nature portfolio

Peer Review File

Regulatory T cells crosstalk with tumor and endothelium through lymphotoxin signaling

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

A version of this paper was originally rejected for publication by Nature Communications, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Piao et al. investigated the function of Treg-derived lymphotoxin (LT) on tumor growth and metastasis. Using in vitro systems, they showed that lymphotoxin signaling classical and nonclassical NFkB signaling, and nonclassical NFkB pathway promotes the proliferation and migratory abilities of tumor cells. Coculturing tumor cells with LT-expressing, but not LT-deficient, Tregs induces NFkB pathway, correlating with increased tumor cell proliferation and transwell migration. Treg coculturing with lymphatic endothelial cells (LECs) also increased their ability to promote tumor cell proliferation and migration in vitro. In vivo, LTbR-deficiency in tumor cells and nonclassical NFkB inhibitor treatment slowed down tumor growth and lymph node metastasis. LTbR knockout in the tumor stroma also prolonged the survival of tumor-bearing mice without impairing tumor growth. Based on these data, the authors conclude that Treg regulate tumor cell and LEC activities through lymphotoxin signaling.

Tregs are well established for their ability to promote cancer growth by suppressing immune responses. This manuscript provides interesting insights on how Tregs could regulate tumor cells and LECs and expands our knowledge on Treg biology.
However, data presented using in vitro systems did not lend sufficient support to conclude that “Tregs crosstalk with tumor cells and ECs via lymphotoxin signaling” in vivo. While in vitro experiments demonstrate the ability of Tregs to alter tumor cell/LEC activities via LT signaling, whether these observations are relevant in vivo is not clear from their experiments. Importantly, it has yet to be established (1) whether lymphotoxins are necessary factors by which Tregs regulate tumor growth/metastasis (via NFkB), (2) whether Tregs are non-redundant sources of lymphotoxin-mediated tumor regulation. Ideal experiments would require mouse models that delete lymphotoxin specifically in Tregs.

The studies focused on LTbR signaling in tumor cells and LECs. What is the rationale for focusing on these cells? Many other stromal cells express the receptor even at higher level than tumor/LECs, such as blood endothelial cells and fibroblasts. Is Treg-mediated LTbR-NFkB axis in tumor/LEC important and nonredundant?

Major concerns:
Analyses of human tumors revealed that pathways upregulated in LTbR-high tumors are enriched in cytokine/chemokine signaling. However, the in vitro and mouse studies in this manuscript are mostly focused on tumor cell migration. Is tumor invasiveness/EMT gene signature also upregulated in humans?

It is not clear why comparisons between the expression level of LTbR and other oncogenes is meaningful or relevant.

While the tumor cell lines used in the study express LT receptor, immunoblot data in Fig 2 showed weak baseline activation of NFkB activation in the absence of stimulus (by LT receptor antibody ligation and Treg coculturing). To what extent is NFkB activated in tumor cells in vivo? Did Treg depletion decrease NFkB signaling in vivo?

The in vitro data relied almost exclusively on antibody ligation to activate LTbR. Do other approaches of LT receptor activation trigger similar patterns of downstream signaling? Such as recombinant lymphotoxin peptides?

The authors previously show that effector T cells express significant levels of lymphotoxins (Piao, Cell Report 2022). Why did coculturing with Teffs (Figure 5) not activate NFkB signaling? What about B cells which have high LT. In other words, are Tregs just a minor source of LT? If so it would call into question the significance of the ex vivo findings.

What's the mechanism for prolonged survival of tumor-bearing LTbR-/- mice? Did the mice have fewer metastases?

In Figure 7 the CD45+ vs. CD45- populations are not well separated, and CD45 vs. live/dead-Aqua is not well compensated; thus the subsequent T cell/MDSC quantifications are questionable.
More importantly, the functional importance of the changes in the immune profile is not clear, and how it is tied to the changes in LEC/tumor responses to LT inhibition?

The authors did not provide data supporting the statement that tumor- or endothelium-derived CXCL1 was suppressed by nciLT: tumor and EC gatings need to be shown. More importantly, the data at best imply the correlation between CXCL1 decrease and G-MDSC reduction, but whether this chemokine is important in the process is not established.

The claim that nciLT inhibits endothelial specific SOX18 and FLRT2 "to reduce tumor angiogenesis" was not established. Correlation was shown but the role of SOX18/FLRT2 was not investigated.

Are CD8+ T cells important?

Reviewer #2

(Remarks to the Author)
Piao W et al found that regulatory T (Treg) cell express high lymphotoxin (LT) α1β2 which preferentially stimulate LTβ receptor (LTβR) and induce non-classical NFκB signaling in tumor cells and lymphatic endothelial cells (LECs), resulting in increased tumor growth and metastasis. In addition, they developed LTβR blocking peptides which selectively inhibit classical (ciLT) or nonclassical (nciLT) NFκB signaling pathways. Especially, blocking peptide targeting nciLT inhibits tumor growth and metastasis through reducing infiltration of suppressive immune cells, such as Treg cells and myeloid-derived suppressor cells, and tumor angiogenesis. They suggest that Treg cells directly promote nciLT NFκB signaling pathways in tumor cells and LECs, which can be targetable by blocking peptides.
It is potentially interesting to note that secreted factors produced by Treg cells directly promote tumor growth and metastasis. However, the impact of this study is severely impaired since there are critical issues with the experimental method, interpretation of the data, and several points were not sufficiently addressed.

General comments;
Although the authors have confirmed LTβR expression in cancer cells (supplementary table 1), it is necessary to present the expression profile of both LTα1β2 and LTβR in multiple cell populations infiltrated in the tumor microenvironment, such as macrophages, B cells, myeloid-derived suppressor cells and fibroblasts. Since single cell RNA sequencing data has been published for various cancer types, the expression analysis of these molecules in tumor-infiltrating immune cells should be shown by utilizing the published data. Based on the results, the author should clarify why this study focuses only on the relationship between LTα1β2 from Tregs and LTβR on both cancer cells and LECs. Furthermore, it is very important to present the actual expression profile of LTα1β2 in Treg cells and activation status of nciLT NFκB signaling pathways in cancer cells and LECs by using human clinical specimens such as FFPE.

In vitro studies of co-culture using cancer cell lines and immune cells were neatly conducted, however, in vivo evaluation is not sufficient. In Figure 5k, CXCL10 and CCL5 are elevated by TLβR stimulation. These chemokines are known to be essential for the infiltration of CD8+T cells (https://pubmed.ncbi.nlm.nih.gov/29579623/ , https://www.nature.com/articles/nri.2017.49). To confirm that CD8+T cells are not associated with this phenotype, the authors should conduct to evaluate antigen-specific CD8+T-cell activation using OVA-expressing cancer cells and tumor growth after CD8+T-cell depletion by antibodies. Furthermore, in vivo analysis using conditional knockout mice such as Treg-cell-specific LTα1β2 depletion and LEC-specific LTβR depletion are needed.

Specific points;
1. In extended data Fig.2, can LTα1β2 secreted by Tregs be detected in culture supernatants?
2. In figure 4c, LTβR stimulation can compensate for cancer cell death by nciLT blocking. On the other hand, in figure 2c, p52 reduction still maintains. Are the results consistent?
3. In figure 7c, % of CD25+Foxp3+ population in CD4+T cells looks reduced by nciLT blocking. What mechanisms are involved?

