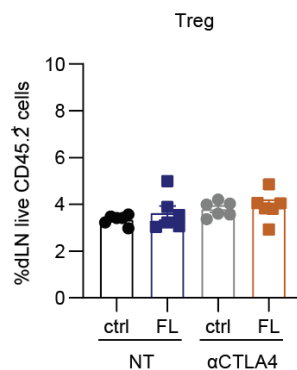
Response: The reviewer raises an interesting question as to whether the effect of anti-CTLA-4 mediating activation of T_{pex} cells is functioning directly or indirectly through the depletion of Tregs. We have performed additional experiments to address this question. These experiments found that anti-CTLA-4 (9H10 clone) treatment reduced the frequency of Tregs within the tumor, in line with our previous observations (PMID 30018045), although the overall number of Tregs was not significantly affected. We have included this data in new **Extended Data Figure 3h**, which is referred to on line 316 of the revised manuscript.

“Given that anti-CTLA-4 has previously been described to deplete Tregs we also analyzed effects on the CD4⁺ population. Consistent with prior work anti-CTLA-4 depleted the frequency of Tregs, although the total number of Tregs was not significantly affected (**Extended data Fig. 3h**). “

We have also added a sentence to the discussion on line 430 highlighting that the effects of CTLA-4 blockade on T_{pex} cells may be direct or indirect.

“It is possible that this effect was mediated through an intrinsic effect of blocking CTLA-4 on the T_{PEX} cells and/or an indirect mechanism through the modulation of other CTLA-4 expressing populations such as Tregs.”

We also assessed the impact of anti-CTLA-4 on Treg frequency in the tumor draining lymph node as requested by the reviewer. This analysis indicated that anti-CTLA-4 treatment did not affect Treg frequency in the tumor draining lymph node (**Reviewer Only Figure 4**). This is included only as a reviewer only figure but can be included in the manuscript upon request.



Reviewer Only Figure 4- CD4⁺foxp3⁺ Treg frequency in AT-3 OVA tumor-draining lymph nodes following anti-CTLA-4 therapy.

6. Is the broadening of the TCR repertoire induced by combination CTLA-4 and Flt3L treatment blocked upon FTY720 treatment?

Response: We thank the reviewer for this suggestion. We have performed this experiment as suggested, which demonstrated that FTY-720 treatment completely abrogated the clonotypic expansion mediated by Flt3L and anti-CTLA4 treatment. This data is shown in new **Figure 6L** and is referred to in the following text on line 313.

“Bulk TCR-Sequencing analysis of CD8⁺ T cells isolated from the tumors of treated mice revealed that the clonotypic expansion mediated by the combination of Flt3L and anti-CTLA-4 was attenuated by FTY-720 treatment, highlighting the critical involvement of egress from the tumor dLN in this effect (Fig. 6l).”

Reviewer #2

(Remarks to the Author)

In this manuscript by Lai, Chan, Armitage et al., the authors dissect the effects of flt3L overexpression in various mouse syngeneic tumor models. They show that enhanced tumor control of flt3 expressing tumors is associated with increased DC, NK and T cell infiltration into tumors, including stem-like CD8 T cells. They also use Ova overexpression to study adoptively transferred tumor specific CD8 T cells to show similar skewing towards a stem-like phenotype in flt3 expressing tumors. They use FTY-720 and CCR7 KO BM chimeras to support the hypothesis that DCs trafficking to LN are required for the generation of these stem-like tumor infiltrating CD8 T cells. They also showed that anti-CTLA4 further improved tumor control of flt3L expressing tumors and was associated with increased tumor specific and diverse clonally expanded T cell responses in dLN and tumors. Overall, this is a carefully executed and described high quality study.

Response: We thank the reviewer for their positive appraisal of our manuscript.

1. My general concern in terms of the impact of this paper for this journal is that the novelty seems to come mostly from some mechanistic dissection (Importance of dLN) for the effect of Flt3 expanded DCs on the induction of stem-like CD8 T cells responses.

There is very little attempt to relate these findings to human studies including those involving the combined use of Flt3L and checkpoint blockade.

Response: We agree with the reviewer that relating the findings to human studies is an important approach to highlight the clinical significance of the work. There is limited clinical data available from patients treated with Flt3L and immune checkpoint blockade. To our knowledge the only published clinical data is of 11 non-Hodgkin's lymphoma patients and limited immune correlates were reported from this study (PMID 30962585).

