nature portfolio

Peer Review File

Alternations in inflammatory macrophage niche drive phenotypic and functional plasticity of Kupffer cells

Corresponding Author: Dr Lin Tian

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
“Availability of an inflammatory macrophage niche drives phenotypic and functional alterations in Kupffer cells”

Summary:
In this manuscript, Han-Ying Huang and Lin Tian define liver macrophage lineages in experimental models of metastatic liver cancer. Using several elegant lineage tracing systems, the authors determine that most macrophages associated with metastatic cancer in the liver are derived from CCR2 dependent peripheral blood monocytes. Interestingly, their evidence also supports a redundant system held in reserve to maintain macrophage metastasis-associated macrophage populations when monocytes are unable to support the demand. In this case, the author’s experimental evidence indicates Kupffer cells, the predominant resident liver macrophage subset, contribute to making up deficiencies in overall macrophage abundance in livers with cancer metastasis. Notably, a small overlap exists with aspects of recent article in Nature by Frederic Geissmann and colleagues (PMC10881399) and the studies presented here. These overlapping aspects are in general agreement and make use of different liver cancer models and similar/complementary genetic systems. Overall, the authors assess a compelling scientific premise using many innovative genetic models and I only have a few concerns:

Specific comments:

• It isn’t always clear that the authors are defining normal liver macrophages (N-macs) as derived from the adjacent healthy tissue from the same livers that have liver cancer. Indeed, the only indications I found are on line 93 and figure 3e. This should not be much more clear, and maybe described at least once in each figure legend and results section. The impact of the manuscript is critically lessened if readers misinterpret N-macs as a comparison of macrophage from healthy control livers and not a comparison with adjacent healthy tissue. For perspective, this misinterpretation happened with my first two readings!
• Some aspects of the manuscript could be better described in the results and/or appropriately referenced. Examples include the metastatic models and the function of Tim4.
• Immunofluorescent experiments are generally lacking in controls. These controls prove signals are above background and not from autofluorescence.
• The Cut&Run and ATAC-seq data “appear” questionable. Genome wide assessments seem valid in 5g. However, ChIP data is expected to be peaky, unlike some of the locus specific examples chosen. For example, the Spp1, Trem2, and Id3 loci all have very distal regulatory activity in liver macrophage data from other labs. And the data from these labs also looks lower quality if the windows are set to a similar size as selected in this manuscript.
• Line 135 – Statement regarding tracing with Clec4f is not correct… “moMacs, which never express Clec4f”. Many studies in other contexts have shown that “moMacs” can express Clec4f when filling a vacant KC niche. Examples include many studies describing Tim4- Kupffer cells during steatohepatitis, and more recently a Science paper from Paul Kubes’s lab describing bone marrow origin syncytial macrophages that turn on Clec4f and take on properties of Kupffer cells.
• Some histology (ED6d) or immunohistochemistry data, if available, would be beneficial. Especially for introduction of the metastasis model at figure 1. I don’t view this as necessary.
• Figure 2d: Depletions efficiencies are not described.
• Figure 2e: Tamoxifen dosing schedule is unclear.
• Figure 3: Analysis of proliferation occurs 12 days after initial clodronate. Is the proliferation rate dynamic?
• Several figure panels are challenging to interpret. Some dot plots have various shading patterns that are challenging to

interpret as their definition is only provided in the methods section under the statistics heading (2b, 4h). Color choices and or line widths for 5h and 5i are challenging to discriminate. Fig 2b and Ext 2b use inconsistent population definitions, which was a little confusing.
• The methods are generally well written and adequately describe approaches used. Sources of mouse models are described, but some primary references may be missing.
• Quality control considerations for 'omics data are not described, and no QC stats are provided.
• There are several minor typos that will be caught by spell check.

Reviewer #2

(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.

Reviewer #3

(Remarks to the Author)
Review of: Availability of an inflammatory macrophage niche drives phenotypic and functional alterations in Kupffer cells

In this study, the authors characterized the myeloid cell pool in liver metastasis, confirmed some of their observations in samples of patients, and showed that the tumor microenvironment consists of LMAMs derived from mo-macs, and that KCs are depleted. These observations are in line with many other publications showing that (1) liver injury, and primary liver cancer, results in KC depletion and monocyte infiltration with mo-mac the main mac population during liver injury and cancer and (2) KC niches can be replenished by local proliferation (minor proportion) and monocyte infiltration (main contributors). The authors also claim that inhibition of monocyte infiltration and mac proliferation opens the opportunity for KC proliferation/infiltration and manipulation of KC differentiation to LMAMs as potential therapeutic strategy. Finally, they present that inhibition of monocyte infiltration and mac proliferation increases the efficacy of ICI.

Overall, the authors performed extensive fate mapping experiments that support the main conclusions of their study. The data are relevant and, although the main insights have been shown (at least in part) in other liver diseases and primary liver cancer, the results provide new insights in the pathogenesis of liver metastasis, essential to expand the therapeutic landscape. However, to claim increased efficacy of ICI, supporting data/figures are necessary.
The main aspects that should be addressed are provided below:

In the first setting, the authors analyzed myeloid cell populations in liver metastasis and adjacent normal tissue. How far at distance was 'normal tissue' collected, and did the authors also investigate the difference between tumor nodule and surrounding microenvironment (which is usually not 'normal' but an infiltrating niche)? A schematic view of sampling would increase clarity in this respect.

The authors characterized the myeloid cell pool in TME in liver metastasis and those results resemble other observations in hepatocellular carcinoma (decreased population of KCs, increase of mo-mac). Do the authors think that common signals in liver metastasis and primary liver cancer are responsible for these observations? In addition, do common signals also drive KC differentiation? Can the authors comment on this in the discussion? At least the findings in primary liver cancer, published by other groups, should be addressed in the discussion.

Line 197: "Although CLs can effectively deplete both circulating monocytes and KCs (Fig. 3c), they cannot cross the tumor vasculature due to their size; thus, macrophages within metastatic nodules are not depleted." How sure are the authors that CLs cannot cross the tumor vasculature? This should be confirmed, i.e. by measurements of tumor vessels. It can also be that LMAMs do not engulf liposomes.

Line 302: The authors claim that dual blockade of Ccr2 and Csf1r increased the efficacy of anti-Pd1 immune checkpoint inhibition compared with that achieved by knockout of Ccr2 alone. However, the only data supporting this, are presented in Fig 6, panel f: "Representative bioluminescence imaging of metastasis-bearing Ccr2GFP/GFP mice and Ccr2GFP/WT littermates treated with PLX5622 and anti-Pd1 antibody (left)". These images do not clearly show the effect of single and dual blockade of Ccr2 and Csfr1 and the increased efficacy of anti-Pd1 in the dual blockade strategy. Please change the figures and images accordingly.

References are used in the abstract, which is unusual.