Reviewer #3

(Remarks to the Author)
The manuscript elegantly elucidates the mechanism by which Tregs utilize lymphotoxin α1β2 to selectively activate LTβR nonclassical NFκB pathway on both tumor cells and LECs, thereby fostering tumor progression and metastasis. The study employs ciLT and nciLT, two decoy peptides with specificity for the LTβR-classical and nonclassical NFκB pathways, to meticulously dissect the distinct contributions of each branch of the bifurcated LTβR signaling in the context of tumor immunology. The manuscript offers a detailed exploration of biochemical, cellular, and in vivo data, clearly showing

important and consistent phenotypes. It demonstrates, through experiments on different tumor cell lines, the crucial role of LTαβ/LTβR pathways in the complex interactions among Tregs, LECs, and tumor cells within the tumor microenvironment. The authors need to clarify this study over previous research (Brinkaman et al., Nat. Commun, 2016; Piao et al., Nat. Commun, 2018; Piao et al., Cell Rep, 2020) for the novelty of this work. Some issues need to be addressed as list below

In details:
1. Fig.3 and 4 show that B16F10 tumor cells rely on LTβR nonclassical NFκB signaling for lymphatic migration and growth. Specifically, Fig.4c reveals that nciLT treatment significantly enhances apoptosis in B16F10 cells over 5 to 16 hours, but LTβR activation negates nciLT-induced apoptosis. Both in vitro and in vivo studies confirm nciLT's tumor growth effects depend on LTβR. Yet, its impact on apoptosis varies without detailed clarification, which only broadly mentions nciLT's promotion of apoptosis.

2. Fig.5 and Extended Data Fig.2a aim to investigate the potential role of Tregs interacting directly with tumor cells. Does the experimental design clearly distinguish whether the interaction between the two is direct or indirect? It is unclear if the current data conclusively prove the essential contribution of LTα from Treg in vivo to signal in LTβR, the conditional knockout of LTα in Treg might be needed.

3. Regarding the validation of differentially expressed genes from bulk RNA-seq data, it is indeed advisable to move beyond RNA-level validation methods such as RT-PCR. Protein-level detection methods such as flow cytometry or imaging offer a more comprehensive validation by reflecting the functional consequences of gene expression changes. The RNA-seq and cDNA microarray data lacks sufficient relevance to subsequent functional validation.

4. The interpretation of differentially expressed genes from bulk RNA-seq data could benefit from a more nuanced approach. For instance, in Fig.5j, it is observed that the CXCL10 signal is significantly upregulated in B16F10 cells following LTβR agonist treatment. However, it is an oversimplification to categorize CXCL10 solely as an angiogenic and immunosuppressive chemokine. To accurately elucidate the function of differentially expressed genes identified by RNA-seq, it is essential to integrate these findings with functional experiments, such as depletion/blocking experiments.

5. Fig.6d and 6e indeed present an intriguing phenotype where in vivo LTβR deficiency does not affect the growth of the primary tumor but leads to reduced survival of tumor-bearing mice. To further elucidate the reasons behind this observation, it is crucial to investigate whether factors beyond the primary tumor, such as metastasis, contribute to the decreased survival rate in LTβR-/- mice. Utilizing metastasis models, such as the B16F10 i.v. model, would be a valuable approach to assess the role of LTβR in metastatic processes.

minor issues with the figures:
a) The color scheme in Fig.1a is inconsistent with the other figures.
b) In line 155, the references should be Fig.2e, 3f, instead of Fig.2d-f.
c) It is recommended to include a schematic diagram of the Transwell assay in Fig.3 due to the various migration assays depicted.
d) The size and weight of B16F10 tumors in Fig.4f are excessively large, violating animal experiment ethics.
e) In line 249, the reference should be Fig.5i, not Fig.5.
f) In the schematic diagrams of in vivo experiments in Fig.4d and Fig.7a, C57/B6 mice should be depicted as dark-colored mice, not light-colored nude mice, to avoid misleading the readers.
g) In Fig.7, for the detection of cytokines secreted by TILs, the positive proportion should be analyzed.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Multiple deficiencies previously pointed out remain insufficiently addressed.

Most prominently, it remains unclear whether the ex vivo findings are relevant in vivo to support the statement “Tregs crosstalk with tumor cells and ECs via lymphotoxin signaling”. The newly added adoptive transfer studies attempt to address this but fall short. The authors report that Tregs co transferred with tumor cells enhance tumor growth and that this depends on Treg LT. But there is no characterization of the effects of Treg LT deficiency on Treg survival, nor any characterization of the tumor environment. In particular they do not determine whether in this model there is a connection in vivo between Treg lymphotoxin and tumor cell/LEC LT signaling.

The functional significance of Treg-mediated LTbR-NFkB axis in tumor cell/LEC is also unclear

Other examples of unaddressed points include the functional significance of immune changes, and the relevance of SOX18/FLRT2 in tumor angiogenesis, among others.

Reviewer #2

(Remarks to the Author)
I apologize for the inadequate interpretation of the data in a couple of Extended Figures.

Although the revised manuscript has been improved by additional analytical data, the following two points still require clarification to substantiate the conclusions of this study.

1. The authors have performed the analysis using human blood samples. However, it is necessary to prove at the protein level that LTα1β2+ Treg cells are actually infiltrating the tumors using human clinical specimens. The authors should further show whether these tumor-infiltrated Treg cells also express CXCR3 to validate the results of the in vitro chemokine analysis.

2. Since the authors state that LT deficient Tregs do not exist and compensatory changes that occur after deletion at early stages of Treg development are likely to mask the role of LT due to many other Treg suppressive mechanisms, the immunosuppressive activity of WT and LT-/- Treg cells may differ. If so, the difference in tumor growth between tumors with WT and LT-/- Treg cells may not be related to LTα1β2 expression in Treg cells. Therefore, they should demonstrate no difference in immunosuppressive activity between WT Treg cells and LT-/- Treg cells. Furthermore, in Extended 5c, Treg cells are administered five times more than tumor cells without other immune cells expressing LTα1β2. This is highly non-physiological for an in vivo experiment.

Reviewer #3

(Remarks to the Author)
The authors have addressed the concerns

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study is much improved with the new data. While it would be of added interest to determine if the effects are necessary (e.g., using FoxP3-Cre: LT flox mice), the authors have addressed our major concerns.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The manuscript has been revised well.I think this manuscript will be acceptable.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewers' comments:

Reviewer #1 (Remarks to the Author): with expertise in cancer immunology and vascular biology

In this manuscript, Piao et al. investigated the function of Treg-derived lymphotoxin (LT) on tumor growth and metastasis. Using in vitro systems, they showed that lymphotoxin signaling classical and nonclassical NFkB signaling, and nonclassical NFkB pathway promotes the proliferation and migratory abilities of tumor cells. Coculturing tumor cells with LT-expressing, but not LT-deficient, Tregs induces NFkB pathway, correlating with increased tumor cell proliferation and transwell migration. Treg coculturing with lymphatic endothelial cells (LECs) also increased their ability to promote tumor cell proliferation and migration in vitro. In vivo, LTbR-deficiency in tumor cells and nonclassical NFkB inhibitor treatment slowed down tumor growth and lymph node metastasis. LTbR knockout in the tumor stroma also prolonged the survival of tumor-bearing mice without impairing tumor growth. Based on these data, the authors conclude that Treg regulate tumor cell and LEC activities through lymphotoxin signaling.