However, to address this point we have analyzed some of our key findings in relationship to three clinical studies involving immune checkpoint blockade; the IMvigor210 trial, which is one of the largest patient datasets treated with immune checkpoint blockade (n =348 patients), an Australian-based study where a cohort of 158 melanoma patients were treated with immune checkpoint blockade (PMID 30753825) and a study of melanoma patients that included scRNA-seq analysis and helped define the key CD8⁺ T cell states required for an effective response to immune checkpoint blockade (PMID 30388456, Sade-Feldman et al. 2018, Cell). Our analysis shows that:

- 1) The Flt3L gene signature derived from bulk RNA-sequencing of control vs Flt3L-expressing tumors is associated with significantly increased survival.
- 2) The gene signature derived from the T04 stem-like CD8⁺ clusters expanded by Flt3L was associated with significantly increased survival in both datasets.
- 3) The T04 cluster shows a strong positive enrichment for genes associated with the CD8⁺ cluster identified by Sade-Feldman *et al.* to be critically required for response to immune checkpoint blockade.
- 4) IL21 is associated with improved response to immunotherapy.

This new data is shown in **Figures 7i-j and p, Extended Data Fig 6a** and is referred to in the following text on lines 352 and 381.

“To place these results in a clinical/human context we compared the gene signatures derived from these analyses to patient datasets. Firstly, we identified that the bulk Flt3L gene signature, characterized by high expression of *Xcr1* and *Il12b* was associated with improved response in patients undergoing immune checkpoint blockade (Extended Data Fig. 6a). Specifically, we analyzed this in the context of the T04 cluster that was expanded upon Flt3L treatment and observed that the T04 gene signature was associated with improved survival in two independent cohorts of patients treated with immune checkpoint blockade (Fig. 7i). Moreover, the T04 gene

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signature was highly enriched for genes associated with a subset of cells previously shown to be correlated with effective outcomes in patients treated with immune checkpoint blockade⁵ (**Fig. 7j**)."

"Moreover, high expression of IL-21 in tumor biopsies was associated with improved responses in patients undergoing immune checkpoint blockade, supporting the potential importance of this pathway (**Figure 7p**)."

Comments:

2. Given that every model is different I think it would be appropriate to mention which mouse models are being used in the abstract if possible.

Response: We have not listed tumor models in the abstract as we do not believe this is standard for publications in *Nature Immunology*, but we would be happy to make this amendment if the editor feels this is warranted. For the time being, in order to make this easier to follow we have instead added additional labelling referring to the different models in the figures.

3. Methods and figure legends do not provide specifics about the number of OT-1 T cells transferred related to Fig. 3.

Response: We apologise for this oversight and have now included these details.

4. Related to Fig. 6f, the authors claim that anti-CTLA4 reduced frequencies of CD8+CD62L+TCF1+ T cells in Flt3 expressing tumors. However, this is not statistically compared in Fig. 6f.

Response: We apologise for the confusion. We had performed this statistical test but this was not indicated on the graph. An updated graph is now included in a revised version.

5. Also, why is SLAMF6 not being used as a marker for these experiments in contrast to earlier figures?

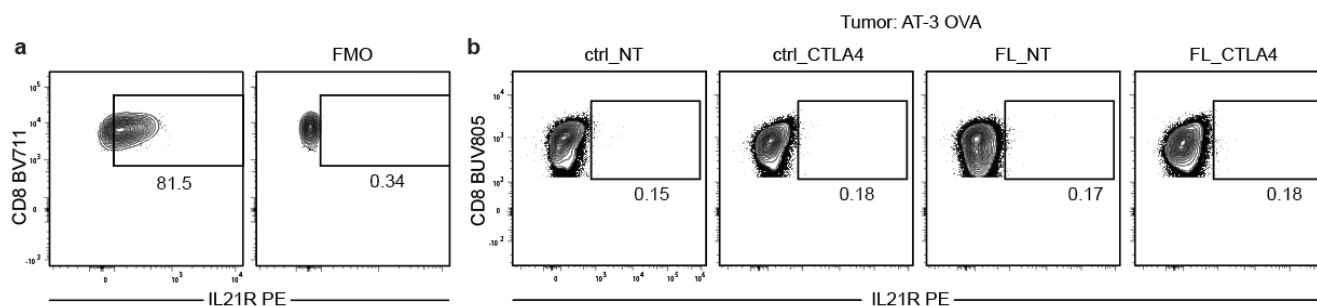
Response: We used SLAMF6 as a surrogate of TCF1 expression as previously described (PMID 32396847) and thus analysing the data by SLAMF6 or TCF1 yields similar results. To highlight this, we have added an additional flow cytometry plot in **Extended Data Figure 3f** highlighting that the SLAMF6⁺CD62L⁺ population is almost 100% TCF1⁺.

