nature portfolio

Peer Review File

Tumor draining lymph nodes connected to cold triple-negative breast cancers are characterized by Th2-associated microenvironment

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this work, Guo et al aim at correlating how TLDN cell contexture and transcriptomic profiles impact lymphocyte infiltration into tumors (cold vs hot). The authors use human paired tissues from breast cancer patients and apply transcriptomics and IF techniques to answer this question. The developed intranodal self-correlation analyses approach is of interest and could represent a methodological report per se. Instead several of the biological conclusions remain correlative, descriptive or overstated, without any functional validation.

Major points:

1) Cell abundance: the authors claim a preferential Th2 skewing in Mast cell rich TLDNcold (section page 8), however as they also state and show Th2 relative abundance is comparable in Cold vs hot TDLN (Supp Fig S3). Please comment.
2) Mast cell – IL4: the authors imply that mast cells are the source of IL4, but no co-staining has been performed and data remain correlative and only at mRNA level. A co-stain CD117 and IL4 should be included in Figure 4.
3) Fig 3. The authors focus on the spatial distribution and distance between mDCs and T cells to propose that shorter distances imply preferred interactions. What is the biological relevance of a distance difference of 0.1 um when comparing mDCs-Th1 and mDCs-Th2 (Fig 3C)?
Moreover, CD8 T cell distance to mDCs seems higher than the one of mDCs to CD4 Th1, even in hot TLDN. Would this mean that CD8 T cells are poorly primed in hot TLDN? Why then the paired tumors are hot and immune response seems efficient in these patients?
4) The authors claim that mast cell are more abundant in TLDN cold vs hot (Fig 4G). However, from the Nanostring cell type abundance analysis the difference is not statistically significant (Fig S1B). Please comment.
5) The main limitation of this study is that the conclusions are based on correlation. The authors use terms such as "demonstrate that interactions are generally weaker", "mDC-T cell interactions were lower", and others similar statements, that are not supported by the data. Please edit.
6) Fig 6. The scheme of the proposed mechanisms in place in TLDN cold vs hot is too hypothetical and not supported by experimental data. The authors don't provide any functional data/validation regarding IL-4 secretion by mast cells, about IL-4 driving preferential Th2 polarization (anyway not observed in Fig S3), about Th2 dependent down-stream effects in tumors (e.g., IL-4 on PDFG+ monocytes, fibroblasts). These data should be provided to confirm the hypotheses stemming from the transcriptomic analyses.

Minor:

1) What is the difference between Fig 4B and Supp Fig S5, TPSAB1 panel?

2) Title: The authors do not provide functional data supporting the mast cell dependent skewing of Th2 cells. They only observed increased mast cells and IL4 in TLDNcold. The title should be modified.

3) Many claims throughout the manuscript are overstated and should be toned down, unless functional validation is provided.

Reviewer #2 (Remarks to the Author):

In this paper by Gue et al. the authors show a correlation between Th2/Th1 skewing in the draining lymph nodes of breast cancer patients and the infiltration of T cells into their primary tumors.
Th2 skewing was found in patients with low infiltration of T cells into the primary tumor (cold tumors). The skewing was influenced by the distances of T cells from dendritic cells and the

presence of mast cells in the lymph nodes. The finding that the proportion of mast cells in the lymph nodes is negatively correlated with the number of TILs in the primary tumor is very interesting. Overall, the paper is well-written and describes some novel findings that are important for the scientific community. The following comments should be addressed in a comprehensive revision.

1. The authors analyzed the LNs. Please clarify whether these are sentinel LNs and give more details about the time of collection? Was it at diagnosis?
2. Figure 1 also includes a summary of the results, I suggest removing it and including only the technology.
3. The authors state in page 5 line 8: "FFPE samples were subjected to NanoString analyses". Nanostring is a company that have different technologies please indicate the specific technology and how it works (e.g. GeoMx-spatial transcriptomics, COSMX etc…). In addition, please indicate the size of the region in the sample from which the RNA was extracted and how was this region determined. If possible, please provide images of these regions.
4. In page 5 line 14 the authors state: "Further analysis of 14 immune cell scores (Supplementary Table 2) provided by NanoString also showed no significantly different immune cell types between TDLNCold vs. TDLNHot ". Please elaborate more about how these scores were calculated.
5. In page 8 and figure 3 the authors describe the multiplex imaging. Some important points should be clarified:
a. The definition of CD8+T cells should be changed to CD3+ CD4- negative cells
b. Please indicate how many samples were analyzed. Moreover, it may be better to represent figures 3C-D as single dots from different samples (like in figure 4A) rather than a bar plot.
c. How many cells were analyzed? what is the size of the imaging region?
d. Images in the figure are from very small regions please provide images with lower magnification to appreciate the location in the lymph nodes where the images were acquired (i.e. T cell zone, B cell zone etc…)
e. Please provide a heatmap of the classified cells and the markers expressed by them.
f. Figure 3C, D show the same exact data, only reordered. This is confusing.
I have some concerns regarding the results presented in Figure 3. First, in the main text the authors indicate that the results are significant but Figure S4 shows no significant difference between hot and cold tumors. Furthermore, even if the results are significant, the differences in distances between the cells are 0.1 - 0.2 microns. It is unclear what is the biological meaning of such a low difference.

6. In page 10 line 12 the authors state: "We found that mast cell densities in TDLNCold were significantly higher than TDLNHot (Fig. 4G, $p = 0.0023$, Data S7) ". Please state what was the image size that was analyzed.
7. The authors state in page 9 line 14; "IL4 from mast cells drives preferred Th2 polarization in TDLNCold". In the data presented in the paper there is only a correlation between Il-4 levels and mast cell densities. Performing double staining of mast cells and Il-4 (FISH or immunofluorescence) would strengthen this point. In any case, no causation is shown and this statement should be toned down. Accordingly, the title of the paper should be toned down to something like: "Mast cell proportions and Th2 skewing in the TDLN is correlated with T cell infiltration into TNBC tumor"
8. In order to strengthen the paper, I suggest performing multiplex IF of mast cells, T cells and DCs simultaneously to understand the interactions between T cells and mast cells.
9. In page 11 line 1 the authors state: "we calculated the Th2/Th1 ratios within tumors based on NanoString signatures (Table S2) from our NanoString dataset (Fig. 5, top row, Data S8), as well as public METABRIC-TNBC data (Fig. 5, bottom row, Data S8) ". How was this ratio calculated?
10. Please provide cell type score, differential expression and correlations for primary tumors also like in Figure S1A, B and Figure 2.
11. In Figure 5 the authors show convincingly a negative correlation between high Th2/Th1 ratio and CD45 expression, I would like to see if there is also a negative correlation with CD3 expression