Reviewer #4

(Remarks to the Author)
In this manuscript, Huang et al. developed and applied multiple lineage tracing models to interrogate the origin of liver metastasis-associated macrophages (LMAMs) in different scenarios. They demonstrated that monocyte-derived macrophages (mo-macs) are the major population of LMAMs, while in monocyte-deficient occasions, LMAMs can be replenished either via increased local macrophage proliferation or by promoting Kupffer cell (KC) infiltration. Further, they proposed therapeutic strategies of dual blockade of monocytes and macrophages as a way to reprogram the

microenvironment of liver metastasis. The scientific question is of great importance and the manuscript is overall well-written. Notably, the models developed in this study are impressive and valuable to decouple the macrophage ontogeny in other diseases. However, there are several key elements missing – the heterogeneity of LMAMs from the single-cell data, the contribution of KCs and other antigen-presenting cells (APCs) in mo-macs sufficient and deficient liver metastasis, the interchange of macrophages and neutrophils and their impacts on T cell dynamics in the therapeutic model. The detailed comments and suggestions are as follows:

• The single cell data shown in Figure 1 clearly exhibit heterogeneity for both normal macrophages (N-mac) and LMAMs. It has also been reported that macrophages in the tumor microenvironment usually contains pro-tumor and anti-tumor phenotypes, which can be distinguished by the expression of angiogenesis (e.g. Spp1) and complement (e.g. C1q) genes. The authors should check if LMAMs in their models also include different subsets and how these subsets change in their multiple lineage tracing models. Also, they should show the expression of Cx3cr1, Ccr2 and other genes they use to generate models.

• Using different lineage tracing models, the authors demonstrated that LMAMs can be replenished either via increased local macrophage proliferation or by promoting Kupffer cell (KC) infiltration. Interestingly, the two circumstances seem to lead to different tumor growth directions – KCs are more active in phagocytosis and tumor-killing. How do they interact with T cell or other lymphocyte populations? Is the contribution/ratio of different origins of LMAMs could be a predictor of tumor metastasis/tumor microenvironment?

• In figure 4a, the changes from Ly6G-low to Ly6G-high of macrophages are particularly interesting. Since Ly6G is a neutrophil marker, is this shift a macrophage to neutrophil alteration? Other papers have reported that neutrophil is also a major regulator of liver metastasis and it promotes metastasis. How does increase of neutrophil in this model contribute to the metastasis?

• For Figure 4e and 4f, the authors should show T cell changes w/o mo-macs depletion to compare the dynamics of T cells upon treatment.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have provided a much-improved manuscript that satisfactorily addresses my main concerns. Overall, this is a nice work using many innovative genetic tools to answer important questions about the function and ontogeny of macrophages in liver metastases. A few minor issues remain that can be addressed in proofing:

The authors use several metastasis models, but it is not always clear which model was used for individual experiments.

Line 116, the added reference for Timd4 is relevant. But it would be helpful to also describe and cite why Tim4 is relevant. Florent Ginhoux has some more recent studies describing Tim4 (along with Lyve and Folr2) as general indicators of tissue residence versus monocyte macrophage. Or the authors can reuse citations more specific to the liver macrophage subsets.

There are still misspellings and areas with grammar issues throughout.

It was still hard for me to see the colors on the legend for 5g. If the line width is increased, I think this won't be a problem. This might be an issue with print outs because the legend colors were easy to interpret on a screen.

Reviewer #2

(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.

Reviewer #3

(Remarks to the Author)
The authors sufficiently addressed my comments and I agree with the revised manuscript.

Reviewer #4

(Remarks to the Author)
The authors have addressed my questions.

We sincerely appreciate reviewers for their constructive comments and invaluable insights. We have seriously taken their critiques and suggestions into account, and have carefully addressed all their concerns, as shown below marked in blue. The corresponding changes/additions to the manuscript are shown in red text. We are thankful for reviewers' willingness to consider the revised manuscript.

Reviewer #1 (Remarks to the Author):
"Availability of an inflammatory macrophage niche drives phenotypic and functional alterations in Kupffer cells"

Summary:
In this manuscript, Han-Ying Huang and Lin Tian define liver macrophage lineages in experimental models of metastatic liver cancer. Using several elegant lineage tracing systems, the authors determine that most macrophages associated with metastatic cancer in the liver are derived from CCR2 dependent peripheral blood monocytes. Interestingly, their evidence also supports a redundant system held in reserve to maintain macrophage metastasis-associated macrophage populations when monocytes are unable to support the demand. In this case, the author's experimental evidence indicates Kupffer cells, the predominant resident liver macrophage subset, contribute to making up deficiencies in overall macrophage abundance in livers with cancer metastasis. Notably, a small overlap exists with aspects of recent article in Nature by Frederic Geissmann and colleagues (PMC10881399) and the studies presented here. These overlapping aspects are in general agreement and make use of different liver cancer models and similar/complementary genetic systems. Overall, the authors assess a compelling scientific premise using many innovative genetic models and I only have a few concerns:

Thank you for the positive feedback and constructive suggestions to our manuscript. We address specific comments below.

Specific comments:

• It isn't always clear that the authors are defining normal liver macrophages (N-macs) as derived from the adjacent healthy tissue from the same livers that have liver cancer. Indeed, the only indications I found are on line 93 and figure 3e. This should not be much more clear, and maybe described at least once in each figure legend and results section. The impact of the manuscript is critically lessened if readers misinterpret N-macs as a comparison of macrophage from healthy control livers and not a comparison with adjacent healthy tissue. For perspective, this misinterpretation happened with my first two readings!

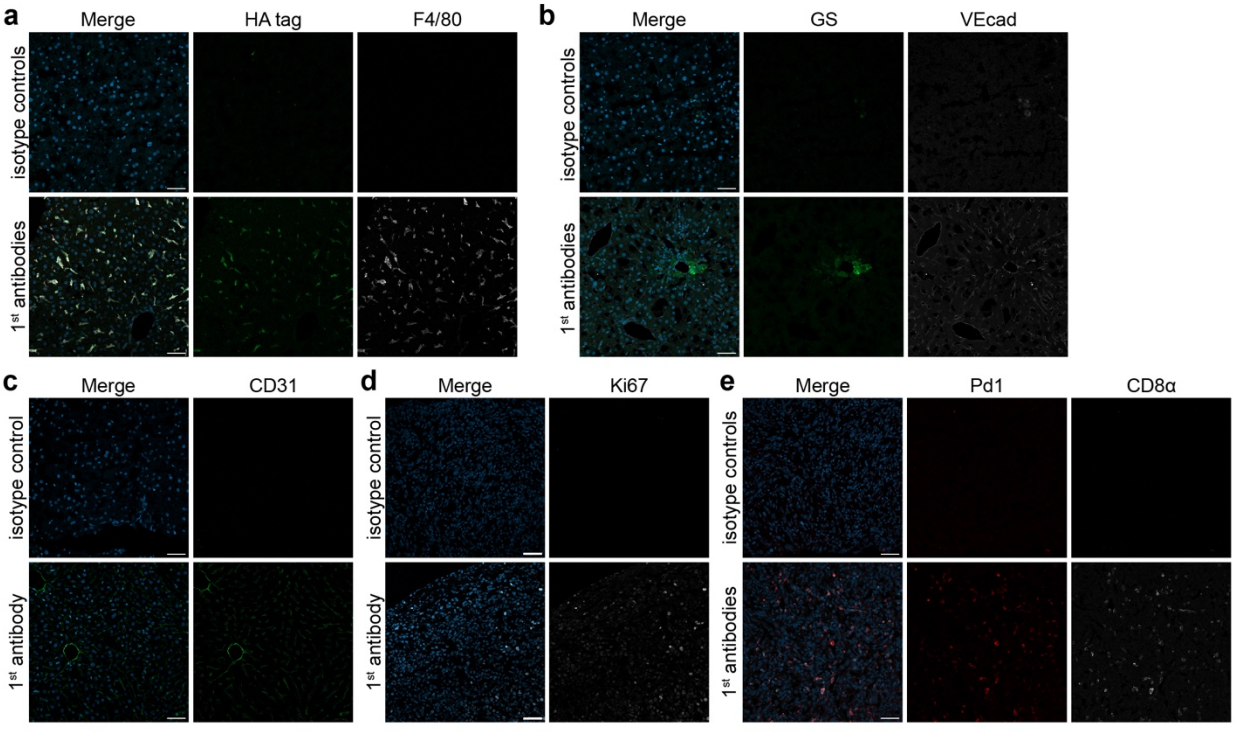
We apologized for the confusing use of N-macs in the main text. When comparing liver metastasis-associated macrophages (LMAMs) with N-macs, we consistently used the adjacent normal liver tissues from the same livers. We have included this information in the result section of in the figure legends.