6. Were comparative statistics performed downstream of the cluster analysis? Were stats performed for Fig. 7d right panels?

Response: To address the reviewer's question, we have now performed a statistical analysis on the graphs shown in Fig 7 and these statistics are included in the revised figure.

7. The authors make a point in the abstract about the appearance of an IL21R expressing cluster of CD8 T cells. However, this seems to come from a single experiment using the AT3-FL-OVA cell line. It is not clear to me how broadly relevant this phenotype might be if not assessed in other tumor models – perhaps assessed using orthogonal methods such as flow cytometry staining of IL21R.

Response: We agree with the reviewer that validation of the importance of the IL-21-IL-21R pathway would enhance the significance of the study. To address this question, we performed flow cytometry on tumour-infiltrating lymphocytes following treatment with anti-CTLA-4. Whilst we validated that this antibody successfully labelled CD8⁺ splenocytes (**Reviewer Only Figure 5a**), we were unable to detect significant expression of IL-21R on tumor-derived CD8⁺ T cells in either the MC38 (data not shown) or AT-3 OVA models (**Reviewer Only Figure 5b**). We believe this may be due to lower expression of IL-21R on tumor-infiltrating lymphocytes and the sensitivity of the antibody. Notably, the IL-21R⁺ T13 cluster identified by scRNA-seq constituted only ~2% of CD8⁺ T cells and therefore we believe this may be difficult to identify by flow cytometry.



Reviewer Only Figure 5 – IL21R fluorochrome antibody staining of (a) activated splenocyte-derived CD8⁺ T cells (b) AT-3 OVA tumor-derived CD8⁺ T cells

We therefore devised an alternative strategy to validate the connection with IL-21. We performed therapeutic studies blocking the IL-21R and observed that IL-21R blockade significantly attenuated the therapeutic benefit of Flt3L and anti-CTLA-4 combination therapy. Moreover, in response to the request for additional human data, we have also interrogated the prognostic value of IL-21 expression in patients undergoing immunotherapy. This data is presented in new **Figure 7o-p** and is referred to in the following text on line 377.

“To evaluate this in the context of Flt3L we performed therapeutic studies with CTLA-4 blockade in the presence of an IL-21R blocking antibody. The efficacy of anti-CTLA-4 was significantly attenuated by IL-21R blockade, highlighting the importance of this cytokine in the therapeutic effect mediated by Flt3L and anti-CTLA-4 combination therapy (**Figure 7o**). Moreover, high expression of IL-21 in tumor biopsies was associated with improved responses in patients undergoing immune checkpoint blockade, supporting the potential importance of this pathway (**Figure 7p**).”

8. Related to Fig. 7, it is difficult to determine what gene expression profiles associated with each of clusters shown and the UMAPs are not well annotated. Therefore, it is not very clear why only particular clusters are highlighted in the narrative and in the figures. For instance, on lines 312-314 two different clusters L05, L08 are described in parallel and it is not clear how the figures show increased expression of these genes for these two clusters. While I believe it, it is not clear from the figures where the Tex and Tpex are supposed to be in Fig. 7l or how this trajectory analysis really shows T13 as an intermediate (except that it is in the middle of the UMAP).

Response: We apologise for the confusion. To address the lack of clarity regarding the identity of each cluster we have now provided a new **Supplementary Table 1** that indicates the top 25 upregulated genes within each cluster. Specifically with regards to the L05 and L08 clusters, we focussed upon these clusters because their frequency was increased in the Flt3L and anti-CTLA-4 condition. To further highlight that these clusters resemble antigen experienced cells we have provided a dot plot analysis (**New Extended Data 4d**), which aids comparison between the clusters. To improve clarity of the pseudotime analysis we have modified the presentation of **Figure 7l** to better highlight this point.

Minor:

Ref 11 and 33 do not have journal names.