12. Figure S3 please change densities to cells/mm2

Reviewer #3 (Remarks to the Author):

Guo et al assessed the immune composition of TDLN from matched cold or hot TNBC primary tumors. Based on a machine learning technique, they suggest a preferential induction of Th2 cells in TDLN of cold tumors, correlating with an enhanced presence of IL-4-expressing mast cells in that location. They correlate this with an enhanced Th2/Th1 balance, enhanced IL-4 and enhanced "healing-type" macrophages in the primary tumors. The rationale is interesting and the findings are intriguing, although some are still rather correlative and/or speculative. Some remarks remain:

- The preferential induction of Th2 in TDLN is, surprisingly, not translated into higher Th2 cell numbers (nor Th2-associated gene expression), as one would expect some level of Th2 cell proliferation upon activation by DC. The authors should assess activation and proliferation markers on CD4+ T cells in the TDLN samples (cold versus hot).
- Another point of divergence, which was not investigated, is the nature of the DC in TDLNs. Is there a difference in the number of cDC1, cDC2 or CCR7+ mature DC? Do Th2 cells mainly make contact with one of these DC subsets?
- Do the mast cells need to be in close contact to CD4+ T cells and DC? Are they not only more numerous, but also in closer contact to T cells/DCs in cold vs hot TDLN?
- The proposed link between a dominant Th2 response in the primary tumor and the relative paucity of T-cell infiltration is rather speculative and impossible to conclude from the data. The enhanced presence of PDGFC+ macrophages in these tumors is no proof. Are there more activated fibroblasts? Is the matrix denser? Do T cells accumulate at the tumor edge?

NCOMMS-23-13759
Detailed responses to editors

We thank the reviewers for insightful comments and suggestions. Based on their feedback, we have conducted additional experiments and made revisions according to the comments. Below are our point-by-point responses. We appreciate the opportunity to submit the attached revised manuscript. We highlighted all the revisions in the manuscript.

Reviewer #1 (Remarks to the Author):

In this work, Guo et al aim at correlating how TLDN cell contexture and transcriptomic profiles impact lymphocyte infiltration into tumors (cold vs hot). The authors use human paired tissues from breast cancer patients and apply transcriptomics and IF techniques to answer this question. The developed intranodal self-correlation analyses approach is of interest and could represent a methodological report per se. Instead several of the biological conclusions remain correlative, descriptive or overstated, without any functional validation.

Major points:

1) Cell abundance: the authors claim a preferential Th2 skewing in Mast cell rich TLDNcold (section page 8), however as they also state and show Th2 relative abundance is comparable in Cold vs hot TDLN (Supp Fig S3). Please comment.

We appreciate the reviewer bringing up this important point, which will be addressed here and in the revised manuscript. Our findings consistently revealed that even when there are no cell abundance differences between TDLNs related to hot vs. cold tumors (except for mast cells), there are gene correlation and spatial relationship differences that are biologically consistent. Our group has published several papers showing that spatial organization, rather than numerical differences, of immune cells within TDLNs[1,2] or tumors[3] correlate with clinical outcomes.

Here, preferential Th2 polarization in $TDLN_{Cold}$ vs. $TDLN_{Hot}$ is suggested by different spatial distributions of mDC-Th cells, rather than overall Th cell abundances. We implemented an unbiased spatial clustering analysis of mDC, Th1, and Th2 cells to quantify the spatial distributions between mDC and Th cells. We found higher mDC-Th2 dominated clusters within $TDLN_{Cold}$ compared with mDC-Th1 dominated clusters within $TDLN_{Hot}$. These spatial findings suggest preferential Th2 polarization even when Th2 and mDC cell abundance are similar between $TDLN_{Cold}$ vs. $TDLN_{Hot}$. Potential mechanism is the rapid egress rate of Th2 cells[4] such that these cells do not accumulate within TDLNs. Higher Th2/Th1 ratio in paired cold primary tumors further supports this hypothesis. We address this point in the revised Discussion (Page 18, Line 1~15).

2) Mast cell – IL4: the authors imply that mast cells are the source of IL4, but no co-staining has been performed and data remain correlative and only at mRNA level.

We have addressed this important point by co-staining IL4 and CD117 (mast cell marker) on 22 TDLN slides (n_{Cold} = 11 and n_{Hot} = 11) from both discovery and validation cohorts. We identified $IL4^+$ mast cells ($IL4^+CD117^+$ cells) within $TDLN_{Cold}$ and $TDLN_{Hot}$ (new Fig. 4I & J). Densities of $IL4^+$ mast cells are significantly higher in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 4K, $p = 0.011$). In addition, we compared

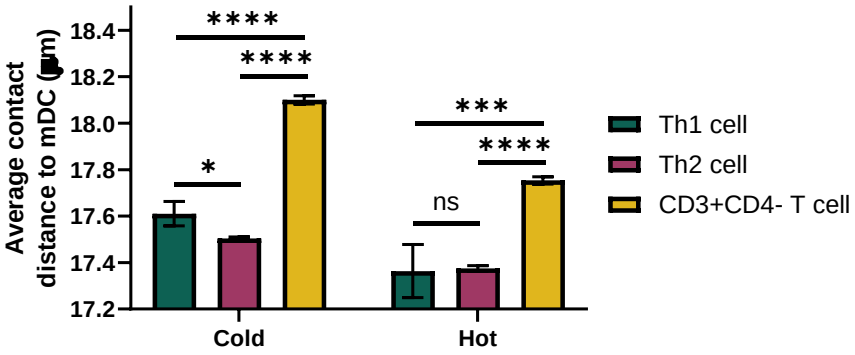
the percentages of $IL4^+$ mast cells to total mast cells. This also showed higher percentages of $IL4^+$ mast cells within $TDLN_{Cold}$ than $TDLN_{Hot}$ (new Fig. 4L, p = 0.006).

3) Fig 3. The authors focus on the spatial distribution and distance between mDCs and T cells to propose that shorter distances imply preferred interactions. What is the biological relevance of a distance difference of 0.1 um when comparing mDCs-Th1 and mDCs-Th2 (Fig 3C)? Moreover, CD8 T cell distance to mDCs seems higher than the one of mDCs to CD4 Th1, even in hot TLDN. Would this mean that CD8 T cells are poorly primed in hot TLDN? Why then the paired tumors are hot and immune response seems efficient in these patients?

