nature portfolio

Peer Review File

Inhibiting B cells enhances the efficacy of STING agonism or immune checkpoint blockade in hepatocellular carcinoma

Corresponding Author: Professor Dan Duda

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)
The manuscript “Inhibiting B-cell-mediated Immunosuppression to Enhance the Immunotherapy Efficacy in Hepatocellular Carcinoma” presents additional evidence that B cells contribute to the resistance to immune checkpoint blockade in hepatocellular carcinoma. Their studies showed that B cells are increased in liver of ICB treated mice, and STING agonism increases circulating IL-10 and intertumoral infiltration of B cells. Further, combination of STING agonism and Tim-1 blockade augments B cell differentiation and improves anti-tumor effects in murine HCC. However, overall B cell population and Tim-1 expression on B cells are very low in the liver tissues of tumor-bearing mice. Also, Tim-1 is expressed in T cells and other immune cells. Therefore, the evidence that Tim-1+ immunosuppressive B cells are a major source of therapy resistance in hepatocellular carcinoma is not very strong. Previous studies have shown that STING agonists result expansion of IL-35+ regulatory B cells in PDAC. B cell infiltration is much higher in pancreas than in liver.
Several other points the authors could address to enrich the manuscript.
1. Fig. 2C showed enrichment score of different immune subsets in control and STING treated mice. It seems that B cells are major immune cells after STING treatment. However, it’s known that myeloid cells are major population of immune cells in liver. Using Flow cytometry analysis of immune cell subsets within CD45+ cells will make data more convincing.
2. Fig. 6G showed Tim-1 expression in B cells with very low levels after STING treatment. Whether Tim-1+ B cells produce more IL-10 and IL-35 remains unknown.
3. B220 can be used as a B cell marker. However, B220 is also used as a marker to identify mouse plasmacytoid dendritic cells. It’s better to use CD19 and B220 to define B cells in Fig. 6E.
4. B cells have multiple subsets depending on their stage of development and function. The effect of B cells on tumor immunity depends on the balance of various B cell subtypes. Many studies have shown that B cells can promote tumor progression in mouse tumor models whereas human studies have shown that B cell infiltration is associated with better prognosis and response to immunotherapy. The translational relevance of findings from B cell depletion are unclear.

Reviewer #2

(Remarks to the Author)
The manuscript investigates the role of B cells, particularly TIM1+ B cells, in the context of PD-1 blockade and STING agonist therapy in murine hepatocellular carcinoma (HCC) models with underlying liver fibrosis. The authors reported that B cell depletion enhances the efficacy of immune checkpoint blockade (ICB) and STING agonism, while TIM1 blockade synergizes with STING agonists to improve antitumor responses. Additionally, they observed that STING agonism exhibits pronounced anti-metastatic activity, unlike ICB. While the study addresses an underexplored aspect of B cell biology in HCC immunotherapy, the depth could be further enhanced for publication.

Suggestions:
The current study reports that B-cell depletion enhances the efficacy of combined ICB and STING agonist therapy. While the authors observe increased B-cell aggregates and TLS-like structures in STING agonist-treated mice, any further analysis to define B-cell subsets (e.g., regulatory vs. effector B cells) or TLS maturity?
1. The study lacks specificity of TIM1 blockade effects. TIM1 is expressed on multiple immune and non-immune cells (e.g., T cells, B cells). The authors use anti-TIM1 antibody therapy but fail to demonstrate TIM1 expression patterns in their model

(e.g., flow cytometry or IHC to show TIM1+ cell distribution). They did not exclude off-target effects on non-B cells (e.g., does TIM1 blockade also affect TIM1+ T cells?).
2. Given that STING agonists can directly activate dendritic cells and reshape the TIME, the observed effects may not be B cell dependent. The authors should validate their findings in B cell-deficient models.
3. Are the expanded B-cell populations identical between PD-1 blockade and STING agonism? What is the proportion of TIM1+ B cells?
4. Does adding VEGFR2 inhibition to PD-1 blockade alter B-cell abundance or polarization?
5. Do STING agonists/TIM1 blockade show similar efficacy in spontaneous models (e.g., Myc; Trp53 KO or DEN-induced HCC)?
6. Human HCC data (e.g., TIM1+ B cell correlates from public datasets) would strengthen the translational impact.
7. The work could be improved by adding data related to single-cell RNA-seq or high-dimensional flow cytometry to dissect B cell subsets and genetic models (e.g., conditional TIM1 KO) to validate B cell-specific roles, or the spatial profiling of TLS and immune crosstalk in the TIME.