Tregs are well established for their ability to promote cancer growth by suppressing immune responses. This manuscript provides interesting insights on how Tregs could regulate tumor cells and LECs and expands our knowledge on Treg biology.
However, data presented using in vitro systems did not lend sufficient support to conclude that "Tregs crosstalk with tumor cells and ECs via lymphotoxin signaling" in vivo. While in vitro experiments demonstrate the ability of Tregs to alter tumor cell/LEC activities via LT signaling, whether these observations are relevant in vivo is not clear from their experiments. Importantly, it has yet to be established (1) whether lymphotoxins are necessary factors by which Tregs regulate tumor growth/metastasis (via NFkB), **The in vitro data show that Treg LTαβ engages tumor and LEC LTβR primarily through the nonclassical NFκB signaling for multiple responses, including transendothelial migration, growth, and cytokine/chemokine production (Fig. 5a-i). We now use four approaches to study Treg cross talk with tumor cells and LECs in vivo: 1) Inoculate LTβR$^{-/-}$B16F10 melanoma into WT C57BL6 mice; or transfer WT B16F10 into LTβR$^{-/-}$ mice, to study regulated Tregs in tumor and LNs. 2) Adoptively transfer WT or LTα$^{-/-}$ Tregs plus B16F10 to C57BL/6 to see the different influences for tumor growth. 3) Adoptively transfer primary LECs together with tumor cells to WT or LTβR$^{-/-}$ mice, to study the impact of LEC LTβR on tumor metastases. 4) Analyze Tregs and LTβR-nonclassical NFκB-driven molecules/cytokines using NFκB classical and nonclassical specific blocking peptides. Since Treg specific LT $^{-/-}$ mice do not exist, we adoptively transferred WT or LTα$^{-/-}$ Tregs to tumors in WTC57BL/6 recipients, showing WT but not LTα$^{-/-}$ Tregs promote melanoma growth. We now add new data to support our hypothesis (see Fig. 4h, Fig. 6e-h, and Extended Data Fig 5c.).**

(2) whether Tregs are non-redundant sources of lymphotoxin-mediated tumor regulation. **We did not claim Tregs as non-redundant source of LT, but could be major source of membrane bound LT, since we demonstrate that both human and mouse Tregs express the highest level of surface LT among all immune cells (Piao et al., *Cell Reports* 2020; 30, 1052–1062, Figure 1B). We also analyzed published single cell RNA sequencing data, and found CD4 Tregs express the highest combined level of LTα and LTβ genes (lines 147-151) (Extended Data Fig.1d-e). We further stress these two points in the Results and Discussion.**

ıdeal experiments would require mouse models that delete lymphotoxin specifically in Tregs. **Unfortunately, LT deficient Tregs (e.g., Foxp3-Cre x LTα-flox) do not exist and compensatory changes that occur after deletion at early stages of Treg development are likely to mask the role of LT due to many other Treg suppressive mechanisms. For example, we have shown that Tregs also engage PD-1/PD-L1 on tumors and LECs (Piao et al., *Nat Commun*. 2022; 13, 2176; Piao et al. J Immunol (2023) 210 (1_Supplement): 86.09). However, we transferred 1x10^6 WT or LTα$^{-/-}$ Tregs together with 0.2x10^6 B16F10 into WT C57BL/6 mice. WT Tregs significantly promoted melanoma growth, while LTα$^{-/-}$ Tregs failed to do so, suggesting the direct role of Treg LTα1β2 on melanoma growth (lines 345-348) (Extended Data Fig. 5c).**

The studies focused on LTbR signaling in tumor cells and LECs. What is the rationale for focusing on these cells?
A: In our previous studies, we found that among T cell subsets, both human and mouse Tregs express the highest level of membrane anchored LTα1β2, suggesting that Tregs are the predominant population to interact with LTβR, which is highly expressed on human and mouse LECs (Piao et al., 2020, Cell Reports). LT deficiency prevents Treg lymphatic

migration to draining LNs and impairs their suppressive function in models of allograft protection. Engagement of LTβR on LECs by LTα1β2+ but not LTα-/- Treg cells induces LEC basal protrusions that support Treg afferent transendothelial migration (Brinkman et al, *Nat Commun*. 2016; 7: 12021).
The majority of solid tumors contain both tumor and stroma cells, including LECs which form lymphatic vessels for transporting metastatic and immune cells to draining LNs. Thus, we focused on LTβR signaling in tumor cells and LECs, using these LTβR decoy peptides to selectively target either classical or nonclassical NFκB signaling pathways in melanoma cells and LECs to dissect the molecular underpinnings related to cancer cell migration and metastasis in the syngeneic mouse melanoma model whose metastasis follow reliable lymphatic routes. Now we stress these rationales for focusing on tumor cell and LECs in the Introduction(100-102), Results (lines 147-151), and Discussion (lines 415-418; 462-465).

Many other stromal cells express the receptor even at higher level than tumor/LECs, such as blood endothelial cells and fibroblasts.
Both LECs and blood endothelial cells (BECs) express comparable levels of LTβR, and fibroblasts have lower levels (Onder et al. J Exp Med. 2013, 210(3):465-73.). We now also include the analyzed LTβR expression level on cancer associated fibroblasts (CAFs) from published single cell RNA sequencing data, showing a lower level than tumor and endothelial cells, especially in human breast cancer and melanoma (Extended Data Fig.1a-c). BEC, especially microvascular BEC, LTβR signaling might be of interest for Treg recruitment to tissue/tumor from blood circulation, which may contribute to the reduced intratumoral Tregs in nciLT treated melanoma. The manuscript is already quite long and including a comprehensive analysis of BEC is beyond the scope of the present study.

Is Treg-mediated LTbR-NFkB axis in tumor/LEC important and nonredundant?
Teffs and B cells also express LTαβ but at lower levels than Tregs. Tregs preferentially stimulate tumor nonclassical NFkB -NIK signaling (Fig. 5a), while Teffs only stimulate tumor cell classical NFkB signaling (Fig.5a, c), and B cells induce no NFκB signaling (see new Extended Data Fig. 3f). Lines 263-266.

Major concerns:
Analyses of human tumors revealed that pathways upregulated in LTbR-high tumors are enriched in cytokine/chemokine signaling. However, the in vitro and mouse studies in this manuscript are mostly focused on tumor cell migration.
We do not find any conflict on this point. Cytokine/chemokine regulation is closely related to cell migration. "The top ten significantly enriched signaling activities (which include cytokine/chemokine signaling) identified in LTβR high cohorts (Fig. 1g) were related to regulation of cellular chemotaxis, immune cell activation and migration, and regulation of inflammatory responses" (lines 139-142).

Is tumor invasiveness/EMT gene signature also upregulated in humans?
As we originally stated, "S100A family genes (such as S100A4 and S100A9) with well-established roles in tumor metastases and invasion" (lines 137-138) are upregulated in LTβR high melanoma cohorts.

It is not clear why comparisons between the expression level of LTbR and other oncogenes is meaningful or relevant.
As we originally stated, "LTβR expression levels were comparable with many oncogenes such as MYC, TP53, and BRCAs for melanoma, breast cancer, and lung cancer (Fig. 1b)" (lines 122-124). This observation supports "High LTβR expression on tumors denotes worse patient prognosis".