• Some aspects of the manuscript could be better described in the results and/or appropriately referenced. Examples include the metastatic models and the function of Tim4.

We appreciate your careful evaluation of our manuscript. We have included reference for the two liver metastasis models (E0771 and MC38) and a spontaneous primary liver cancer model (**Ext Fig. 1a**). Additionally, we also added reference for CITE-seq, Cd68, Clec4f, Timd4, Cypher5E, liposomal clodronate, and DTR in the revised manuscript.

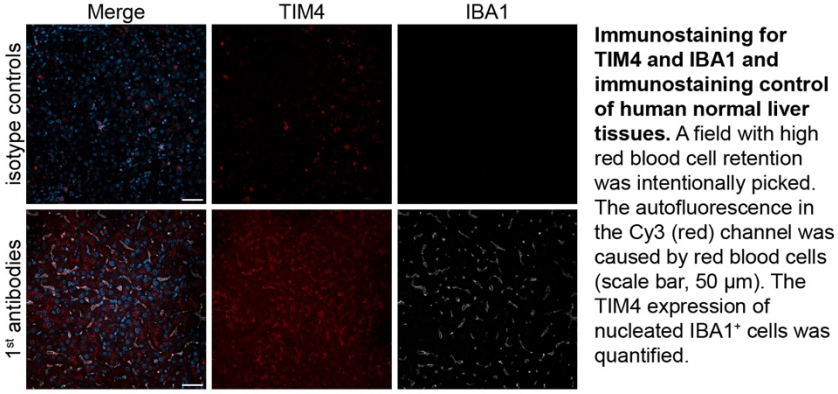
• Immunofluorescent experiments are generally lacking in controls. These controls prove signals are above background and not from autofluorescence.

Thanks for your valuable suggestions. Indeed, normal liver tissues exhibit high autofluorescence. For mouse tissues, we used two strategies, whole animal perfusion and TrueVIEW Autofluorescence Quenching Kit, to reduce this autofluorescence. To confirm that the immunofluorescence signals were not from autofluorescence, we used our fluorescent reporter mice *Clec4f*$^{tdT\text{-}Cre}$, *CD68*^{EGFP} and *R26*$^{LSL\text{-}RST\text{-}tdT\text{-}DTR}$ to evaluate the antibodies including anti-Clec4f, anti-tdTomato, anti-GFP and anti-hHB-EGF (DTR). In addition, we performed isotype controls for the other antibodies (anti-F4/80, anti-HA Tag, anti-Glutamine Synthetase, anti-VE cadherin, anti-CD31, anti-Ki67, anti-Pd1, and anti-CD8α). The results were shown below.



Immunostaining controls for antibodies used in the study. a) anti-HA tag and anti-F4/80 immunostaining of normal liver tissues from *Clec4f*$^{tdT\text{-}Cre}$;*R26*$^{LSL\text{-}UPRT\text{-}HA\text{-}sfGFP\ mice}$; **b, c)** anti-Glutamine Synthetase (GS), anti-VEcad and anti-CD31 immunostainings of normal liver tissues from *wild-type* mice. The CD31 expression level of heptic central veins was significantly higher than that of sinusoidal vessels. **d,e)** anti-Ki67, anti-Pd1 and anti-CD8α immunostainings of liver metastasis from *wild-type* mice. The control and the stained slides were imaged with same parameters. The contrast and brightness were not adjusted (scale bar, 50 μm).

For clinical samples of human liver tissues, the autofluorescence from red blood cells is strong, and the controls are particularly useful to distinguish real signals from the background autofluorescence. The results were shown below and were added to **Supplementary Data 3**.



Immunostaining for TIM4 and IBA1 and immunostaining control of human normal liver tissues. A field with high red blood cell retention was intentionally picked. The autofluorescence in the Cy3 (red) channel was caused by red blood cells (scale bar, 50 μm). The TIM4 expression of nucleated IBA1$^+$ cells was quantified.

• The Cut&Run and ATAC-seq data "appear" questionable. Genome wide assessments seem valid in 5g. However, ChIP data is expected to be peaky, unlike some of the locus specific examples chosen. For example, the Spp1, Trem2, and Id3 loci all have very distal regulatory activity in liver macrophage data from other labs. And the data from these labs also looks lower quality if the windows are set to a similar size as selected in this manuscript.

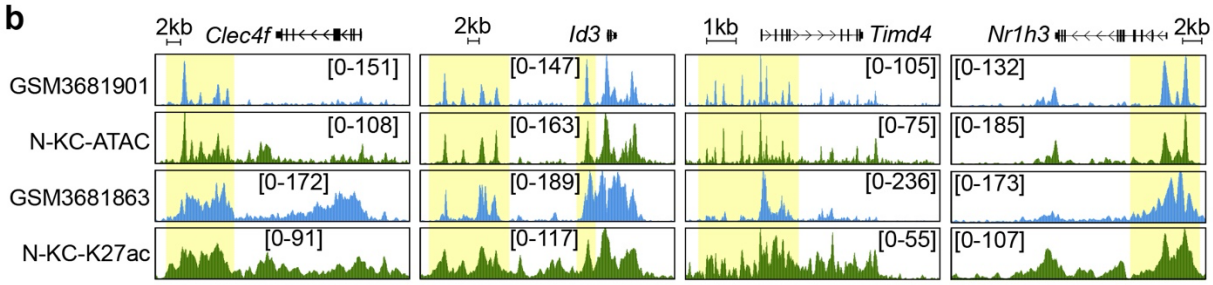
Thank you for the question regarding the quality of our epigenetic profiling data. Indeed, because of cross-linking and sonication during library preparation for chromatin immunoprecipitation (ChIP), ChIP-seq is characterized by high background peaky data and large number of cell input (more than 1 million). Compare with ChIP-seq, Cleavage Under Targets and Tagmentation (CUT&Tag) or Cleavage Under Targets and Release Using Nuclease (CUT&RUN) requires much less cells and generates data with high signal-to-noise ratio (Henikoff lab, ***Elife***, 2017, PMID 28079019; ***Nature Communications***, 2019, PMID 31036827). As we only isolated around 5×10^4 Kupffer cell-derived liver metastasis-associated macrophages from the metastatic nodules (~ 0.01 gram), CUT&Tag was chosen for profile macrophage enhancer landscapes in this study.