Reviewer #3

(Remarks to the Author)
In this manuscript, Liu et al. explore the role of B cells in modulating the efficacy of immunotherapy in hepatocellular carcinoma (HCC). Using mouse models, they show that treatment with the STING agonist BMS-986301 promotes the expansion of a tumor-supportive B cell subset and TLS-like structures in an immune checkpoint inhibitor (ICI)-sensitive model. B cell depletion enhanced the response of both ICI-resistant and ICI-sensitive tumors to STING agonist treatment and/or checkpoint blockade. Additionally, combining the STING agonist with TIM-1 blockade resulted in synergistic tumor suppression. While the manuscript is generally well written and the data are presented clearly, there are significant limitations in data interpretation and mechanistic insight that should be addressed.
Major points.
- The experiments in Figure 4 lack a single-arm B cell depletion control, which is necessary to demonstrate whether the efficacy of the STING and checkpoint inhibitor combination is dependent on B cells.
- In Figure 6, the experiments should be repeated using a B cell-deficient background or under conditions of conditional STING and/or TIM-1 deletion specifically in B cells to establish a mechanistic link. This is particularly important given that TIM-1$^+$ cells represent only ~1% of the B cell population. What accounts for their disproportionate effect? Are these cells antigen-specific?
- Related to the above point, it remains unclear which immune cell populations are responding to STING agonist and/or anti-PD-1 treatment. Targeted depletion of T and/or B cells could help clarify the cellular contributors to the observed therapeutic effects.
- In Figure 3D, the potential impact of fibrosis on B cells has not been addressed; an additional control arm is needed. Furthermore, the metastasis data in Figures 3G and 3H are confounded by differences in primary tumor growth, which may influence the results.
- To strengthen the generalizability of the findings, the study should ideally include more than one STING agonist.
Minor points
- Numbers of mice and error bars need to be added for all the graphs. Some in vivo experiments have quite low n (3-4) and the sample size needs to be increased.
- B220 is not specific for B cells, CD19 or CD20 should be used (Figure 2E, F)
- Please specify which media and/or supplements were used for B cell culture.
- B cell depletion should also be confirmed within the tumor tissue, as intratumoral B cells are typically more resistant to depletion than those in peripheral compartments.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors have addressed my concerns.

Reviewer #2

(Remarks to the Author)
The authors have addressed all my concerns. Thank you very much for the detailed revision.

Reviewer #3

(Remarks to the Author)
Unfortunately, most of the pertinent points were not addressed experimentally, but rather referred to prior studies (which cannot address B cell/STING aspect) or provided transcriptomic based data, which is not sufficient to address the questions at hand.

RESPONSE TO REVIEWERS' COMMENTS

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

The manuscript "Inhibiting B-cell-mediated Immunosuppression to Enhance the Immunotherapy Efficacy in Hepatocellular Carcinoma" presents additional evidence that B cells contribute to the resistance to immune checkpoint blockade in hepatocellular carcinoma. Their studies showed that B cells are increased in liver of ICB treated mice, and STING agonism increases circulating IL-10 and intertumoral infiltration of B cells. Further, combination of STING agonism and Tim-1 blockade augments B cell differentiation and improves anti-tumor effects in murine HCC. However, overall B cell population and Tim-1 expression on B cells are very low in the liver tissues of tumor-bearing mice. Also, Tim-1 is expressed in T cells and other immune cells. Therefore, the evidence that Tim-1+ immunosuppressive B cells are a major source of therapy resistance in hepatocellular carcinoma is not very strong. Previous studies have shown that STING agonists result expansion of IL-35+ regulatory B cells in PDAC. B cell infiltration is much higher in pancreas than in liver.
Several other points the authors could address to enrich the manuscript.

Response: We thank the reviewer for the expert evaluation of our manuscript and for succinctly summarizing our findings. Our study highlights the immunosuppressive role of B cells in the context of acquired resistance to immunotherapy.