While the tumor cell lines used in the study express LT receptor, immunoblot data in Fig 2 showed weak baseline activation of NFkB activation in the absence of stimulus (by LT receptor antibody ligation and Treg coculturing). To what extent is NFkB activated in tumor cells in vivo? Did Treg depletion decrease NFkB signaling in vivo?
LTβR-classical NFκB signaling in resting tumor cell lines is at a low level of activation (line 156). However, overall classical NFκB is constitutively activated in most tumor cells. In vivo, both NFκB pathways are overall constitutively activated in tumor cells. However, Tregs specifically enhance LTβR nonclassical NFkB signaling (Fig.5a,c). Thus, Treg depletion would not affect overall classical or nonclassical NFkB signaling in vivo.

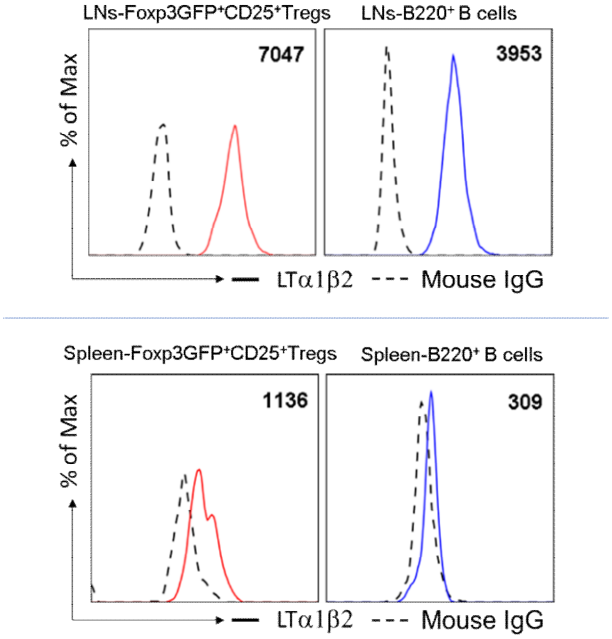
The in vitro data relied almost exclusively on antibody ligation to activate LTbR. Do other approaches of LT receptor activation trigger similar patterns of downstream signaling? Such as recombinant lymphotoxin peptides?

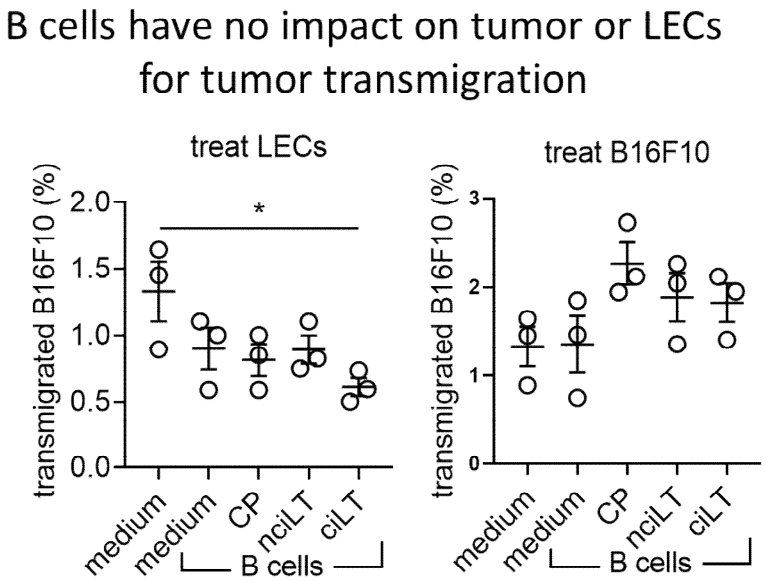
The approach of using agonist anti-mouse LTβR mAb (such as clone 3C8) to activate LTβR signaling in various cells has been well established (Dejardin, E., et. al. (2002), Immunity. 17(4):525-35) and is more stable than recombinant mouse LTα1β2 protein. LIGHT could be used for LTβR activation, but it also activates HVEM. However, we also used recombinant human LTα1β2 protein (R&D Systems) to stimulate human A375 melanoma cells (Fig. 2e,f).

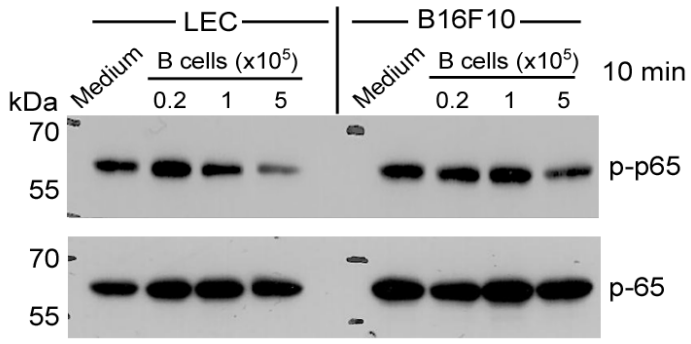
The authors previously show that effector T cells express significant levels of lymphotoxins (Piao, Cell Report 2022).
We previously showed that effector T cells (activated CD4 T cells) express significantly lower levels of lymphotoxin than Tregs (Fig. 1b and Fig. 5a, MFI: Tregs:1375; activated CD4: 719. Piao, Cell Reports 2020: https://www.sciencedirect.com/science/article/pii/S2211124719317486?via%3Dihub

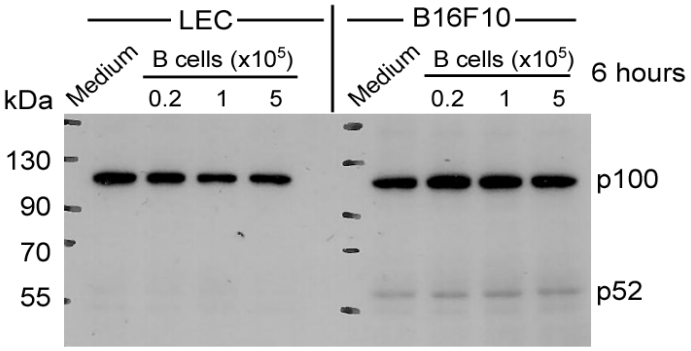
Why did coculturing with Teffs (Figure 5) not activate NFkB signaling?
Coculturing with Teffs (Figure 5a) did not activate nonclassical NFκB signaling in B16F10 but induced strong classical NFκB p65 (Extended Data Fig. 3b).

What about B cells which have high LT. In other words, are Tregs just a minor source of LT? If so it would call into question the significance of the ex vivo findings.
B cells have proinflammatory and proimmune functions, not suppressive functions. We now provide new data that B cell LTα1β2 is expressed at lower levels than Treg LTα1β2, and neither induces NFκB signaling nor promotes tumor cell migration (Extended Data Fig. 3e-g, and see below).