The regulatory elements of *Clec4f* and *Id3* we highlighted are indeed very distant from encoding regions (**Fig. 5i**). For *Clec4f*, the super enhancers are located at 10kb downstream to transcriptional termination site; for *Id3*, an enhancer is located at 20kb upstream to transcriptional start site. We also compared our ATAC-seq and CUT&Tag-seq data with published epigenetic profiling data of normal and fibrotic Kupffer cells from Dr. Chris Glass lab (Sakai et al, ***Immunity***, 2019, GSE128662; Seidman et al, ***Immunity***, 2020, GSE128338). Despite the use of different library preparation methods and sequencing platforms in the studies, the peak positions and RPKM-normalized signal intensities showed a high degree of similarity. This comparison results were shown below and were added to **Supplementary Data 4**.

a

sample name	assay	number of cells	read length	library	study	tissue
GSM3681901	ATAC-seq	50,000	75bp	single-end	Sakai et al 2019	normal liver
N-KC-ATAC	ATAC-seq	10,000	150bp	pair-end	this manuscript	normal liver
GSM3681863	ChIP-seq H3K27ac	500,000	75bp	single-end formaldehyde fixation	Sakai et al 2019	normal liver
N-KC-K27ac	CUT&Tag H3K27ac	10,000	150bp	pair-end	this manuscript	normal liver

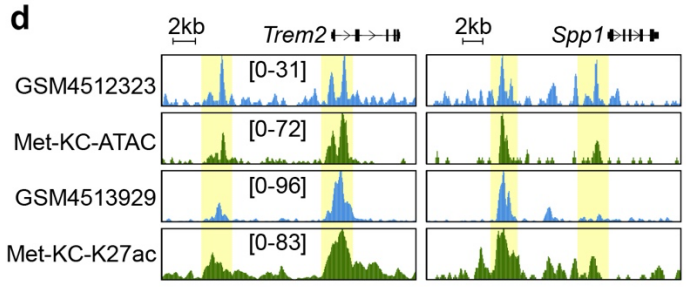
b



c

sample name	assay	number of cells	read length	library	study	tissue
GSM4512323	ATAC-seq	50,000	75bp	single-end	Seidman et al 2020	NASH liver
Met-KC-ATAC	ATAC-seq	10,000	150bp	pair-end	this manuscript	Liver Met
GSM4513929	ChIP-seq H3K27ac	500,000	75bp	single-end formaldehyde fixation	Seidman et al 2020	NASH liver
Met-KC-K27ac	CUT&Tag H3K27ac	10,000	150bp	pair-end	this manuscript	Liver Met

d



Comparison of epigenetic profiling data of Kupffer cells. **a,b)** overview of ATAC-seq data and CUT&Tag-seq for H3K27ac data of Kupffer cells isolated from normal liver and comparing peaks of KC-identity genes; **c,d)** overview of ATAC-seq data and CUT&Tag-seq for H3K27ac data of Kupffer cells isolated from liver diseases and comparing peaks of scar-associated macrophage genes. Enhancers were highlighted in yellow and the ranges of reads per kilobase million (RPKM)-normalized values were shown. (NASH, nonalcoholic steatohepatitis)

• Line 135 – Statement regarding tracing with Clec4f is not correct… “moMacs, which never express Clec4f”. Many studies in other contexts have shown that “moMacs” can express Clec4f when filling a vacant KC niche. Examples include many studies describing Tim4- Kupffer cells during steatohepatitis, and more recently a Science paper from Paul Kubes’s lab describing bone marrow origin syncytial macrophages that turn on Clec4f and take on properties of Kupffer cells.

Thank you for pointing out this problem. We agree that this statement was misleading. In addition to the Science paper you mentioned (Peiseler et al, ***Science***, 2023, PMID 37676943), Dr. Martin Guilliams lab and Dr. Chris Glass lab employed *Clec4f*^{DTR} transgenic mice to ablate

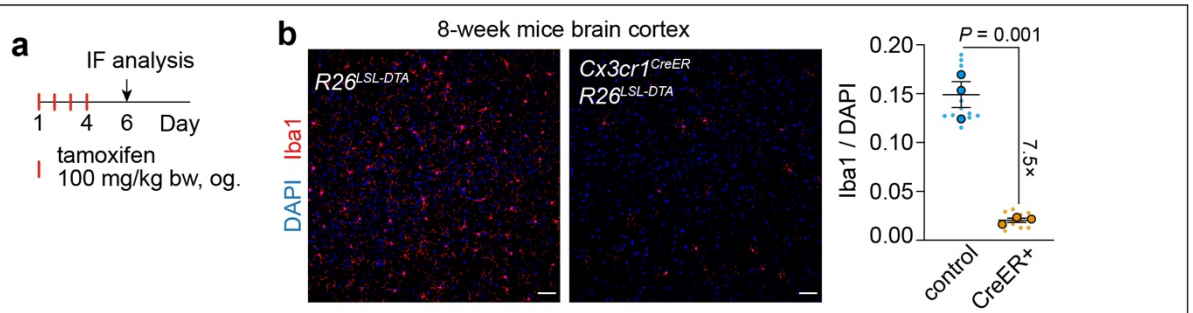
Kupffer cells, and found that the circulating monocytes can infiltrate into liver and differentiate into Clec4f^{+} Kupffer cells (Scott et al, ***Nature Communications***, 2016, PMID 26813785; Bonnardel et al, ***Immunity***, 2019, PMID 31561945; Sakai et al, ***Immunity***, 2019, PMID 31587991). This inappropriate statement was replaced with "*mo-macs, which are derived from Clec4f^{-} monocytes*" in the revised manuscript (Line 144).

• Some histology (ED6d) or immunohistochemistry data, if available, would be beneficial. Especially for introduction of the metastasis model at figure 1. I don't view this as necessary.

Thank you for this valuable suggestion. We added an introduction panel of tumor models used in this study (**Ext Fig. 1a**).

• Figure 2d: Depletions efficiencies are not described.

Because KCs are independent of Cx3cr1, we chose microglia to test the depletion efficiencies of *Cx3cr1^{CreERT2};R26$^{LSL-DTA}$* (**Fig. 2d**), in addition to the inflammatory monocyte mentioned in the manuscript (**Fig. 2i** and **Fig. 2j**). Microglia of adult mice are positive for Cx3cr1 and can be fluorescently labeled using tamoxifen inducible Cre recombinase driven by endogenous *Cx3cr1* promoter (Yona et al, ***Immunity***, 2013, PMID 23273845). Similar to others (Liu et al, *Nature Communications*, 2021, PMID 34330901), a four-dose tamoxifen treatment resulted in around 7.5-fold decrease of Iba1^{+} microglia in the cortex region of brain, suggesting this model is highly efficient for genetically ablate Cx3cr1^{+} cells.