We agree that a distance difference of 0.1 um, while statistically significant, may not represent a significant biological relevance. Instead, we have replaced this distance-based spatial analysis with spatial clustering analysis as an unbiased quantitative metric of cellular relationships. We implemented DBSCAN (Density-Based Spatial Clustering of Applications with Noise), a commonly used spatial clustering algorithm, on mDC, Th1, and Th2 cells to identify mDC-Th1 vs. mDC-Th2 clusters within each TDLN. We defined cluster types based on the cell compositions within each cluster. Specifically, Th1 dominated cluster was defined as a cluster with existence of mDC and a larger Th1 cell population than Th2 cells, while Th2 dominated cluster was defined as a cluster with existence of mDC and a larger Th2 cell population than Th1 cells. Further details on this methodology are provided in a new subsection in Methods.

In the new Fig. 3, we compared the relative abundances of Th1- vs. Th2-dominated clusters (number of Th1- or Th2- dominated clusters divided by total cluster number) within cold vs. hot TDLNs. In new Fig 3C&D, we show that Th2-dominated clusters are higher in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (p = 0.05), while Th1-dominated clusters are similar in $TDLN_{Cold}$ and $TDLN_{Hot}$ (p = 0.53). We also provide a heatmap of the average values in a 2-by-2 comparison which shows that Th2 clusters are highest within $TDLN_{Cold}$ (new Fig 3E). These cold-vs-hot TDLN comparisons further support that Th2 cell polarization is preferred in $TDLN_{Cold}$.

For CD8+ T cells, we rename “CD8+ T cell” with “CD3+CD4- cell” since reviewer 2 correctly pointed out as these are not necessarily the same. We compared the average contact distances between mDC with Th1, Th2, or CD3+CD4- cells (see figure below). This showed that CD3+CD4- cells have the longest distance to mDC as compared to Th1 or Th2 cells. We agree that this is an interesting finding which might indicate that CD3+CD4- cells are less actively engaged with mDC compared to Th cells within TDLNs. Also of potential interest, average CD3+CD4- cells to mDC distance is shorter in hot TDLNs as compared to cold TDLNs.



4) The authors claim that mast cell are more abundant in TLDN cold vs hot (Fig 4G). However, from the Nanostring cell type abundance analysis the difference is not statistically significant (Fig S1B). Please comment.

This is another important point that we addressed with additional data. In NanoString, cell type abundances are estimated by average NanoString gene expression - KIT, CMA1, TPSAB1, MS4A2, and PRG2 based on NanoString panel annotation and methodology for mast cells. Low abundance of mast cells within TDLNs renders such quantification via representative genes not as accurate as IHC staining with quantitative cell counts. Thus, we implemented IHC staining of our original samples which showed that mast cells were enriched in $TDLN_{Cold}$ in the original Fig 4G. To further resolve this conflict from NanoString, we collected an additional 20 TDLNs paired with 10 cold and 10 hot primary tumors as an independent validation cohort. These samples were analyzed via IHC staining and confirmed that mast cells are enriched in $TDLN_{Cold}$ (Fig S10C [original Fig S7B], Fig 4H).

We also went back to our NanoString data and closely examined the data in original FigS1B (current Fig S2B) with outlier analysis (Grubbs method with alpha = 0.05). We identified an outlier in the mast cell data series. After outlier removal, mast cell abundance from NanoString indeed also became statistically significant ($p = 0.007$, Mann Whitney test). We address this important point in the revised Discussion (Page 17, Line 3~9).

5) The main limitation of this study is that the conclusions are based on correlation. The authors use terms such as “demonstrate that interactions are generally weaker”, “mDC-T cell interactions were lower”, and others similar statements, that are not supported by the data. Please edit.

We appreciate the reviewer’s comment. We have toned down statements/terms that did not have solid data to support in the manuscript. We indicate these toned-downed statements with yellow underlined text highlighted in the revised manuscript.

6) Fig 6. The scheme of the proposed mechanisms in place in TLDN cold vs hot is too hypothetical and not supported by experimental data. The authors don’t provide any functional data/validation regarding IL-4 secretion by mast cells, about IL-4 driving preferential Th2 polarization (anyway not observed in Fig S3), about Th2 dependent down-stream effects in tumors (e.g., IL-4 on PDFG+ monocytes, fibroblasts). These data should be provided to confirm the hypotheses stemming from the transcriptomic analyses.

In this revision, we have provided additional data to support these proposed mechanisms in the following three aspects. These are covered in the above responses but will be summarized again below.

First, to support the IL4 expression from mast cells, we co-stained IL4 and CD117 on 22 TDLN slides ($n_{Cold} = 11$ and $n_{Hot} = 11$) from both discovery and validation cohorts. We identified $IL4^+$ mast cells ($IL4^+CD117^+$ cells) in both $TDLN_{Cold}$ and $TDLN_{Hot}$ (new Fig. 4I & J). The cell densities of $IL4^+$ mast cells were significantly higher in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 4K, $p = 0.011$). In addition, we also calculated the percentages of $IL4^+$ mast cells to total mast cell numbers. This also showed that there were higher $IL4^+$ mast cell percentages in $TDLN_{Cold}$ than $TDLN_{Hot}$ (new Fig. 4L, $p = 0.006$).

Secondly, IL4 is a well-known signal to drive Th2 polarization[5]. While Th2 abundances within $TDLN_{Cold}$ and $TDLN_{Hot}$ were similar in Fig S6 (S3 originally), we showed that the spatial distributions of mDC-Th cells were different. By replacing the original distance-based metric with an unbiased spatial clustering

metric, we found there were more mDC-Th2 clusters in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 3C~E) while mDC-Th1 clusters were similar. This spatial finding represents a novel and more distinct approach to support the Th2 polarization preference within $TDLN_{Cold}$.

Third, the mechanism of IL4/Th2-dependent fibrosis through macrophage polarization within TME has been reported as a potential hurdle for immune infiltration[6–9]. In a recent murine study of breast cancer, inhibition of Th2 function significantly improved infiltration of $CD8^+$ T cells and reduced M2 like macrophages[10]. To look for similar evidence of this in our study, we quantified the gene expression of an additional 730 genes from our paired primary tumors with NanoString nCounter Cancer Pathway panel to cover tissue-repair related genes (new Fig S13C&D). We found that multiple markers for the tissue repairing phenotype were upregulated, including PPARGC1A, VEGFA, INHBB, HOXA11, ERBB2, and LAMA5 within cold tumors (new Fig S13C&D and new Data file S10). Additionally, we estimated the abundances of tissue repairing macrophages (TsRpM) and cancer associated fibroblast (CAF) using the gene deconvolution method, MCP-counter, and corresponding gene signatures (Table S2) for both in-house NanoString data and public METABRIC-TNBC data. We found strong correlations between Th2 and TsRpM, as well as TsRpM and CAF cell type scores (new Fig 5C, G, J, and N).