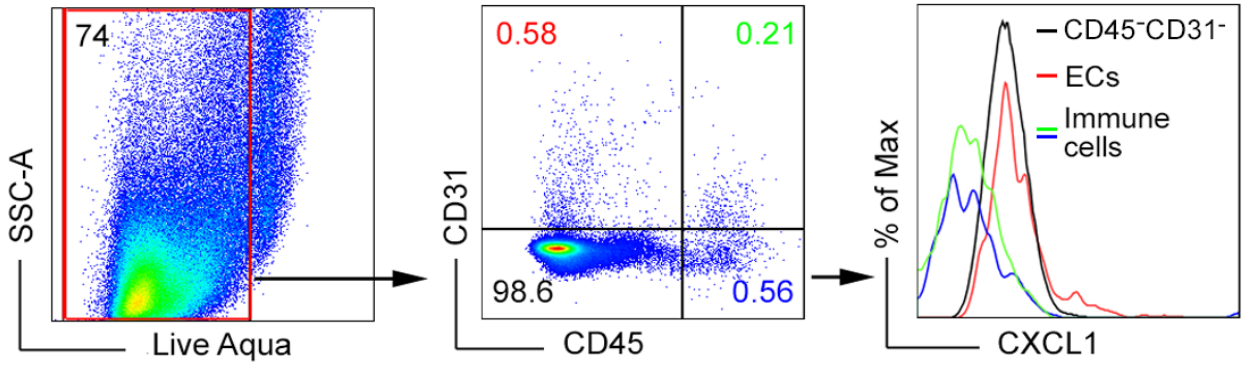
What's the mechanism for prolonged survival of tumor-bearing LTbR-/- mice? Did the mice have fewer metastases?
We reanalyzed the melanoma model in LTβR-/- mice and found reduced tumor growth compared to the WT mice. Tumor-bearing LTβR-/- mice have significantly reduced intratumoral Tregs which may contribute to the prolonged survival, and we added this data to Fig. 6d-g LTβR-/- mice do not have peripheral LNs, therefore we could not directly

assess tumor metastasis to the draining LNs. However, we did test pulmonary metastasis in LTβR-/- mice after intravenous injection and found significantly reduce lung metastases in LTβR-/- mice. Importantly, we adoptively transferred primary LECs together with B16F10 intravenously into LTβR-/- mice and found the transferred LEC increased pulmonary metastases (Fig. 6h).

In Figure 7 the CD45+ vs. CD45- populations are not well separated, and CD45 vs. live/dead-Aqua is not well compensated; thus the subsequent T cell/MDSC quantifications are questionable.
Now adjusted the gating strategy by first gating on live cells (Aqua/dead-) then gating CD45+ cells. The subsequent T cell/MDSC frequencies remain unchanged (see the gating strategy in Fig. 7d).

More importantly, the functional importance of the changes in the is not clear, and how it is tied to the changes in LEC/tumor responses to LT inhibition?
These immune profile changes of Tregs, MDSC, CD4 T cells, and CD8 T cells in TILs are very well known throughout the literature (https://www.cell.com/cancer-cell/pdf/S1535-6108(23)00042-9.pdf , https://www.nature.com/articles/s41571-023-00846-y , https://www.nature.com/articles/s43018-023-00521-2 https://www.nature.com/articles/s41416-020-01048-4) to highly determine immune and tumor outcomes. We more thoroughly present these interpretations in the Discussion.

The authors did not provide data supporting the statement that tumor- or endothelium-derived CXCL1 was suppressed by nciLT: tumor and EC gatings need to be shown
In our original manuscript we showed nciLT suppressed CXCL1 production in human Tregs cocultured A375 melanoma cells or LECs (Extended Data Fig. 3c-d). In Fig. 7d, the gated CD45 negative population of melanoma cells had significantly suppressed CXCL1 production in nciLT treated melanoma. We have now analyzed the $CD45^-$ $CD31^-$ population of WT melanoma at day 13 (was not included in our original manuscript), and this showed that CXCL1 was expressed by more than 98% of $CD45^-CD31^-$ tumor cells including fibroblast cells (see below and Extended Data Fig. 3e). Further, we also gated $CD45^-$ $Lyve\text{-}1^+$ (LEC) population for CXCL10 expression (Fig7d).



Since the $CD45^-CD31^-$ cells include fibroblast cells, we analyzed tumor CXCL1 expression by transplanting B16F10-GFP melanoma, however, the transferred B16F10-GFP did not show a distinct population.

More importantly, the data at best imply the correlation between CXCL1 decrease and G-MDSC reduction, but whether this chemokine is important in the process is not established.
CXCL1 (melanoma growth-stimulatory activity/growth-regulated protein α) is major myeloid cell chemoattractant, and is highly expressed in melanoma cells. The role of CXCL1 in tumorigenesis of melanoma is well established (Richmond & Thomas, 1988; Thomas & Richmond, 1988; Chen et al., 2001; Geiser, Dewald , 1993; Moser, Schumacher, von Tscharner, Clark-Lewis, & Baggiolini, 1991. https://www.sciencedirect.com/science/article/pii/S0092867412006435
The claim that nciLT inhibits endothelial specific SOX18 and FLRT2 "to reduce tumor angiogenesis" was not established. Correlation was shown but the role of SOX18/FLRT2 was not investigated.
We believe the correlation is highly relevant and well supported by extensive literature (https://elifesciences.org/articles/24238; https://aacrjournals.org/cancerres/article/72/12/3105/575575/Genetic-Ablation-of-SOX18-Function-Suppresses ; https://www.sciencedirect.com/science/article/pii/S1044579X1830141X ; https://pubmed.ncbi.nlm.nih.gov/35104247/ ; https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2023.1229227/full). SOX18 is an important

transcription factor involved in the development of cardiovascular and lymphatic vessels during embryonic development. The SOX18 protein has been suggested to be a significant diagnostic and prognostic marker in various types of cancer. In addition, it is involved in the progression of atherosclerosis and wound healing processes. It has been observed that its level is higher in a number of cancer types, including melanoma, pancreas, stomach, liver, breast, lung, ovarian and cervical cancer. Lines 485-486 discuss that our results are "in line with SOX18-deficient mice having suppressed B16F10 lymphangiogenesis and metastases (Duong T et al. Cancer Res 72, 3105-3114)".

Are CD8+ T cells important?
The literature well supports that CD8 infiltration in the tumor is critical for anti-tumor immunity (https://www.nature.com/articles/s41577-021-00574-3; https://www.sciencedirect.com/science/article/pii/S1074761323004107?via%3Dihub). Now we discuss more about how nciLT impacts intratumoral CD8 which corelates to Treg recruitment (lines 492-494). Blocking endothelial LTβR signaling inhibits tumor egress of all immune cells, including CD8 T cells. Since tumor cell-derived CXCL10 and CCL5 recruit both Tregs and CD8 T cells, blockade of the initial prompt recruitment of Tregs to the tumor bed may play a critical role for tumor regression, which unleashes CD8 proliferation.

Reviewer #2 (Remarks to the Author): with expertise in cancer immunology, Tregs

Piao W et al found that regulatory T (Treg) cell express high lymphotoxin (LT) α1β2 which preferentially stimulate LTβ receptor (LTβR) and induce non-classical NFκB signaling in tumor cells and lymphatic endothelial cells (LECs), resulting in increased tumor growth and metastasis. In addition, they developed LTβR blocking peptides which selectively inhibit classical (ciLT) or nonclassical (nciLT) NFκB signaling pathways. Especially, blocking peptide targeting nciLT inhibits tumor growth and metastasis through reducing infiltration of suppressive immune cells, such as Treg cells and myeloid-derived suppressor cells, and tumor angiogenesis. They suggest that Treg cells directly promote nciLT NFκB signaling pathways in tumor cells and LECs, which can be targetable by blocking peptides.
It is potentially interesting to note that secreted factors produced by Treg cells directly promote tumor growth and metastasis.
LTα1β2 is membrane bound protein and is not a secreted factor. Our experimental model specifically showed that direct cell contact was required (Extended Data Fig. 5a-b). Tregs directly use their surface LTα1β2 to bind its cognate receptor LTβR on tumor cell or endothelial cells, to promote tumor growth and metastasis (Fig. 5d-i). We also showed that "the soluble secretomes from LECs did not affect tumor growth, suggesting cell surface molecules on LECs interact with receptors or ligands on tumor cells for proliferation (line 336-338)".