Microglia Depletion efficiency of *Cx3cr1^{CreER};R26$^{LSL-DTA}$*. a) Schematic of oral gavage (og.) feeding of tamoxifen; **b)** immunofluorescence staining and quantification of Iba1^{+} microglia after tamoxifen treatment in *Cx3cr1^{CreER};R26$^{LSL-DTA}$* mice and littermate Cre-negative controls.
Smaller dots are values from individual fields. Outlined circles are mean values taken over multiple fields/sections from the same mouse. *P* value was calculated by comparing individual animals using two-tailed paired Student's *t*-test.

• Figure 2e: Tamoxifen dosing schedule is unclear.

Thank you for this feedback. We used oral gavage feeding for tamoxifen treatment (100 mg/kg body weight), which was less toxic than intraperitoneal Injections (Donocoff et al, ***Sci Rep***, PMID 32943672). The tamoxifen dosing schedule was added to the corresponding figure (**Fig. 2e**, **Fig. 3f**, **Fig. 5c**) and method in the revised manuscript (Line 503).

• Figure 3: Analysis of proliferation occurs 12 days after initial clodronate. Is the proliferation rate dynamic?

Thank you for the question. Based on our bioluminescence imaging data, micro-metastasis started to form around Day12 post-portal vein injection, which we believed is an appropriate time point to start to record macrophage proliferation.

An earlier time point (e.g., Day 6 post-tumor engraftment) may record monocyte or macrophage proliferation before the formation of metastatic colonization. As the progenies of these proliferated macrophages will also be labeled with sfGFP, this results in an overestimation of the proliferation rate of LMAMs. On the other side, a later time point (e.g., Day 18 post-tumor engraftment) may miss the local macrophage proliferation during incipient metastatic outgrowth.

• Several figure panels are challenging to interpret. Some dot plots have various shading patterns that are challenging to interpret as their definition is only provided in the methods section under the statistics heading (2b, 4h). Color choices and or line widths for 5h and 5i are challenging to discriminate. Fig 2b and Ext 2b use inconsistent population definitions, which was a little confusing.

Thank you for your feedback. **1)** For immunofluorescence, smaller dots without an outline are values from individual fields (~0.4 mm^2 fields), and circles that are outlined represent mean values taken over 4−5 fields from the same mouse. We now added this description in each figure legends. **2)** For **Fig. 5h** and **5i**, we have changed the color panel and used traditional IGV track plots other than mean-averaged lines. **3)** For **Fig 2b,c** and **Ext Fig 2b,c**, we have used a consistent population definition (the percent of HA^+ or $sfGFP^+$ in $F4/80^+$ cells).

• The methods are generally well written and adequately describe approaches used. Sources of mouse models are described, but some primary references may be missing.

Thank you for this comment. We now added reference for mouse models *Clec4f*$^{tdT\text{-}Cre}$, *CD68*^{EGFP}, *Cx3cr1*^{CreERT2}, and *Ccr2*^{GFP} in “Animals used” section of **Method**.

• Quality control considerations for ‘omics data are not described, and no QC stats are provided.

Thank you for the feedback. We have added “sequencing depth” information to the “single cell RNA-seq” (Line 699-724), “single cell TCR-seq” (Line 749-774) and “Epigenetic profiling” (Line 781-785) in the revised manuscript.

• There are several minor typos that will be caught by spell check.

Thank you for the feedback. We have double checked the spelling and corrected some typos.

Reviewer #1 (Remarks on code availability):

CITE-seq describes plot creation only. Not mapping/filtering/clustering/etc.

Thank you for your evaluation on our CITE-seq data. We agree that the original CITE-seq analysis was not informative enough. We have re-analyzed the data and performed clustering for the LMAMs with a higher resolution and identified 5 major clusters of populations (Spp1, Cxcl9, Ccl5, Vcan, Stmn1), suggesting the heterogeneity within LMAMs (**Fig. 1e**). The clustering results were largely consistent with previously reported macrophage scRNA-seq data (Qi et al, ***Nature Communications***, 2022, PMID 35365629; Wu et al, ***Cancer Discovery***, 2022, PMID 34417225; Bill et al, ***Science***, 2023, PMID 37535729). Notably, this clustering result of CITE-seq was largely consistent with an independent scRNA-seq experiment shown in **Fig. 4c**.

The parameters for mapping, filtering and clustering were added to Method section (Line 699-708). The full codes were added to the GitHub (https://github.com/lintian0616/KC_plasticity).

Reviewer #2 (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.

We appreciate Reviewer 2 for their feedback.

Reviewer #3 (Remarks to the Author):
Review of: Availability of an inflammatory macrophage niche drives phenotypic and functional alterations in Kupffer cells

In this study, the authors characterized the myeloid cell pool in liver metastasis, confirmed some of their observations in samples of patients, and showed that the tumor microenvironment consists of LMAMs derived from mo-macs, and that KCs are depleted. These observations are in line with many other publications showing that (1) liver injury, and primary liver cancer, results in KC depletion and monocyte infiltration with mo-mac the main mac population during liver injury and cancer and (2) KC niches can be replenished by local proliferation (minor proportion) and monocyte infiltration (main contributors). The authors also claim that inhibition of monocyte infiltration and mac proliferation opens the opportunity for KC proliferation/infiltration and manipulation of KC differentiation to LMAMs as potential therapeutic strategy. Finally, they present that inhibition of monocyte infiltration and mac proliferation increases the efficacy of ICI.

Overall, the authors performed extensive fate mapping experiments that support the main conclusions of their study. The data are relevant and, although the main insights have been shown (at least in part) in other liver diseases and primary liver cancer, the results provide new insights in the pathogenesis of liver metastasis, essential to expand the therapeutic landscape. However, to claim increased efficacy of ICI, supporting data/figures are necessary.
The main aspects that should be addressed are provided below:

Thank you for the positive feedback and careful review of our manuscript. Your suggestions helped us to improve the accuracy of our manuscript. We address specific comments below.

In the first setting, the authors analyzed myeloid cell populations in liver metastasis and adjacent normal tissue. How far at distance was 'normal tissue' collected, and did the authors also investigate the difference between tumor nodule and surrounding microenvironment (which is usually not 'normal' but an infiltrating niche)? A schematic view of sampling would increase clarity in this respect.

Thank you for the comment. A schematic view was added to the revised manuscript (**Fig. 1d**).

The authors characterized the myeloid cell pool in TME in liver metastasis and those results resemble other observations in hepatocellular carcinoma (decreased population of KCs, increase of mo-mac). Do the authors think that common signals in liver metastasis and primary liver cancer are responsible for these observations? In addition, do common signals also drive KC differentiation? Can the authors comment on this in the discussion? At least the findings in primary liver cancer, published by other groups, should be addressed in the discussion.