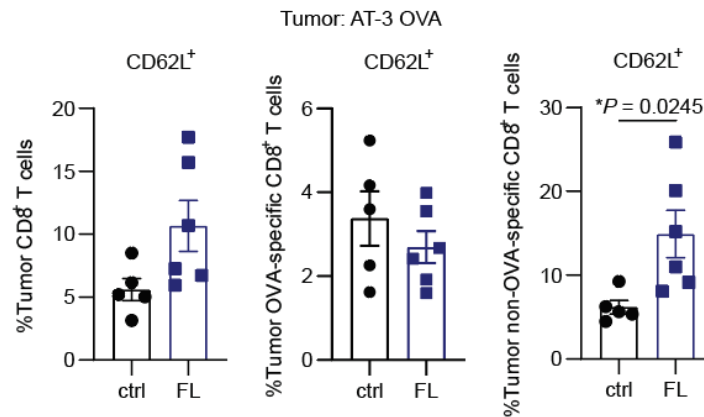
Response: We thank the reviewer for highlighting this error. We have now corrected this.

In figure 2, why not use tetramers to look see if any of these T cell populations are Ova/tumor specific?

Response: We thank the reviewer for this question. To address this question, we performed an experiment to analyse the proportion of CD8⁺ T cells expressing CD62L in ctrl or Flt3L-overexpressing AT-3 OVA tumors and then further subdivided the CD8⁺ T cells using a SIINFELK-loaded tetramer. This analysis revealed that the majority of CD62L⁺ cells did not bind the SIINFELK tetramer and that Flt3L specifically enhanced the frequency of the CD62L⁺tetramer⁺ cells (**Reviewer Only Figure 6**). We posit that this may be because Flt3L is more adept at maintaining the stem-like population of low affinity TCR clones (as highlighted by the OT-3 data). However, as this is speculative we have not included this data in the manuscript but this can be included upon request.

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Reviewer Only Figure 6 – Impact of Flt3L on the phenotype of SIINFEKL-tetramer positive and negative CD8⁺ T cells isolated from AT-3 OVA tumors. *p<0.05 unpaired t test.

Line 228-229 is not clear in terms of the comparison being made between high and low affinity Ova-specific T cells maybe add “... in comparison to OT-I”?

Response: We thank the reviewer for this suggestion. We have now amended this text, located on line 228, as follows.

“Consistent with our hypothesis, OT-3 cells exhibited a significantly enhanced proportion of CD62L+SLAMF6+ cells relative to OT-I cells, and this population was further enhanced in the context of Flt3L (Fig. 3h-i).”

Reviewer #3

(Remarks to the Author)

The present study intended to define how to enhance stem-like T cell responses in tumor immunotherapy via the use of Flt3L-expanded conventional type I dendritic cells 1 (cDC1). cDC1 are known (1) to efficiently cross present tumor antigens to activate CD8⁺ T cell responses, (2) to promote TPEX maintenance and activation, and (3) to enhance the number of Tcf1+SLAMF6⁺ CD8⁺ T cells within tumors. Profiling of Flt3L overexpressing versus control 24JK tumors via single cell RNA sequencing revealed the presence of significantly enriched DC populations characterized by high expression of Xcr1 (cluster 8), Cd209 (cluster 4), or Ccr7 and Il12 (cluster 7) and it correlated with an increase in the proportion of CD8⁺ T cells with a stem-like/memory phenotype. Analysis of Flt3L overexpressing or control AT-3 OVA and MC38 tumors yielded similar results and showed increased proportion of CD8⁺CD44⁺ T cells expressing surface markers characterizing TPEX cells endowed with superior stem-like potential. Convergent results were also obtained using AT-3 OVA tumor-bearing mice intraperitoneally injected with a single dose of recombinant Flt3L-Fc prior to analysis 8-9 days later. Using XCR1-DTR mice, the authors formally established a correlation between the presence of specifically reduced numbers of tumor cDC1 and of CCR7+PD-L1⁺ DC numbers and a reduction of the stem-like CD8⁺ T cell subset. Using a model involving adoptive transfer of congenic OTI T cells into Flt3L-Fc preconditioned mice, the authors showed that Flt3Lpre-conditioning