In addition to the above additional data and analyses, we toned-down statements/arguments as the reviewer suggested (underlined texts in the revised manuscript). We also provided additional discussion on our proposed mechanisms supported by previous studies (Fig. S15, Page 20, line 3~17).

Minor:

1) What is the difference between Fig 4B and Supp Fig S5, TPSAB1 panel?

Fig 4B removed an outlier identified by the Grubb's method (alpha < 0.01), while FigS5 TPSAB1 panel included all the original data points with outliers.

2) Title: The authors do not provide functional data supporting the mast cell dependent skewing of Th2 cells. They only observed increased mast cells and IL4 in TLDNcold. The title should be modified.

We have modified our title: "IL4+ mast cells bias Th2 polarization within TDLNs associated with cold TNBC tumors."

3) Many claims throughout the manuscript are overstated and should be toned down, unless functional validation is provided.

We have toned down overstated statements/terms in this manuscript. We indicate these toned-downed statements with yellow underlined text in the revised manuscript.

Reviewer #2 (Remarks to the Author):

In this paper by Gue et al. the authors show a correlation between Th2/Th1 skewing in the draining lymph nodes of breast cancer patients and the infiltration of T cells into their primary tumors.
Th2 skewing was found in patients with low infiltration of T cells into the primary tumor (cold tumors).
The skewing was influenced by the distances of T cells from dendritic cells and the presence of mast cells

in the lymph nodes. The finding that the proportion of mast cells in the lymph nodes is negatively correlated with the number of TILs in the primary tumor is very interesting. Overall, the paper is well-written and describes some novel findings that are important for the scientific community. The following comments should be addressed in a comprehensive revision.

We appreciate these positive comments from the reviewer.

1. The authors analyzed the LNs. Please clarify whether these are sentinel LNs and give more details about the time of collection? Was it at diagnosis?

Based on pathological reports, all LNs in this study were sentinel LNs collected at diagnosis without neoadjuvant treatment. We clarified this in the Methods section.

2. Figure 1 also includes a summary of the results, I suggest removing it and including only the technology.

We have removed the results summary in Fig 1 and updated it with our additional experiments.

3. The authors state in page 5 line 8: "FFPE samples were subjected to NanoString analyses". Nanostring is a company that have different technologies please indicate the specific technology and how it works (e.g. GeoMx-spatial transcriptomics, COSMX etc…). In addition, please indicate the size of the region in the sample from which the RNA was extracted and how was this region determined. If possible, please provide images of these regions.

We used the NanoString nCounter technology which can accurately measure the bulk RNA expression from FFPE slides:
https://nanostring.com/products/ncounter-analysis-system/ncounter-systems-overview/#howitworks
We specifically used their Pan-Cancer Immune panel and Cancer Pathway panel.
We provide the area (in mm^2) of regions from which RNA was extracted in new Data file S1. These regions were selected by a clinical pathologist (Dr. Schmolze) to include the whole TDLN. We also provide screenshots in the new Fig S1.

4. In page 5 line 14 the authors state: "Further analysis of 14 immune cell scores (Supplementary Table 2) provided by NanoString also showed no significantly different immune cell types between TDLNCold vs. TDLNHot ". Please elaborate more about how these scores were calculated.

Immune cell type scores were calculated by the nSolver v4.0 software from NanoString. Detailed methodology from their documentation: https://nanostring.com/support/knowledge-base-faqs/

"The cell type score itself is calculated as the mean of the log2 expression levels for all the probes included in the final calculation for that specific cell type. Because the scores are dependent on probe-specific counting and capturing efficiencies, these should only be interpreted as relative cell abundance values compared to the same cell type within other samples or groups of samples. The scores should not be used as measures for the abundance of a cell type relative to other cell types within the same sample, nor should it be used to quantitate cell abundance within a single sample."

We have provided more details about how these cell type scores were calculated in the Methods section as well as the figure legend for Fig S2.

5. In page 8 and figure 3 the authors describe the multiplex imaging. Some important points should be clarified:
a. The definition of CD8+T cells should be changed to CD3+ CD4- negative cells

We agree that CD8+ T cell is not necessarily the same as CD3+CD4- T cell. We have replaced CD8+ T cell(s) with CD3+CD4- cell(s) in the revised manuscript.

b. Please indicate how many samples were analyzed. Moreover, it may be better to represent figures 3C-D as single dots from different samples (like in figure 4A) rather than a bar plot.

The original distance comparison in Figure 3 included cell-pair-wise comparisons from 7 cold TDLN and 7 hot TDLN. In other words, each data point in this plot was a mDC-cell pair. These cell pair numbers were: mDC-Th1 cell (n_{Cold} = 9476, n_{Hot} = 2140); mDC-Th2 cell (n_{Cold} = 690054, n_{Hot} = 194240); mDC-CD3+CD4- cell (n_{Cold} = 74318, n_{Hot} = 102519); mDC-mDC (n_{Cold} = 516218, n_{Hot} = 74550). Since all reviewers commented on the marginal difference of the original distance analysis, we replaced the distance analysis with spatial clustering analysis on each sample (new Fig 3C~E). Updated Fig 3C~E provide all the original data points.

c. How many cells were analyzed? what is the size of the imaging region?

Total cell numbers and the area (in mm^2) of analyzed region for each TDLN are provided in new Data file S1.

d. Images in the figure are from very small regions please provide images with lower magnification to appreciate the location in the lymph nodes where the images were acquired (i.e. T cell zone, B cell zone etc…)

We appreciate these suggestions and now provide regions with lower magnification in new Fig. 3 and whole TDLN slides with their corresponding regions (from new Fig. 3) in new Fig. S4. B cell zones, T cells zones, and sinuses can be observed in both the images with lower magnification and whole TDLN slides. Our spatial clustering analysis was implemented on whole TDLN slides to include as much information as possible. Details on region size and cell numbers are provided in Data file S1.

e. Please provide a heatmap of the classified cells and the markers expressed by them.

We now provide a heatmap of classified cells and markers expressed by them for each slide (new Fig. S5 & S11) of both panels. For improved visualization, we also provide ridge plot showing the marker expression for each cell type.