However, the impact of this study is severely impaired since there are critical issues with the experimental method, interpretation of the data, and several points were not sufficiently addressed.
General comments;
Although the authors have confirmed LTβR expression in cancer cells (supplementary table 1), it is necessary to present the expression profile of both LTα1β2 and LTβR in multiple cell populations infiltrated in the tumor microenvironment, such as macrophages, B cells, myeloid-derived suppressor cells and fibroblasts. Since single cell RNA sequencing data has been published for various cancer types, the expression analysis of these molecules in tumor-infiltrating immune cells should be shown by utilizing the published data. **We added the expression profile of both LTα1β2 and LTβR in multiple cell populations infiltrated in the tumor microenvironment of melanoma, breast cancer, and prostate cancer patients, using published single cell RNA sequencing data. (lines 147-151) (Extended data Fig.1)**

Based on the results, the author should clarify why this study focuses only on the relationship between LTα1β2 from Tregs and LTβR on both cancer cells and LECs.
We added additional rationales for our focus in the Introduction (lines 77-88).

Furthermore, it is very important to present the actual expression profile of LTα1β2 in Treg cells
In our original manuscript, we provided these in Extended Data Fig. 3a that human Tregs express significantly higher LT α1β2 than effector T cells with flow cytometric analysis.

(and activation status of nciLT NFκB signaling pathways in cancer cells and LECs by using human clinical specimens such as FFPE

We believe It is almost impossible to study NFκB signaling in FFPE human clinical samples, and the differential expression levels of signaling molecules related to NFκB signaling would not inform about the LTβR-specific NFκB signaling.

In vitro studies of co-culture using cancer cell lines and immune cells were neatly conducted, however, in vivo evaluation is not sufficient. In Figure 5k, CXCL10 and CCL5 are elevated by TLβR stimulation. These chemokines are known to be essential for the infiltration of CD8+T cells (https://pubmed.ncbi.nlm.nih.gov/29579623/ , https://www.nature.com/articles/nri.2017.49). To confirm that CD8+T cells are not associated with this phenotype, the authors should conduct to evaluate antigen-specific CD8+T-cell activation using OVA-expressing cancer cells and tumor growth after CD8+T-cell depletion by antibodies. Furthermore, in vivo analysis using conditional knockout mice such as Treg-cell-specific LTα1β2 depletion and LEC-specific LTβR depletion are needed

Our bulk RNA sequencing and RT-PCR data showed CXCL10 and CCL5 are driven by LTβR-nonclassical NFkB signaling, and are specifically blocked by nciLT. We analyzed these chemokines for expression in tumor or EC cells in peptide treated melanoma to better support our hypothesis (Fig 7d). However, employing an entirely new model to evaluate CD8 cells is beyond the scope of the current report. Unfortunately, LT deficient Tregs (i.e., LTα-flox) do not exist and compensatory changes that occur after deletion at early stages of Treg development are likely to mask the role of LT due to many other Treg suppressive mechanisms. For example, we have shown that Tregs also engage PD-1/PD-L1 on tumors and LECs (Piao et al., *Nat Commun*. 2022; 13, 2176; Piao et al. J Immunol (2023) 210 (1_Supplement): 86.09). LEC specific depletion of LTβR is incomplete and associated with either compensatory changes or failure of lymphatics to develop (Lucas Onder et al. Immunity 2017; 47,80-92) (Saxena et al., Cell Rep.2022;3, 110727). We performed additional new experiments where we adoptively transfer WT or LT-/- Tregs together with B16F10 to C57BL/6 recipients and also adoptively transfer primary LECs together with tumors into LTβR-/- recipients. The results from these experiments showed the critical roles of in vivo Treg LTα1β2 and LEC LTβR signaling in tumor immunity (Extended Fig. 5c, Fig. 6h).

Specific points;

1. In extended data Fig.2, can LTα1β2 secreted by Tregs be detected in culture supernatants?

In the "Introduction" line 53-54: "LTβR has two known ligands: the membrane bound lymphotoxin (LT) heterotrimer (LTa1β2)…" There is no secreted LTα1β2.

2. In figure 4c, LTβR stimulation can compensate for cancer cell death by nciLT blocking. On the other hand, in figure 2c, p52 reduction still maintains. Are the results consistent?

LTβR stimulation induced both classical and nonclassical NFkB signaling. In Fig.4c, nciLT inhibited constitutively activated nonclassical NFkB signaling and induced tumor cell apoptosis which was compensated by LTβR stimulation, indicating that LTβR-mediated classical NFkB promoted tumor growth. Now we clarify this in the Results (lines 223-225). There is no conflict with the results in Fig. 2c, that nciLT slightly inhibited low level constitutively active NIK signaling (p100/p52 processing) and blocked fully activated LTβR nonclassical NFkB signaling.

3. In figure 7c, % of CD25+Foxp3+ population in CD4+T cells looks reduced by nciLT blocking. What mechanisms are involved?

We acknowledge the confusion. To clarify and precise this mechanism, we edited the sentences In the Discussion line 489: "Tumor and LEC derived CXCL1, CXCL5, and CXCL10 recruit CXCR2+ MDSCs and CXCR3+Tregs (Moreno Ayala, M. A. et al, Immunity. 2023 Jul 11;56(7):1613-1630), the major immune suppressive components in TME for tumor growth, neovascularization, and metastasis. Thus, the suppression of these chemokines by nciLT are likely key attributes for the reduced immune suppressive cells in tumor". In addition, in the original manuscript, we indicated and discussed the reduced CXCL1 expression in tumors and LECs in nciLT treated melanoma (Fig. 7d), supporting possible mechanisms for the reduction in tumor Tregs at day 13. Since Treg recruitment to tissues or tumors is a rapid and early event in immunity, the early inhibition of Treg recruitment may be more critical than reduced Treg egress from tumor.

Reviewer #3 (Remarks to the Author): with expertise in cancer immunology, lymphotoxin signaling

The manuscript elegantly elucidates the mechanism by which Tregs utilize lymphotoxin α1β2 to selectively activate LTβR nonclassical NFκB pathway on both tumor cells and LECs, thereby fostering tumor progression and metastasis. The study employs ciLT and nciLT, two decoy peptides with specificity for the LTβR-classical and nonclassical NFκB pathways, to meticulously dissect the distinct contributions of each branch of the bifurcated LTβR signaling in the context of tumor immunology. The manuscript offers a detailed exploration of biochemical, cellular, and in vivo data, clearly showing important and consistent phenotypes. It demonstrates, through experiments on different tumor cell lines, the crucial role of LTαβ/LTβR pathways in the complex interactions among Tregs, LECs, and tumor cells within the tumor microenvironment. The authors need to clarify this study over previous research (Brinkaman et al., Nat. Commun, 2016; Piao et al., Nat. Commun, 2018; Piao et al., Cell Rep, 2020) for the novelty of this work. Some issues need to be addressed as list below

In details:

1. Fig.3 and 4 show that B16F10 tumor cells rely on LTβR nonclassical NFκB signaling for lymphatic migration and growth. Specifically, Fig.4c reveals that nciLT treatment significantly enhances apoptosis in B16F10 cells over 5 to 16 hours, but LTβR activation negates nciLT-induced apoptosis. Both in vitro and in vivo studies confirm nciLT's tumor growth effects depend on LTβR. Yet, its impact on apoptosis varies without detailed clarification, which only broadly mentions nciLT's promotion of apoptosis.