Thank you for the insightful suggestions. Indeed, the phenomenon that KCs were replaced by mo-macs in liver metastasis was also reported in primary liver cancer (Lefere et al, ***Journal of Hepatology***, 2019, PMID 31213365), and was shown to be mediated in a CCR2–CCL2-dependent manner (Li et al, ***Nature Reviews Cancer***, 2021, PMID 34326518). To experimentally confirm this, we added a spontaneous Myc overexpression (Myc^{OE}) and TP53 knockout ($TP53^{KO}$) hepatocellular carcinoma (HCC) model via hydrodynamic tail vein injection (HDTV) (Galarreta et al, ***Cancer Discovery***, 2019, PMID 31186238) in the revised manuscript (Line 86-88). Consistent with our observations in liver metastasis models, the number of $CD11b^{low}$ $F4/80^{+}$ KCs was reduced in HCC, while the number of $CD11b^{high}$ $F4/80^{+}$ mo-macs was increased in HCC (**Fig. 1b,c**). We have added the other groups' observations and our findings of primary liver cancer in the section of **Discussion** (Line 356-360, Line 375-377).

We believed that the common signal drives the KC trans-differentiation in HCC. Because the niche interactions among statelet cells, sinusoidal endothelial cells, hepatocytes are critical for maintaining the expression of KC-identify genes (Bonnardel et al, ***Immunity***, 2019, PMID 31561945; Sakai et al, ***Immunity***, 2019, PMID 31587991), we hypothesized that the disruption of this normal liver niche in tumor microenvironment drives the loss of KC-identity genes and trigger the differentiation towards mo-macs. To test this hypothesis in HCC, we used HDTV to generate the Myc^{OE};$TP53^{KO}$ primary liver cancer in our KC-tracing mouse model. Consistent with our findings in liver metastasis models, we observed more KC-derived tumor-associated macrophages in the monocyte-deficient background (*Clec4f*$^{tdT\text{-}Cre}$*;R26*$^{LSL\text{-}UPRT\text{-}HA\text{-}sfGFP}$*;Ccr2*$^{ko/ko}$) in comparison with the littermate controls (*Clec4f*$^{tdT\text{-}Cre}$*;R26*$^{LSL\text{-}UPRT\text{-}HA\text{-}sfGFP}$*;Ccr2*$^{ko/wt}$) (**Fig. 4e**). And these KC-TAMs also exhibit phenotypic changes by decreasing expression of KC identity genes such as Clec4f and Timd4 (**Fig. 4j,k**).

Line 197: "Although CLs can effectively deplete both circulating monocytes and KCs (Fig. 3c), they cannot cross the tumor vasculature due to their size; thus, macrophages within metastatic nodules are not depleted." How sure are the authors that CLs cannot cross the tumor

vasculature? This should be confirmed, i.e. by measurements of tumor vessels. It can also be that LMAMs do not engulf liposomes.

Thanks for this critical question. We agree that the statement "*they cannot cross the tumor vasculature due to their size*" is arbitrary. For example, it is possible that CL can cross tumor vessel, but cannot be uptaken by LMAMs. Therefore, we replace this inappropriate statement with "*they cannot be engulfed by LMAMs, as revealed by the red fluorescent DiI-encaupsulated liposomes, which have similar size with CLs (**Fig. 3d,e**); thus, macrophages within metastatic nodules are not depleted*" in the revised manuscript (Line 211-212).

Line 302: The authors claim that dual blockade of Ccr2 and Csf1r increased the efficacy of anti-Pd1 immune checkpoint inhibition compared with that achieved by knockout of Ccr2 alone. However, the only data supporting this, are presented in Fig 6, panel f: "Representative bioluminescence imaging of metastasis-bearing $Ccr2^{GFP/GFP}$ mice and $Ccr2^{GFP/WT}$ littermates treated with PLX5622 and anti-Pd1 antibody (left)". These images do not clearly show the effect of single and dual blockade of Ccr2 and Csfr1 and the increased efficacy of anti-Pd1 in the dual blockade strategy. Please change the figures and images accordingly.

We agree that it would be more rigid to test the effect of the single and dual blockade of Ccr2 and Csf1r on efficacy of anti-Pd1 treatment. Interestingly, knockout of Ccr2 alone can significantly improve the efficacy of anti-Pd1 treatment, as evidence by an increase of effector T cells and a decrease of terminally exhausted T cells (**Ext Fig. 9**). And combined blockade of Ccr2 and Csf1r further increased response rates and decreased liver metastasis burden compared to single blockade of Ccr2 (**Fig. 6f**). The data was added to the revised manuscript (Line 333-351).

References are used in the abstract, which is unusual.

Thank you for the comment. We have removed the references in the abstract, according to formatting instructions of Nature Communications.

Reviewer #4 (Remarks to the Author):

In this manuscript, Huang et al. developed and applied multiple lineage tracing models to interrogate the origin of liver metastasis-associated macrophages (LMAMs) in different scenarios. They demonstrated that monocyte-derived macrophages (mo-macs) are the major population of LMAMs, while in monocyte-deficient occasions, LMAMs can be replenished either via increased local macrophage proliferation or by promoting Kupffer cell (KC) infiltration. Further, they proposed therapeutic strategies of dual blockade of monocytes and macrophages as a way to reprogram the microenvironment of liver metastasis. The scientific question is of great importance and the manuscript is overall well-written. Notably, the models developed in this study are impressive and valuable to decouple the macrophage ontogeny in other diseases. However, there are several key elements missing – the heterogeneity of LMAMs from the single-cell data, the contribution of KCs and other antigen-presenting cells (APCs) in mo-macs sufficient and deficient liver metastasis, the interchange of macrophages and neutrophils and

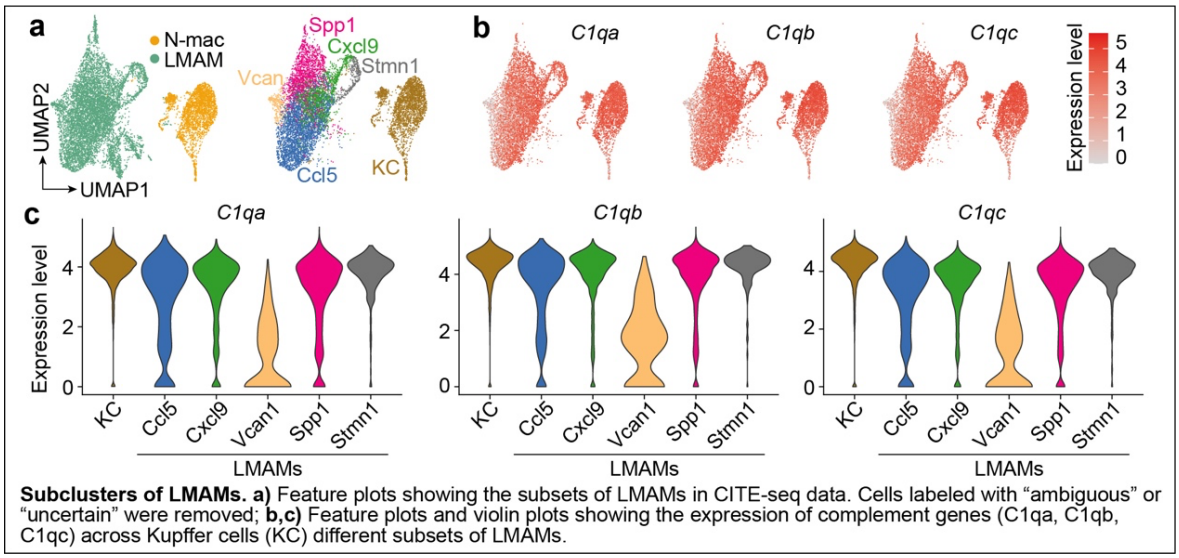
their impacts on T cell dynamics in the therapeutic model. The detailed comments and suggestions are as follows:

Thank you for the positive feedback and careful review of our manuscript. We appreciate your questions regarding liver metastasis-infiltrating T cells, which helped to increase the impact of the story and to reach a broader audience. We address specific comments below.