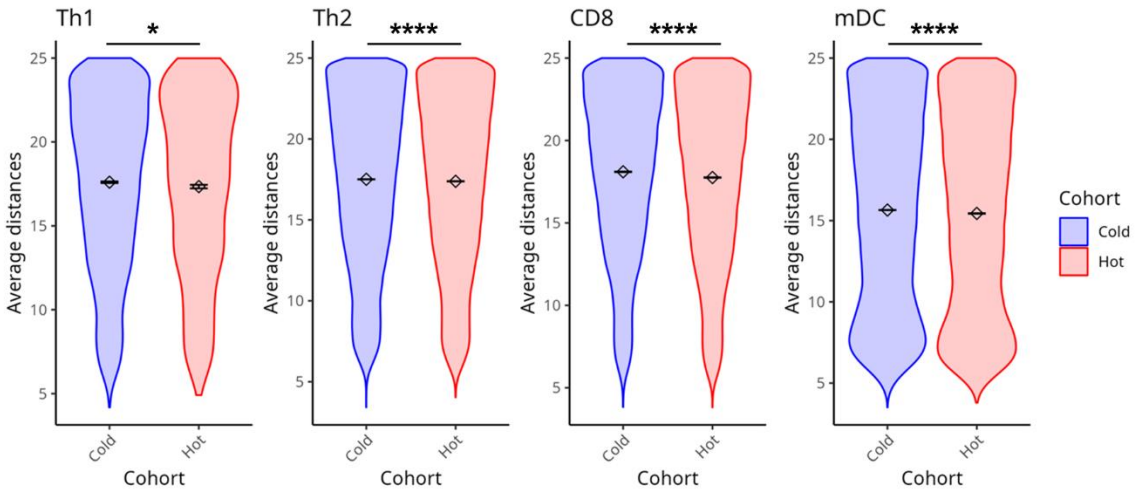
f. Figure 3C, D show the same exact data, only reordered. This is confusing.
I have some concerns regarding the results presented in Figure 3. First, in the main text the authors indicate that the results are significant but Figure S4 shows no significant difference between hot and cold tumors. Furthermore, even if the results are significant, the differences in distances between the cells are 0.1 - 0.2 microns. It is unclear what is the biological meaning of such a low difference.

We appreciate these points. As addressed above, we agree that a distance difference of 0.1 um, while statistically significant, may not represent a significant biological relevance. Instead, we have replaced this distance-based spatial analysis with spatial clustering analysis as another unbiased quantitative metric of cellular relationships. We implemented DBSCAN (Density-Based Spatial Clustering of

Applications with Noise), a commonly used spatial clustering algorithm, on mDC, Th1, and Th2 cells to identify mDC-Th1 vs. mDC-2 clusters within each TDLN. We defined cluster types based on the cell compositions within each cluster. Specifically, Th1 dominated cluster was defined as a cluster with existence of mDC and a larger Th1 cell population than Th2 cells, while Th2 dominated cluster was defined as a cluster with existence of mDC and a larger Th2 cell population than Th1 cells. Further details on this methodology are provided in a new subsection in Methods.

In the new Fig. 3, we compared the relative abundances of Th1- vs. Th2-dominated clusters (number of Th1 or Th2 dominated clusters divided by total cluster number) within cold vs. hot TDLNs. In new Fig 3C&D, we showed that Th2-dominated clusters are higher in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ ($p = 0.05$), while Th1-dominated clusters are similar in $TDLN_{Cold}$ and $TDLN_{Hot}$ ($p = 0.53$). We also provide a heatmap of the average values in a 2-by-2 comparison which shows that Th2 clusters are highest within $TDLN_{Cold}$ (new Fig 3E). These cold-vs-hot TDLN comparisons further supported that Th2 cell polarization is preferred in $TDLN_{Cold}$.

Lastly, we apologize for the oversight in Fig. S4 and have corrected this as follows:



6. In page 10 line 12 the authors state: “We found that mast cell densities in TDLNCold were significantly higher than TDLNHot (Fig. 4G, p = 0.0023, Data S7) “. Please state what was the image size that was analyzed.

Whole TDLN slide images were used to identify mast cells. Total cell number and area (in mm^2) of the analyzed regions for each TDLN are now provided in the new Data file S1. We also provide screenshots for each stained slide in a new Fig. S9.

7. The authors state in page 9 line 14; “IL4 from mast cells drives preferred Th2 polarization in TDLNCold”. In the data presented in the paper there is only a correlation between Il-4 levels and mast cell densities. Performing double staining of mast cells and Il-4 (FISH or immunofluorescence) would strengthen this point. In any case, no causation is shown and this statement should be toned down. Accordingly, the title of the paper should be toned down to something like: “Mast cell proportions and Th2 skewing in the TDLN is correlated with T cell infiltration into TNBC tumor”

As addressed above, we co-stained IL4 and CD117 on 22 additional TDLN slides (n_{Cold} = 11 and n_{Hot} = 11) from both discovery and validation cohorts. We identified $IL4^+$ mast cells ($IL4^+CD117^+$ cells) within $TDLN_{Cold}$ and $TDLN_{Hot}$ (new Fig. 4I & J). Densities of $IL4^+$ mast cells are significantly higher in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 4K, p = 0.011). In addition, we compared the percentages of $IL4^+$ mast cells to total mast cells. This also showed higher percentages of $IL4^+$ mast cells within $TDLN_{Cold}$ than $TDLN_{Hot}$ (new Fig. 4L, p = 0.006).

In addition, we agree with the reviewer and have toned down the overstated statement (highlighted in yellow with underlines in the revised manuscript). The title has also been toned down as: "IL4+ mast cells bias Th2 polarization within TDLNs associated with cold TNBC tumors."

8. In order to strengthen the paper, I suggest performing multiplex IF of mast cells, T cells and DCs simultaneously to understand the interactions between T cells and mast cells.

We agree with the reviewer that investigating the interactions between mast cell, T cell, and DCs would further strengthen this manuscript. By considering the potential of IL4 to diffuse across different regions within TDLNs, we hypothesized that IL4 producing mast cells may not need to be directly adjacent to T cells or DCs to influence their biology. This view was supported by a new co-author Dr. Joo Song, who is a pathologist with clinical and research expertise on lymph nodes. Dr. Song initially examined the spatial distribution of mast cells using H-DAB-stained slides (new Fig. S9) and confirmed that mast cells are present within sinuses and T cell zones of TDLNs. Based on these preliminary observations, we co-stained CD3, IL4, and CD117 in 22 additional TDLNs from both discovery and validation cohort (new Fig S1). 30 ROIs were selected for detailed analysis (new Data file S9). Guided by the clinical lymphoma pathologist, a pixel classifier from QuPath was trained to distinguish the B cell zone, T cell zone, and sinus for each ROI (new Fig 4I & J). Detailed region sizes and cell numbers are also provided in Data file S1.

First, to further validate the pathological observation of mast cell presentation in sinuses and T cell zones from H-DAB staining, we compared mast cell densities within B cell zones, T cell zones, and sinuses. We confirmed higher mast cell densities within sinuses ($p < 0.0001$) and T cell zones ($p < 0.0001$) compared to B cell zones (new Fig. S10D). There was a trend for higher mast cell density within sinuses compared to T cell zones (p = 0.07, new Fig. S10D). Densities of $IL4^+$ mast cells followed the same spatial distributions (i.e., sinuses > T cell zones > B cell zones, $p < 0.0001$, new Fig 4M). Again, a marginal significance was also found between sinuses and T cell zones (p = 0.08, new Fig. 4M).