LTβR stimulation induces both classical and nonclassical NFkB signaling. nciLT inhibited constitutively activated nonclassical NFkB signaling and induced tumor cell apoptosis which was compensated by LTβR stimulation, indicating that LTβR-mediated classical NFkB promoted tumor growth. Now we clarify this in the Results (lines 223-225).

2. Fig.5 and Extended Data Fig.2a aim to investigate the potential role of Tregs interacting directly with tumor cells. Does the experimental design clearly distinguish whether the interaction between the two is direct or indirect? It is unclear if the current data conclusively prove the essential contribution of LTα from Treg in vivo to signal in LTβR, the conditional knockout of LTα in Treg might be needed.

In vitro, we showed that LTα1β2 is required for Treg regulation of tumor growth and migration. In complex in vivo systems, any LT-expressing immune cell could trigger LTβR signaling in tumors or LECs. Our previous studies revealed that Tregs which have the highest level of LT, rapidly migrate to the tumor and influence other immune cells lymphatic migration, thus Treg direct crosstalk with tumors and LECs is critical for tumor growth and metastasis. In the tumor TME, the other LTβR ligand LIGHT, which is secreted by tumors or other immune cells (e.g., CD8 T cell) can activated LTβR signaling, therefore the impact of in vivo LTβR blockade might not be specific even in Treg-specific LTKO mice. To examine the direct contribution of Treg LTα1β2 for in vivo LTβR signaling, we adoptively transferred WT or LTα KO Tregs together with B16F10 into C57BL/6 recipients and found WT but not LTα KO Tregs promoted tumor growth (Extended Fig. 5c).

3. Regarding the validation of differentially expressed genes from bulk RNA-seq data, it is indeed advisable to move beyond RNA-level validation methods such as RT-PCR. Protein-level detection methods such as flow cytometry or imaging offer a more comprehensive validation by reflecting the functional consequences of gene expression changes. The RNA-seq and cDNA microarray data lacks sufficient relevance to subsequent functional validation.

We conducted bulk RNA sequencing and cDNA microarray to identify tumor and LEC LTβR signaling-regulated down-stream molecules. We validated each candidate molecule by RT-PCR (Fig.5k, 5m; 6j), flow cytometry (Fig. 4h; Fig. 7d) , and immunohistochemistry (Fig. 7e, f; Extended Data Fig. 4c-d).

4. The interpretation of differentially expressed genes from bulk RNA-seq data could benefit from a more nuanced approach. For instance, in Fig.5j, it is observed that the CXCL10 signal is significantly upregulated in B16F10 cells following LTβR agonist treatment. However, it is an oversimplification to categorize CXCL10 solely as an angiogenic and immunosuppressive chemokine. To accurately elucidate the function of differentially expressed genes identified by RNA-seq, it is essential to integrate these findings with functional experiments, such as depletion/blocking experiments.

The identified genes (such as CXCL1, CXCL10) from RNA-seq were validated at the protein level in immunohistochemistry of peptide influenced tumor and LECs in vitro (Extended Data Fig. 4c-d), and by flow cytometry analysis of tumor cell/LEC-derived CXCL1 or LEC-derived CXCL10 expression (Fig. 7d) to further support our hypothesis.

5. Fig.6d and 6e indeed present an intriguing phenotype where in vivo LTβR deficiency does not affect the growth of the primary tumor but leads to reduced survival of tumor-bearing mice. To further elucidate the reasons behind this observation, it is crucial to investigate whether factors beyond the primary tumor, such as metastasis, contribute to the decreased survival rate in LTβR-/- mice. Utilizing metastasis models, such as the B16F10 i.v. model, would be a valuable approach to assess the role of LTβR in metastatic processes.

We may have created some confusion: We now analyze more melanoma bearing LTβR$^{-/-}$ mice and observe reduced tumor growth (now in Fig. 6d). We further analyzed tumor and TILs of these mice at day 13 by immunohistochemistry and flow cytometric analysis and observed reduced tumor vascularization (Fig. 6e) and TIL-Tregs (Fig. 6g) which may contribute to the prolonged survival (Fig. 6f). LTβR$^{-/-}$ mice do not have peripheral LNs, therefore we could not directly assess tumor metastasis to the draining LNs. However, we studied pulmonary metastasis in LTβR$^{-/-}$ mice after intravenous injection and found reduced tumor lung metastasis in LTβR$^{-/-}$mice (Fig. 6h)

minor issues with the figures:

a) The color scheme in Fig.1a is inconsistent with the other figures. **We unified the color scheme.**

b) In line 155, the references should be Fig.2e, 3f, instead of Fig.2d-f. **This has been corrected.**

c) It is recommended to include a schematic diagram of the Transwell assay in Fig.3 due to the various migration assays depicted. **We added this to Fig. 3a.**

d) The size and weight of B16F10 tumors in Fig.4f are excessively large, violating animal experiment ethics. **Now it's within the limit approved by our IACUC.**

e) In line 249, the reference should be Fig.5i, not Fig.5. **This has been corrected.**

f) In the schematic diagrams of in vivo experiments in Fig.4d and Fig.7a, C57/B6 mice should be depicted as dark-colored mice, not light-colored nude mice, to avoid misleading the readers. **We changed to black color mice.**

g) In Fig.7, for the detection of cytokines secreted by TILs, the positive proportion should be analyzed. **We analyzed CXCL1 expression in the CD45^{+} proportion (immune cells) and found much less level than that of CD45^{-} proportion (Extended Data Fig. 4e).**

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Multiple deficiencies previously pointed out remain insufficiently addressed. Most prominently, it remains unclear whether the ex vivo findings are relevant in vivo to support the statement “Tregs crosstalk with tumor cells and ECs via lymphotoxin signaling”. The newly added adoptive transfer studies attempt to address this but fall short. The authors report that Tregs co transferred with tumor cells enhance tumor growth and that this depends on Treg LT. But there is no characterization of the effects of Treg LT deficiency on Treg survival

Our previous publication (Brinkman, C. C. et al. Treg engage lymphotoxin beta receptor for afferent lymphatic transendothelial migration. Nat Commun 7, 12021, doi:10.1038/ncomms12021 (2016)) showed that WT and LT-deficient Treg have equal suppressive function. There were also no significant differences in expression of IL-10, TGFβ1, perforin, granzyme B, IFNγ, CD39, CD73 and CTLA4 between WT and LTα -deficient Treg. LTα-deficient Treg had no defect in proliferation or viability compared with WT Treg when stimulated with anti-CD3ε and anti-CD28 in vitro. This information is now added in main text lines 502-503.

nor any characterization of the tumor environment. In particular they do not determine whether in this model there is a connection in vivo between Treg lymphotoxin and tumor cell/LEC LT signaling.

We provide new evidence that Tregs directly impact LTβR- nonclassical signaling in tumor cells and in LECs in vivo. First, we intravenously transferred WT or LTα-deficient Tregs to LTβR-depleted (KO) mice (whose LECs have no LTβR expression) and inoculated with WT B16F10. Thus, in this system, Tregs are only allowed to ligate or stimulate LTβR signaling in tumor cells. The WT Tregs significantly promoted melanoma growth, while LTα-deficient Treg failed to do so. There was also increased CXCL1, CXCL10, and CCL5 expression in melanoma cells in mice injected with WT but not LTα-deficient Tregs, clearly showing Treg LTα1β2 can directly activate tumor cell LTβR nonclassical NFkB signaling. We updated this information in the main text lines332-339, and moved “Genes driven by tumor LTbR NFkB signaling” in front of the new data.