led to a significant increase in the proportion of CD62L+SLAMF6+ OT-I T cells inside tumors which showed reduced expression of genes associated with exhaustion. Analysis of the lineage-specific gene regulatory networks of the stem-like/memory CD8+ T cells induced by Flt3L identified several transcription factors whose expression was specifically increased in these cells relative to other CD8+ subsets and the authors specifically focused on Myb. Achieving CRISPR/Cas9 mediated knockout of Myb in OT-I cells and transferred them into mice bearing AT-3 OVA tumors, the lack of Myb led to a significant reduction in the proportion of CD62L+SLAMF6+CD8+ T cells. The authors assessed next whether the LN draining the Flt3L-preconditioned tumors were the source of the CD62L+SLAMF6+ T cells. Administration of FTY-720 suggested that the tumor DLN were necessary for the priming of the CD62L+SLAMF6+ CD8+ T cells found within the Flt3L-expressing tumors. Using a model permitting to delete CCR7 specifically from myeloid cells led to a reduction in the number of CD45.2+ DCs in the dLN of mice bearing Flt3L expressing tumors and in turn in the proportion of CD62L+SLAMF6+CD8+ T cells in the tumor. Using the AT-3 OVA model and performing bulk TCR sequencing on the tumor DLN following anti-CTLA-4 treatment, the authors suggested that such treatment broaden the anti-tumor immune response and correlated with a novel clonally expanded CD8+ subset of effector T cells denoted as cluster T13. Cluster T13 exhibited features of both stem like/memory populations (Foxo1, CD28, Runx2) and effector like populations (Zeb1, Ctla4 and Pdcd1) and it exhibited reduced expression of gene signatures associated with exhaustion. Cells belonging to cluster T13 cells expressed high levels of IL 21R, a finding in line with recent reports indicating the importance of IL-21 in the therapeutic effect of CTLA-4 blockade.

In conclusion, this thorough and convincing study provides a series of novel insights into the mechanism by which Flt3L promotes the differentiation of TPEX and improves responses to immunotherapy combining Flt3L and anti-CTLA-4.

Specific comments:

1/ In a former study (10.1080/2162402X.2015.1019198), one of the authors of the present paper stressed the importance of proper tumor cutaneous inoculation to avoid metastatic tumor cells to reach the tumor DLN and in turn impedes proper analysis of the role of the migration of DC from the tumor to the tumor DLN. Have the authors followed the protocol described in Ref. for all the experiments performed in the present paper and more specifically those in paragraph 'Draining lymph nodes are a key site for the emergence of stem-like memory T cells induced by Flt3L'.

Response: In the publication referred to by the reviewer the authors employed an epicutaneous inoculation protocol to provide an orthotopic model of melanoma. This is not relevant to the tumor lines used in the current study (AT3- breast, 24JK- sarcoma and MC38- Colon) given that these particular lines are not metastatic and so do not interfere with DC priming in the dLNs.

1/ Is there a role for CD4+ T cells in the models used in the present study (see 10.1126/sciimmunol.adi9517) ?

Response: To address this question we have performed additional analyses on the CD4⁺ population. These studies highlight that the combination of Flt3L and anti-CTLA-4 significantly enhances the number (**Extended Data 3d**) and cytokine production (**Extended Data 3g**) of tumor-infiltrating CD4⁺ T cells. These new data are discussed on lines 294 and 308.

Reviewer #1 (Remarks to the Author):

The authors have gone to substantial lengths to respond to my previous comments, providing new evidence that Flt3L increases trafficking of tumour antigen-expressing cDC1 cells to tumour-draining lymph nodes (New Ext Data 2) and that these cells are presenting peptide antigens (Rebuttal Fig 3), providing better definition of the role of CCR7 in enabling expansion of TPEX cells within tumours upon FLT3L expression (Fig 4e-f), and showing that lymph node egress is required for increased polyclonality of TPEX cells within tumours upon Flt3L expression (Fig 6I).

It would be helpful to include the data presented in rebuttal figure 3 in the main paper as evidence that Flt3L promotes antigen presentation of Ova model antigen expressed by the tumour in dLNs is useful in interpreting the findings.

With these additions to the manuscript, I believe the study findings are now suitable for publication.

Response: We thank the reviewer for their positive appraisal of our manuscript and new data. As requested, we have added the data from Rebuttal Figure 3 to the manuscript. This data is located in Extended Data Figure 2-c-d.

Reviewer #2 (Remarks to the Author):

I appreciate the additional analysis and agree that the revisions have nicely improved the manuscript. My comments have all been adequately addressed.

Response: We thank the reviewer for their positive appraisal of our manuscript and new data.