Indeed, the overall proportion of Tim-1$^+$ B cells in liver tumors is not high, so we do not claim that they are important as a mechanism of immune evasion during HCC progression. However, we consistently found substantial increases in this population following STING agonist treatment across different murine HCC models. Importantly, targeting Tim-1$^+$ B cells enhanced the efficacy of immunotherapy. Despite their low abundance, emerging evidence suggests that specific subsets of immunoregulatory B cells can exert disproportionately strong effects on the tumor immune microenvironment. Previous studies, including our work, have shown that in vivo, Tim-1 has a predominant effect in B cells rather than T cells in mice, and the data from Tim-1-deficient or conditional knockout mice in B cells exhibit markedly reduced IL-10 production by regulatory B cells (Bregs) [1, 2, 3, 4, 5], highlighting the essential role of Tim-1 in the induction and maintenance of IL-10 expression in Bregs. In our study, we corroborated these findings in HCC murine models, showing that Tim-1 marks a functionally important fraction of IL-10$^+$ immunosuppressive B cells in the tumor microenvironment.

Furthermore, our bulk RNA-seq analysis of murine tumors treated with STING agonist versus control (**Figure 5A**) revealed increased expression of both IL-10 and IL-35 (encoded by Il12a and Ebi3), consistent with the expansion of immunoregulatory B cell subsets as pointed out by the reviewer. Taken together, these data support our conclusion that even a small fraction of Tim-1$^+$ B cells can contribute meaningfully to acquired resistance to immunotherapy in HCC and may serve as an indicator of immunoregulatory B cell expansion.

We have incorporated these clarifications and additional data into the revised manuscript as detailed below.

1. Fig. 2C showed enrichment score of different immune subsets in control and STING treated mice. It seems that B cells are major immune cells after STING treatment. However, it's known

that myeloid cells are major population of immune cells in liver. Using Flow cytometry analysis of immune cell subsets within CD45+ cells will make data more convincing.

Response: We thank the reviewer for this insightful comment and apologize for the confusion caused by the data displayed in Fig. 2C. As the reviewer pointed out, the data presented are derived from xCell analysis, which calculates enrichment scores rather than absolute cell proportions. These scores are not directly comparable across different immune cell types; therefore, the higher score for B cells after STING treatment does not indicate that B cells are the dominant immune population in the liver tumor microenvironment. We have revised the figure presentation by grouping cell types separately (**Fig. R1A** and **Supplementary Fig. 4**) and added clarifying statements in both the figure legend and Methods (Page 18, Line 638-639) to explicitly explain this feature of xCell analysis.

We fully agree with the reviewer that myeloid cells are a major infiltrating population in liver cancer, as supported by our own prior work[6]. In this study, however, our transcriptional analyses highlighted differences in B cells and B-cell-related pathways, which motivated our focus on B cells. To more accurately assess the composition of immune cell subsets, we performed flow cytometry analysis of CD45$^+$ immune cells from a time-matched HCA-1 model cohort. Given that STING agonists are known to activate dendritic cells[7, 8], we used CD11c and MHC II as markers to identify this population. Our results showed that dendritic cells were indeed more abundant than B cells across all treatment groups. However, we did not observe any significant changes in the proportion of dendritic cells between STING-treated and control groups (**Fig. R1B**). These data suggest that in this HCA-1 model, STING activation may not substantially alter dendritic cell frequencies.