• The single cell data shown in Figure 1 clearly exhibit heterogeneity for both normal macrophages (N-mac) and LMAMs. It has also been reported that macrophages in the tumor microenvironment usually contains pro-tumor and anti-tumor phenotypes, which can be distinguished by the expression of angiogenesis (e.g. Spp1) and complement (e.g. C1q) genes. The authors should check if LMAMs in their models also include different subsets and how these subsets change in their multiple lineage tracing models. Also, they should show the expression of Cx3cr1, Ccr2 and other genes they use to generate models.

Thank you for your evaluation on our CITE-seq and scRNA-seq data. Indeed, macrophages are heterogeneous and plastic, and can exert either anti-tumor or pro-tumor functions. After using a higher resolution to re-cluster the liver metastasis-associated macrophages (LMAMs) in our CITE-seq data, we identified 5 major clusters of populations (Spp1, Cxcl9, Ccl5, Vcan, Stmn1), which were largely consistent with previously reported macrophage scRNA-seq data (Qi et al, ***Nature Communications***, 2022, PMID 35365629; Wu et al, ***Cancer Discovery***, 2022, PMID 34417225; Bill et al, ***Science***, 2023, PMID 37535729). The LMAM clustering data was added to the revised manuscript (**Fig. 1e** and **Fig. 4c,d**).

We checked the expression of complement genes (*C1qa, C1qb, and C1qc*) in LMAMs, which has been shown to related with anti-tumor functions of macrophage in human colorectal cancers (Qi et al, ***Nature Communications***, 2022, PMID 35365629). However, we found both N-macs and LMAMs have high expression of these complement genes. For example, C1q genes were highly expressed even in Spp1^{+} pro-tumor macrophages, suggesting some discrepancies of macrophages in the primary cancer and in the metastatic cancer.



Subclusters of LMAMs. a) Feature plots showing the subsets of LMAMs in CITE-seq data. Cells labeled with "ambiguous" or "uncertain" were removed; **b,c)** Feature plots and violin plots showing the expression of complement genes (C1qa, C1qb, C1qc) across Kupffer cells (KC) different subsets of LMAMs.

We also checked the expression of *Cd68*, *Clec4f*, *Cx3cr1* and *Ccr2*, which we used to generate tracing models. Indeed, both *Cx3cr1* and *Ccr2* were upregulated in LMAMs, thereby providing the rationales for the models we used (e.g., $Cx3cr1^{Cre};R26^{LSL\text{-}DTA}$ and $Ccr2^{GFP/GFP}$) to inhibit mo-macs. This was added to the revised manuscript (**Fig. 1e**).

• Using different lineage tracing models, the authors demonstrated that LMAMs can be replenished either via increased local macrophage proliferation or by promoting Kupffer cell (KC) infiltration. Interestingly, the two circumstances seem to lead to different tumor growth directions – KCs are more active in phagocytosis and tumor-killing. How do they interact with T cell or other lymphocyte populations? Is the contribution/ratio of different origins of LMAMs could be a predictor of tumor metastasis/tumor microenvironment?

Thank you for this insightful question. The KCs in normal liver are important immune component that eliminates tumor cells either by phagocyting live tumor cells or by recruiting cytotoxic $CD8^+$ T cells (Deng et al, ***Nature***, 2024, PMID 38326607). However, KCs exhibited phenotypic changes and started to express pro-tumor genes (**Fig. 4d-f**) and failed to eliminate apoptotic cells efficiently (**Fig. 5e,f**) when infiltrating into liver metastasis nodules. Therefore, we think the KC-derived LMAMs represent as an intermediate state between normal KC and mo-macs.

To investigate whether a higher ratio of KC-derived LMAMs could change T cell population, we conducted single-cell TCR sequencing combined with scRNA-seq on $CD8^+$ T cells infiltrating liver metastases from $Ccr2^{GFP/WT}$ background or $Ccr2^{GFP/GFP}$ background. This approach enabled us to investigate whether the presence of liver metastasis-associated macrophages from different origins could serve as a potential indicator of the tumor microenvironment. We found that the exhausted T cells were highly expanded regardless of Ccr2 backgrounds (**Ext Fig. 9c**). However, a higher percent of effector T cells and lower percent of terminally exhausted T cells were observed in monocyte deficient $CCR2^{GFP/GFP}$ background, which was correlated with a higher ratio of KC-derived LMAMs to mo-macs (**Ext Fig. 9e**).

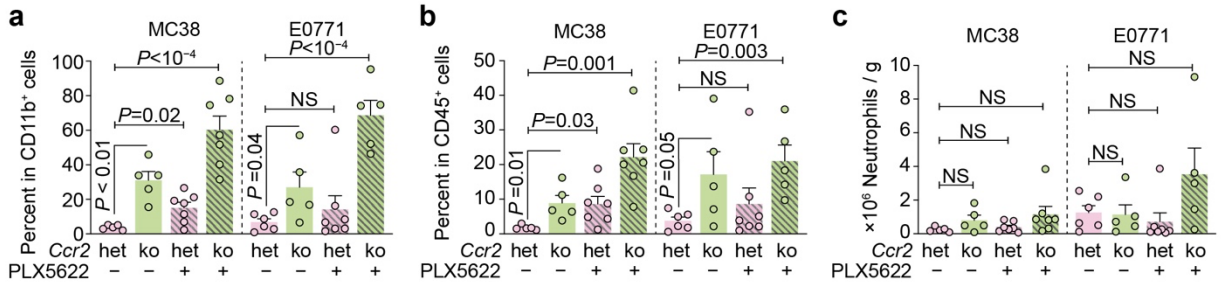
In addition to analyzing T cells and macrophages in the mouse models, it would be intriguing to investigate if the origins of LMAMs could serve as a predictor for the tumor microenvironment using a large cohort of clinical samples. However, since KC-derived LMAMs phenotypically resemble mo-macs, distinguishing the origins of LMAMs from transcriptome data poses a challenge. Because the KC-derived LMAMs maintained epigenetic imprinting (**Fig. 5g-i**), an integrated analysis of single-cell transcriptome and chromatin accessibility data will be essential to address the question. This discussion was added to the revised manuscript (Line 387-395).

• In figure 4a, the changes from Ly6G-low to Ly6G-high of macrophages are particularly interesting. Since Ly6G is a neutrophil marker, is this shift a macrophage to neutrophil alteration? Other papers have reported that neutrophil is also a major regulator of liver metastasis and it promotes metastasis. How does increase of neutrophil in this model contribute to the metastasis?