Secondly, we intravenously transferred WT or LTα-deficient Tregs to WT mice inoculated with LTβR-depleted (KO) B16F10. In this system, Tregs cannot ligate or stimulate LTβR signaling in tumor cells but only in stroma cells, including LECs. Although WT Tregs did not significantly promote LTβR-deficient melanoma growth, they did significantly enhance LEC expression of Sox18, FLRT2, and Clec14, as well as LEC-derived CXCL10 expression, while LTα-deficient Tregs failed to do so. We included these results in the main text lines 379-396.

The functional significance of Treg-mediated LTbR-NFkB axis in tumor cell/LEC is also unclear

We updated the Summary and the Results to clearly show that Tregs LTα1β2 predominantly activate LTβR-non classical NFkB signaling on tumor cells and LECs to promote tumor growth and metastasis. We identified important chemokines CXCL1 and CXCL10 in tumor cells, which are responsible for immunosuppressive Treg and MDSC recruitment to tumor, and also identified endothelial specific and angiogenesis related proteins such as SOX18, FLRT2, and Clec14 in LECs. We proved all these genes are driven by LTβR-nonclassical signaling, and induced by Treg LTα1β2 in vitro and in vivo. Finally, we proved that these genes are specifically down regulated by LTβR-nonclassical NFkB blockade in vitro and in vivo.

Other examples of unaddressed points include the functional significance of immune changes, and the relevance of SOX18/FLRT2 in tumor angiogenesis, among others.

The transcription factor SOX18 has been identified as a critical switch for lymphangiogenesis in the mouse embryo. SOX18 is also critical for tumor-induced lymphangiogenesis. Suppressing SOX18 function is sufficient to impede tumor metastasis. SOX18 is re-expressed during tumor-induced neolymphangiogenesis. B16F10 melanoma had reduced rate of metastasis to the regional draining lymph node in Sox18-deficient mice (Cancer Res (2012) 72 (12): 3105–3114, cited as Ref#32). Role of the SOX18 in neoplastic processes has been confirmed in many studies (Ref#18, #32, #34).

High expression of FLRT2 has been shown in malignant abnormal vessels of advanced human colon cancers, and has been included in prognostic signature genes for bladder cancer (Ref#38), early-

stage stomach cancer (Ref#36), and prostatic adenocarcinoma (Ref#37). Endothelial cell-specific deletion of Flrt2 in mice selectively pruned abnormal vessels and suppressed tumor metastasis (Ando et al, J Clin Invest (2022) 135(5):584–90. doi: 10.1172/JCI153626, Ref#35).

Given all these important studies which demonstrated SOX18 and FLRT2 are critical genes for tumor-induced angiogenesis/neolymphangiogenesis, our cDNA microarray data and RT-PCR in LTβR stimulated WT LECs, NIK$^{-/-}$ LECs, and IKK$^{-/-}$ LECs showed these genes are specifically driven by LTβR-nonclassical but not classical NFkB signaling. We further observed SOX18 and FLRT2 protein were highly expressed in tumor-associated lymphatic vessels in the mouse B16F10 melanoma model, and were down regulated by nonclassical NFkB signaling blockade, which also significantly reduced angiogenesis. Adoptively transferred WT Tregs to WT mice with LTβR$^{-/-}$ B16F10 melanoma (which allow Treg LTα1β2 to only ligate and activate LTβR on stroma cells but not tumor cells) significantly induced SOX18 and FLRT2 expression on lymphatic vessels, while LTα$^{-/-}$ Tregs failed to do so (Extended Data Fig.6a). Thus, as the down-stream molecules of LTβR-nonclassical NFkB signaling, SOX18 and FLRT2 regulated expression sufficiently address the main topic on which we focused: Tregs dialogue with tumor and endothelium through lymphotoxin signaling.

Reviewer #2 (Remarks to the Author):
I apologize for the inadequate interpretation of the data in a couple of Extended Figures.
Although the revised manuscript has been improved by additional analytical data, the following two points still require clarification to substantiate the conclusions of this study.

1. The authors have performed the analysis using human blood samples. However, it is necessary to prove at the protein level that LTα1β2+ Treg cells are actually infiltrating the tumors using human clinical specimens.
   By flow cytometry analysis we showed LTα1β2 high expressing Treg cells in TIL of mouse melanoma (now Extended Data Fig. 4e) which fully support our hypothesis. We confirmed high level lymphotoxin expression on human Tregs in TILs of breast cancer and prostate cancer (Extended Data Fig.1d and 1e) from published single cell RNA sequencing data, which high likely reflects the protein level.

   The authors should further show whether these tumor-infiltrated Treg cells also express CXCR3 to validate the results of the in vitro chemokine analysis.
   We assessed by flow cytometry for T cell CXCR3 and MDSC CXCR2 expression in tumor and draining LNs on day 13 WT B16F10 melanoma. The results show more than 80% of Foxp3+ CD4+ TIL-Tregs and around 18% of TIL-CD8 T cells express CXCR3. In the draining LNs, only 18% of Tregs and 10%of CD8 T cells express CXCR3. Most MDSC express CXCR2 but with low MFIs. These data are now presented in Extended Data Fig.7b, main text lines 523-525.

2. Since the authors state that LT deficient Tregs do not exist and compensatory changes that occur after deletion at early stages of Treg development are likely to mask the role of LT due to many other Treg suppressive mechanisms, the immunosuppressive activity of WT and LT-/- Treg cells may differ. If so, the difference in tumor growth between tumors with WT and LT-/- Treg cells may not be related to LTα1β2 expression in Treg cells. Therefore, they should demonstrate no difference in immunosuppressive activity between WT Treg cells and LT-/- Treg cells.
   This is similar to the concern of Reviewer 1 above. LT-deficient Treg have no defect in immune suppressive function (Brinkman, C. C. et al. Treg engage lymphotoxin beta receptor for afferent lymphatic transendothelial migration. Nat Commun 7, 12021, doi:10.1038/ncomms12021 (2016)). There were also no significant differences in expression of IL-10, TGFβ1, perforin, granzyme B, IFNγ, CD39, CD73 and CTLA4 between WT and LTα -deficient Treg. LTα -deficient Treg had no defect in proliferation or viability compared with WT Treg when stimulated with anti-CD3ε and anti-CD28 in vitro. This information is now added in main text lines 502-503.

   Furthermore, in Extended 5c, Treg cells are administered five times more than tumor cells without other immune cells expressing LTα1β2. This is highly non-physiological for an in vivo experiment.

We apologize for a typographical error, now corrected to $0.5x10^6$ WT or LT$\alpha^{-/-}$Tregs intravenously transferred to mice injected with $0.2x10^6$ WT B16F10. In the additional Treg adoptive transfer assays, we used a 1:1 ratio of Tregs to tumor cells (Fig. 5n, Fig. 6g).

Reviewer #3 (Remarks to the Author):
The authors have addressed the concerns.

Reviewer #4 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The study is much improved with the new data. While it would be of added interest to determine if the effects are necessary (e.g., using FoxP3-Cre: LT flox mice), the authors have addressed our major concerns.
Thanks Reviewer#1, your suggestions have greatly improved the quality of the manuscript.

Reviewer #2 (Remarks to the Author):

The manuscript has been revised well. I think this manuscript will be acceptable.
Thanks Reviewer#2, your suggestions have greatly improved the quality of the manuscript.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.