Second, we compared densities of $IL4^+$ mast cells between $TDLN_{Cold}$ and $TDLN_{Hot}$. Regardless of the TDLN subregion, we found higher densities of $IL4^+$ mast cells in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 4K, p = 0.01). Focusing on TDLN subregions, densities of T cell zone and sinus $IL4^+$ mast cells were significantly higher in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 4N: $p_{Tcellzone}$ = 0.0014, p_{Sinus} = 0.01).

Besides the cell densities of $IL4^+$ mast cells, we also quantified the percentage of $IL4^+$ mast cells to total mast cells. Considering the entire TDLNs, we found higher percentages of $IL4^+$ mast cells in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 4L, p = 0.0064). Focusing on TDLN subregions, only the percentage within sinus, but not T cell zone, $IL4^+$ mast cells were significantly higher in $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 4O, $p_{Tcellzone}$ = 0.22, p_{Sinus} = 0.011).

9. In page 11 line 1 the authors state: "we calculated the Th2/Th1 ratios within tumors based on NanoString signatures (Table S2) from our NanoString dataset (Fig. 5, top row, Data S8), as well as public METABRIC-TNBC data (Fig. 5, bottom row, Data S8) ". How was this ratio calculated?

Gene signatures for Th1 and Th2 cells were from the NanoString Pan-Cancer Immune Profiling panel and IO360 panel[11] and used for both NanoString and METABRIC-TNBC datasets. For our NanoString dataset, the cell type scores of Th1 and Th2 cells were calculated by nSolver v4.0 software, of which more details can be found here: https://nanostring.com/support/knowledge-base-faqs/. For METABRIC-TNBC dataset, the abundances of Th1 and Th2 cells were estimated using the MCP-Counter algorithm[12] with the NanoString signatures. The Th2/Th1 ratios were calculated by dividing the Th2 cell type score to the Th1 cell type score for each sample.

10. Please provide cell type score, differential expression and correlations for primary tumors also like in Figure S1A, B and Figure 2.

The corresponding analysis for primary tumors is provided in new Fig S13 and updated in the main manuscript (Page 12, line 15~ Page 13, line 10).

11. In Figure 5 the authors show convincingly a negative correlation between high Th2/Th1 ratio and CD45 expression, I would like to see if there is also a negative correlation with CD3 expression

We have calculated the correlation coefficients between CD3D expression and Th2/Th1 ratio in updated Fig 5B&I. The correlations in both in-house NanoString data and public METABRIC data are significantly negative ($r = -0.769$ and $r = -0.739$).

12. Figure S3 please change densities to cells/mm2

The y axis label of original Fig. S3A (current Fig. S6A) has been changed to cells/mm^2.

Reviewer #3 (Remarks to the Author):

Guo et al assessed the immune composition of TDLN from matched cold or hot TNBC primary tumors. Based on a machine learning technique, they suggest a preferential induction of Th2 cells in TDLN of cold tumors, correlating with an enhanced presence of IL-4-expressing mast cells in that location. They correlate this with an enhanced Th2/Th1 balance, enhanced IL-4 and enhanced "healing-type" macrophages in the primary tumors. The rationale is interesting and the findings are intriguing, although some are still rather correlative and/or speculative. Some remarks remain:

- The preferential induction of Th2 in TDLN is, surprisingly, not translated into higher Th2 cell numbers (nor Th2-associated gene expression), as one would expect some level of Th2 cell proliferation upon activation by DC. The authors should assess activation and proliferation markers on CD4+ T cells in the TDLN samples (cold versus hot).

We appreciate the reviewer's comment. We provide the expression profiles of all genes related to T cell activation and T cell proliferation based on the NanoString Immune Panel Annotation in two heatmaps with hierarchical clustering (new Fig. S2C & D). No clear separation of $TDLN_{Cold}$ and $TDLN_{Hot}$ was found. We also directly compared the expression of each gene related to T cell activation or proliferation (new Fig S2E & F) and no significant differences were found as well (Page 5, line 14~Page 6, line 2). One possible explanation is that the activation or proliferation status of Th2 cells may not alter once they become polarized. As such, we turned to spatial differences showing enrichment of mDC-Th2 cell clusters specifically within $TDLN_{Cold}$ compared to $TDLN_{Hot}$ (new Fig. 3D&E).

The egress rate of Th2 cells from lymph nodes is known to be high[4]. It is possible that newly polarized Th2 cells do not accumulate locally but exit the TDLN and traffic to tumors. The higher Th2/Th1 ratio in paired cold primary tumors supports this hypothesis. We added these hypotheses into the Discussion section to clarify this observation (Page 18, Line 1~15).

- Another point of divergence, which was not investigated, is the nature of the DC in TDLNs. Is there a difference in the number of cDC1, cDC2 or CCR7+ mature DC? Do Th2 cells mainly make contact with one of these DC subsets?

We agree that the nature of DCs within TDLNs should be further investigated. To start, we used gene signatures of cDC1, cDC2, and CCR7+ mature DCs from a single-cell RNA-seq dataset[13]. We estimated the cell abundances of these 3 DC subtypes with our NanoString bulk transcriptomics data using nSolver v4.0 software. No cell abundance differences were found for these 3 DC subtypes (new Fig. S2G). From the additional experiments already described above, we confirmed that $IL4^+$ mast cells are associated with preference on Th2 polarization.

- Do the mast cells need to be in close contact to CD4+ T cells and DC? Are they not only more numerous, but also in closer contact to T cells/DCs in cold vs hot TDLN?

We agree with the reviewer that spatial relationships between mast cell, T cell, and DCs are important. By considering the potential of IL4 to diffuse across different regions within TDLNs, we hypothesized that IL4 producing mast cells may not need to be directly adjacent to T cells or DCs to influence their biology. This view was supported by our new co-author Dr. Joo Song, who is a pathologist with clinical and research expertise on lymph nodes. Dr. Song initially examined the spatial distribution of mast cells using H-DAB-stained images (Fig. S9) and confirmed that mast cells are present within sinuses and T cell zones of TDLNs. Based on these preliminary observations, we co-stained CD3, IL4, and CD117 in 22 additional TDLNs from both discovery and validation cohort (new Fig S1). 30 ROIs were selected for detailed analysis (new Data file S9). Guided by the clinical lymphoma pathologist, a pixel classifier from QuPath was trained to distinguish the B cell zone, T cell zone, and sinus for each ROI (new Fig 4I & J). Detailed region sizes and cell numbers are also provided in new Data file S1.