Indeed, neutrophil has been shown to promote liver metastasis though its extracellular traps (Yang et al, ***Nature***, 2020, PMID 32528174; Xia et al, ***Nature Communications***, 2022, PMID

35197446). In our study, we did find that the percent of Ly6G$^+$ neutrophil was increased in both CD11b$^+$ myeloid cells and CD45$^+$ leukocytes when Ccr2 and Csf1r were blocked (**Fig. 6a**), which was largely due to the substantial decrease of the monocytes and LMAMs. To mitigate this "monocyte/macrophage to neutrophil shift", we think it will be an interesting direction in the future to simultaneously impair monocytes, macrophages, and neutrophils (e.g., anti-GM-CSF or anti-Ly6G).

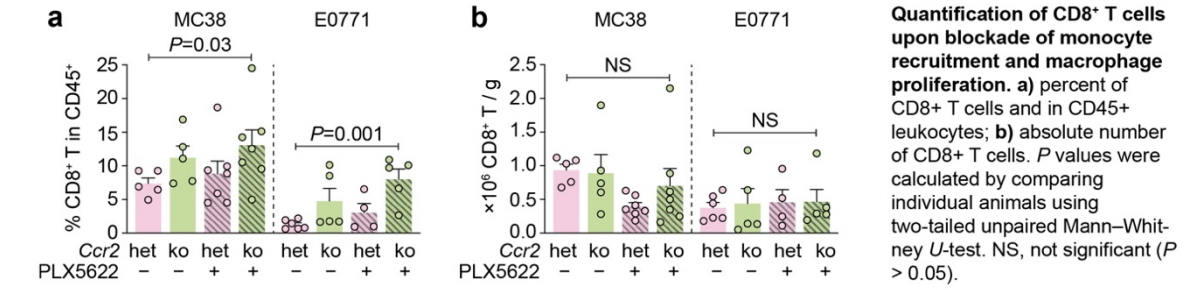
However, the increase of the absolute numbers of LyG$^+$ neutrophils was NOT statistically significant. The results were shown below and was added to **Ext Data Fig. 8a-c** (Line 320-325). Furthermore, dual blockade of monocyte recruitment and LMAM proliferation markedly improved the efficacy of anti-Pd1 treatment (**Fig. 6f**). Therefore, we concluded that net effect of Ccr2 and Csf1r inhibition was to drive a more immunostimulatory tumor microenvironment that enhanced immune checkpoint blockade therapy.



Quantification of CD11b$^+$ LyG$^+$ neutrophil upon blockade of monocyte recruitment and macrophage proliferation. a,b) percent of neutrophils in CD11b+ myeloid cells and in CD45+ leukocytes; **c)** absolute number of neutrophils. *P* values were calculated by comparing individual animals using two-tailed unpaired Mann–Whitney *U*-test. NS, not significant ($P > 0.05$).

• For Figure 4e and 4f, the authors should show T cell changes w/o mo-macs depletion to compare the dynamics of T cells upon treatment.

We used flow cytometry to quantify the numbers of CD8$^+$ T cells with mo-macs depletion and PLX5622 treatment. Due to the profound decrease of mo-macs, it is not surprising that the percent of CD8$^+$ T cells was significantly increased in liver metastasis of when Ccr2 and Csf1r were blocked. However, the absolute number of CD8$^+$ T cells was unchanged. Although metastasis-associated macrophages can prevent T cell infiltration in lung (Peranzoni et al, ***PNAS***, 2018, PMID 29632196) and bone (Xu et al, ***bioRxiv***, 2024, PMID 38765966), our data suggested that LMAMs did not function to exclude CD8$^+$ T cells. The results were shown below and was added to **Ext Data Fig. 8d,e** (Line 335). Instead, our data support the model that liver metastasis could siphon T cells from primary site and LMAMs function to induce T cell apoptosis subsequently (Yu et al, ***Nature Medicine***, 2021, PMID 33398162).



Quantification of CD8$^+$ T cells upon blockade of monocyte recruitment and macrophage proliferation. a) percent of CD8+ T cells and in CD45+ leukocytes; **b)** absolute number of CD8+ T cells. *P* values were calculated by comparing individual animals using two-tailed unpaired Mann–Whitney *U*-test. NS, not significant ($P > 0.05$).

Reviewer #4 (Remarks on code availability):

The code is not completed yet.

We apologize for missing some codes. The full codes were added to the GitHub (https://github.com/lintian0616/KC_plasticity).

We would like to express our sincere gratitude to the reviewers for their efforts and time in evaluating our revised manuscript.

Reviewer #1 (Remarks to the Author):

The authors have provided a much-improved manuscript that satisfactorily addresses my main concerns. Overall, this is a nice work using many innovative genetic tools to answer important questions about the function and ontogeny of macrophages in liver metastases. A few minor issues remain that can be addressed in proofing:

We appreciate these suggestions, which have further enhanced the clarity of data presentation in the manuscript.

The authors use several metastasis models, but it is not always clear which model was used for individual experiments.

In all our experiments, we used mouse colorectal cancer cells (MC38) and mouse breast cancer cells (E0771). To investigate whether Kupffer cells are absent in liver cancer and whether they can occupy macrophage niches left vacant by monocyte depletion in primary liver cancer, we also employed a spontaneous hepatocellular carcinoma model, as illustrated in **Fig. 1b,c** and **Fig. 4e,j,k**.

Therefore, we believe that our observations are not limited to a single mouse model. Rather, the concept that tissue-resident macrophages compete with recruiting monocytes for limited inflammatory macrophage niches can be generalized across multiple models.

Line 116, the added reference for Timd4 is relevant. But it would be helpful to also describe and cite why Tim4 is relevant. Florent Ginhoux has some more recent studies describing Tim4 (along with Lyve and Folr2) as general indicators of tissue residence versus monocyte macrophage. Or the authors can reuse citations more specific to the liver macrophage subsets.

Thank you for the suggestion. We have incorporated the recent work of Prof. Ginhoux and his collaborators (Dick SA, et al, ***Nature Immunology***, 2019, PMID 30538339; Dick SA, et al, ***Science Immunology***, 2022, PMID 34995099) when introducing Timd4 (Line 107 in the revised manuscript).

There are still misspellings and areas with grammar issues throughout.

We sincerely apologize for the misspellings in the manuscript. We have thoroughly reviewed the document and made every effort to correct these errors.

It was still hard for me to see the colors on the legend for 5g. If the line width is increased, I think this won't be a problem. This might be an issue with print outs because the legend colors were easy to interpret on a screen.

Thank you for bringing this issue to our attention. We have increased the thickness of the profile plots for transcriptional start sites in **Fig. 5g**.

Reviewer #2 (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.

We appreciate your suggestions, which have contributed to the overall quality of our manuscript.

Reviewer #3 (Remarks to the Author):

The authors sufficiently addressed my comments, and I agree with the revised manuscript.

Thank you for approving our manuscript.

Reviewer #4 (Remarks to the Author):

The authors have addressed my questions.

Thank you for approving our manuscript.