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- The proposed link between a dominant Th2 response in the primary tumor and the relative paucity of T-cell infiltration is rather speculative and impossible to conclude from the data. The enhanced presence of PDGFC+ macrophages in these tumors is no proof. Are there more activated fibroblasts? Is the matrix denser? Do T cells accumulate at the tumor edge?

We appreciate the reviewer's comments/questions. We first utilized differential gene expression analysis (new Fig. S13A&B, new Data file S10) and compared the immune cell type scores (new Fig. S13F) between cold vs. hot primary tumors. From the DGE analysis, PDGFC and VEGFA, typical markers for tissue repairing macrophages, were the most differentially expressed genes with strongest p-values (new Fig. S13A). Immune cell comparisons, as may be expected, showed that all immune cell types were significantly enriched in hot primary tumors - except for mast cells.

Next, we conducted new experiments to quantify the expression of an additional 730 genes for our paired primary tumors with the NanoString nCounter Cancer Pathway panel, which covers many tissue-repair related genes (new Fig S13C&D). By directly comparing the gene expressions in this cancer panel, we found that multiple markers for the tissue repairing phenotype are upregulated in cold tumors, including PPARGC1A, VEGFA, INHBB, HOXA11, ERBB2, and LAMA5 (new Fig S13C&D and Fig S10). Additionally, we estimated the abundances of tissue repairing macrophages (TsRpM) and cancer associated fibroblast (CAF) using the gene deconvolution method MCP-Counter, and corresponding gene signatures (Table S2) for both in-house NanoString data and public METABRIC-TNBC data. We found significant positive correlations between Th2 and TsRpM, as well as TsRpM and CAF cell type scores (new Fig 5C, G, J, and N).

Lastly, to confirm the more fibrotic TME associated with Th2 cells in cold tumors, we re-analyzed the public transcriptomic datasets (GSE88715) as a new computation experiment. In this published study, the authors collected 38 TNBC primary tumors and stratified these tumors into four groups (i.e., immune desert [ID], marginal restricted [MR], stromal restricted [SR] and fully inflamed [FI]) based on the immune infiltration levels and locations from clinical pathologists. They micro-dissected the slides into epithelium and stroma regions and quantified their gene expression by using microarrays for each region. To align their groups with our cold/hot definitions based on stromal TIL percentages (i.e., TIL% > 60% as hot, while TIL% < 10% as cold), we considered ID and MR as the cold tumors (n = 16) since limited lymphocytes should exist in stroma, and SR and FI as the hot tumors (n = 22). We compared the gene expression profiles between cold and hot tumors within stroma (new Fig. S14A, new Data file S12) and epithelium (new Fig. S14B, new Data file S12) regions. Within the stroma regions (new Fig. S14C), cold tumors upregulated tissue repairing and fibrosis related genes (i.e., FGF1, COL10A1, MMP11, COL5A2), while cytotoxicity and $CD8^+$ T cell markers were upregulated in hot tumors (i.e., CD8A, CD8B, GZMB, CXCL13). In contrast, the expression profiles of epithelium regions were similar between cold and hot tumors (new Fig. S14B). From the cell abundances estimated by MCP-Counter, T cells ($p = 0.0009$) and $CD8^+$ T cells ($p = 0.0004$) were significantly enriched in stroma regions of hot tumors, while CAF ($p = 0.0011$) was the only significantly upregulated cell type in stroma regions of cold tumors (new Fig. S14D, new Fig. 5Q&R). These results suggest more stromal CAFs in cold tumors compared to hot tumors. To address the question whether T cells accumulated within stroma regions, we compared the T cell scores between epithelium and stroma regions within cold primary tumors. We found a significantly higher T cell score in stroma region than epithelium regions (new Fig. S14E, $p = 3.1\times10^{-5}$).

We also provide more discussion about the type 2 immunity and T cell infiltration in the revised Discussion section (Page 20, Line 3 ~ 17).

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I thank the authors for the additional work on revising the original manuscript.
I appreciate the co-staining for IL-4 and CD117 to identify IL-4+ mast cells and for the implementation of the DBSCAN method to define the spatial localization of cells of interest.

Unfortunately, I remain not convinced about the claim that "IL-4+ mast cell bias Th2 polarization", given the absence of any mechanistic/experimental validation. Several statements in the manuscript currently rely on correlative and/or speculative findings and arguments.

As stated by the authors, Th2 cell abundance is the same in both types of LNs. With IL-4 mast cells being enriched in TDLNs "cold", a "bias" in Th2 polarization should result in an increased number of Th2 cells. If not, how would IL-4 bias Th2 polarization, as stated in the title and throughout the manuscript (P12: "to impact Th2 polarization in direct and indirect manners". P18: "In our study, we uncovered a pro-tumor mechanism of MCs within TDLNs for the first time: MCs secrete IL4 and skew CD4 T cell polarization to a Th2-preferred manner, leading to type 2 immunity within the TME and limited lymphocyte infiltration"; P19: "we hypothesize that mast cells in TLDNs migrated from the primary tumor")? These statements are in my opinion not supported by data.
Do the authors think that mast-cell dependent Th2 bias is due to IL-4, being responsible for influencing the spatial distribution of Th cells (0.1 um difference) ? If this is the hypothesis, then the authors should test it experimentally, given that it is the main claim of this work.
Further, the hypothesis of a different egress/exit of Th2 cells from is interesting, or a migration of mast cells from the tumor to the TDLN are interesting, but remain speculative. Is there any difference in chemokine receptors in Th2 cells from TDLN "cold" versus "hot", or in mast cell chemoattractants?
On a similar line of thought, the findings in the tumors (cold vs hot) as compared to the TDLNs remain correlative. The mechanisms of a potential link between primary tumor and TDLN composition are hypothetical. Particularly, the observation of a pro-fibrotic enrichment in cold tumor has previously been reported, and could be completely unrelated to the TLDN "DC-Th2 cluster" observations. The authors are profiling cold vs hot primary tumors, in terms of fibrosis and immune infiltrate.

Lastly, although I fully agree with Reviewer 2 on the nomenclature concern for CD8 T cells (renamed CD3+CD4- cells by the authors), no biological explanation has been provided related to the difference in spatial distribution of these cells in the 2 TDLN settings.

Reviewer #2 (Remarks to the Author):

The authors have addressed most concerns raised in the previous review satisfactorily. The manuscript has been substantially improved and is suitable for publication.
I have a few minor points that may be addressed by the authors:

1.The title is missing the word "is"
2.Figure S5, S11 please provide in higher resolution.
3.Data S5 and S9 are not available on figure share
4.Some supplementary figure legends are missing, e.g. figure.S5

Reviewer #3 (Remarks to the Author):

The authors sufficiently addressed my concerns

Reviewers' comments:

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Unfortunately, I remain not convinced about the claim that "IL-4+ mast cell bias Th2 polarization", given the absence of any mechanistic/experimental validation. Several statements in the manuscript currently rely on correlative and/or speculative findings and arguments.

Our findings and proposed mechanisms are derived from human samples. As such, findings are drawn from many consistent correlations pieced together from available patient materials rather than direct mechanistic experiments that could be done in animals. To address this comment, we have further toned down our title and specific statements appropriately to clarify the correlative nature of our human findings. These toned-down statements are highlighted in red in the revised manuscript.

As stated by the authors, Th2 cell abundance is the same in both types of LNs. With IL-4 mast cells being enriched in TDLNs "cold", a "bias" in Th2 polarization should result in an increased number of Th2 cells. If not, how would IL-4 bias Th2 polarization, as stated in the title and throughout the manuscript (P12: "to impact Th2 polarization in direct and indirect manners". P18: "In our study, we uncovered a pro-tumor mechanism of MCs within TDLNs for the first time: MCs secrete IL4 and skew CD4 T cell polarization to a Th2-preferred manner, leading to type 2 immunity within the TME and limited lymphocyte infiltration"; P19: "we hypothesize that mast cells in TLDNs migrated from the primary tumor")? These statements are in my opinion not supported by data.

We agree that it is important to demonstrate a numerical change in Th2 cells to support biased Th2 polarization within $TDLN_{Cold}$. To address this valid concern, we analyzed the Th2/Th1 ratios (new Fig. 3C) and Th2 percentages to total $CD4^+$ T cells (new Fig. 3D) between $TDLN_{Cold}$ and $TDLN_{Hot}$. We indeed found that Th2/Th1 ratios and Th2% are significantly higher in $TDLN_{Cold}$ to support biased Th2 polarization. Different from our previous analysis on cell densities (normalized to area) and relative abundances (normalized to total cell numbers), Th2/Th1 ratios are calculated by using the absolute cell numbers of Th1 and Th2 cells within each TDLN, representing the balance between Th1 and Th2 polarization within individual lymph nodes. Th2% to total $CD4^+$ T cells further reflect the preference of Th2 cells among all $CD4^+$ T cells within each TDLN. Both of these new analyses have been added to our updated manuscript.

In addition, we have appropriately toned down the statements reviewer mentioned here.

Do the authors think that mast-cell dependent Th2 bias is due to IL-4, being responsible for influencing the spatial distribution of Th cells (0.1 um difference) ? If this is the hypothesis, then the authors should test it experimentally, given that it is the main claim of this work.

Further, the hypothesis of a different egress/exit of Th2 cells from is interesting, or a migration of mast cells from the tumor to the TDLN are interesting, but remain speculative. Is there any difference in chemokine receptors in Th2 cells from TDLN "cold" versus "hot", or in mast cell chemoattractants?

The trivial difference of average mDC-Th2 contacting distances was already removed in our previous revision and replaced with spatial clustering analysis (DBSCAN). This is a more comprehensive and well-recognized approach to represent spatial distribution in imaging analysis. With the DBSCAN method, we found that the relative percentage of Th2-mDC spatial cluster was significantly ($p \leq 0.05$) and substantially (average fold change = 1.79) higher within $TDLN_{Cold}$, while Th1-mDC spatial clustering was similar. It is impossible to manipulate TDLNs and tumors in live patients to directly validate these findings.

As Reviewer 1 suggested, we examined the gene expression of all chemokine receptors related to Th2 cell egress as well as mast cell migration. We found similar expression levels of these genes in the differential gene expression, supporting this hypothesis. We emphasized this finding in the updated manuscript (Supplementary Dataset 2).

On a similar line of thought, the findings in the tumors (cold vs hot) as compared to the TDLNs remain correlative. The mechanisms of a potential link between primary tumor and TDLN composition are hypothetical. Particularly, the observation of a pro-fibrotic enrichment in cold tumor has previously been reported, and could be completely unrelated to the TLDN "DC-Th2 cluster" observations. The authors are profiling cold vs hot primary tumors, in terms of fibrosis and immune infiltrate.

The importance and novelty of our manuscript is unraveling the impact of TDLNs, beyond cancer cells or tumor microenvironment, on immune infiltration into human breast tumors. TDLNs have been largely ignored in human studies of tumor immunology because they are more difficult to obtain for research. Our study is purely human research, which provides a more direct and precise representation of human biology than animal studies. It is otherwise impossible to manipulate TDLNs and tumors in live patients to further validate our findings. While there are inbred mouse models with cold vs. hot tumors, these are of different tumor types with different mutational burden. Our findings are on a single tumor type, triple negative breast cancer, with different levels of immune infiltration.

Lastly, although I fully agree with Reviewer 2 on the nomenclature concern for CD8 T cells (renamed CD3+CD4- cells by the authors), no biological explanation has been provided related to the difference in spatial distribution of these cells in the 2 TDLN settings.

We agree that $CD8^+$ T cell activation is a critical process within TDLN and should directly impact lymphocyte infiltration into tumors. Somewhat surprising, we did not find any signal on CD8 from our gene expression analysis of TDLNs. Rather, our findings revealed for the first-time changes in mast cells and Th1/Th2 balance with TDLNs. For future studies, we agree that further

investigation into $CD8^+$ T cells will be helpful using more advanced high-plex single-cell spatial techniques (e.g., Akoya CODEX).

In sum, we appreciate Reviewer 1's additional comments. We provide additional analyses supporting our conclusions and have toned down our manuscript appropriately. We also added a new paragraph to discuss the limitations of our study, including the correlative nature of our human findings. The major contribution of our manuscript is to unravel the potential impact of TDLNs, which have been largely ignored in human studies of tumor immunology.

Reviewer #2 (Remarks to the Author):
The authors have addressed most concerns raised in the previous review satisfactorily. The manuscript has been substantially improved and is suitable for publication.

We appreciate the reviewer's insightful comments and acknowledgement on our additional experiments and insights to address these comments.

I have a few minor points that may be addressed by the authors:
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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I acknowledge the additional work performed by the authors to support their conclusion and the paragraph on the limitations of the study. I maintain my concerns regarding the purely associative/correlative nature of this study, which limit in my opinion the impact of the work.

Please rephrase the title, since it contains twice the word Association.

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We appreciate Reviewer #1’s acknowledgements on our additional work and discussion of the limitations.

Please rephrase the title, since it contains twice the word Association.

We appreciate the rephrased and shortened title suggested by the editor.