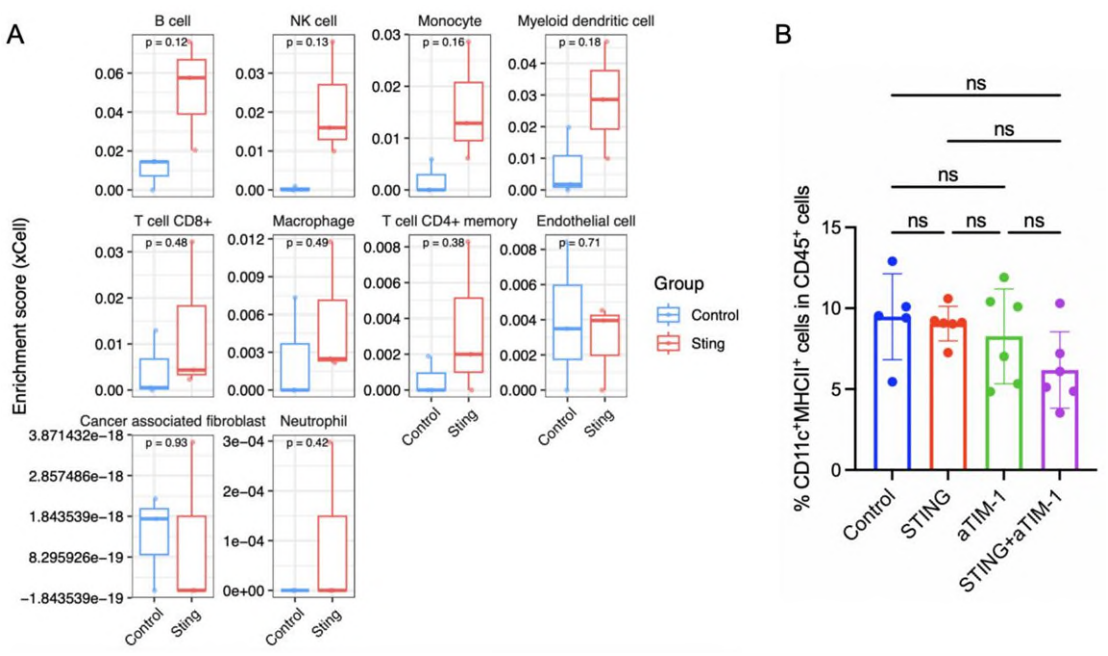

Fig. R1: Immune cell subsets in the HCA-1 murine HCC tissues.
(A) The enrichment score of major cell types in the HCA-1 tumor samples from STING agonist and control groups, as calculated by xCell. P values were calculated by Student's t-test. (B) The proportion of CD11c$^+$MHCII$^+$ cells across different treatment groups in the murine HCA-1 HCC model. Different treatment groups included IgG control, STING agonist, anti-TIM-1, and a combination of STING agonist and anti-TIM-1 antibody.

2. Fig. 6G showed Tim-1 expression in B cells with very low levels after STING treatment. Whether Tim-1+ B cells produce more IL-10 and IL-35 remains unknown.

Response: We appreciate the reviewer's thoughtful comment on this important point. As noted, the proportion of Tim-1$^+$ B cells is indeed low following STING treatment—approximately 1% of total B cells—but this level is significantly elevated compared to the control group. It is technically challenging due to the low cell proportion and the difficulty in detecting cytokine mRNA.

To address whether Tim-1$^+$ B cells produce IL-10 or IL-35, we attempted co-staining for Tim-1 and IL-10/IL-35 in our flow cytometry experiments. However, due to the limited number of Tim-1$^+$ B cells, it was technically challenging to reliably quantify the double-positive populations (Tim-1$^+$IL-10$^+$ or Tim-1$^+$IL-35$^+$). Nevertheless, in our previous study, we found that Tim-1-expressing B cells express higher levels of checkpoint molecules and IL-10, supporting a role for these cells in modulating anti-tumor immunity[9].

Additionally, we analyzed publicly available single-cell RNA-seq and spatial transcriptomic data from HCC patients treated with neoadjuvant anti-PD-1 therapy and from murine B cells[10, 11, 12]. In these datasets, we found that these Tim-1$^+$ B cells can also express IL10 and IL35 (encoded by IL12A and EBI3) (**Fig. R2A, B**). Importantly, not all IL10$^+$ Bregs expressed Tim-1, indicating that Tim-1$^+$ B cells represent only a subset of the broader Breg population. Consistent with this, we detected colocalization of B-cell markers (CD79A, CD79B) with HAVCR1 (the gene encoding Tim-1) (**Fig. R2C**). However, we did not observe colocalization between HAVCR1 and IL10 transcripts in spatial transcriptomic data (**Fig. R2D**), likely reflecting the well-recognized technical challenges of detecting cytokine transcripts at the single-cell level. The transient and low-abundance nature of cytokine mRNAs such as IL-10 often limits their capture in single-cell or spatial transcriptomic datasets[13, 14]. We have incorporated these new data into the Results section of the revised manuscript (Page 11, Line 354-368).

Despite these limitations, prior in vivo studies strongly support the role of Tim-1 in regulating IL-10 production by Bregs. Specifically, Tim-1 is preferentially expressed on B cells rather than T cells in mice, and Tim-1 deficiency markedly impairs IL-10 production in Bregs [1, 2, 3, 4]. Together, these findings suggest that Tim-1$^+$ B cells, although rare, represent an immunoregulatory subset with the potential to contribute to IL-10-mediated suppression in the tumor microenvironment. To further clarify this point, we have now added these points to the revised Discussion section (Page 14, Line 493-505).

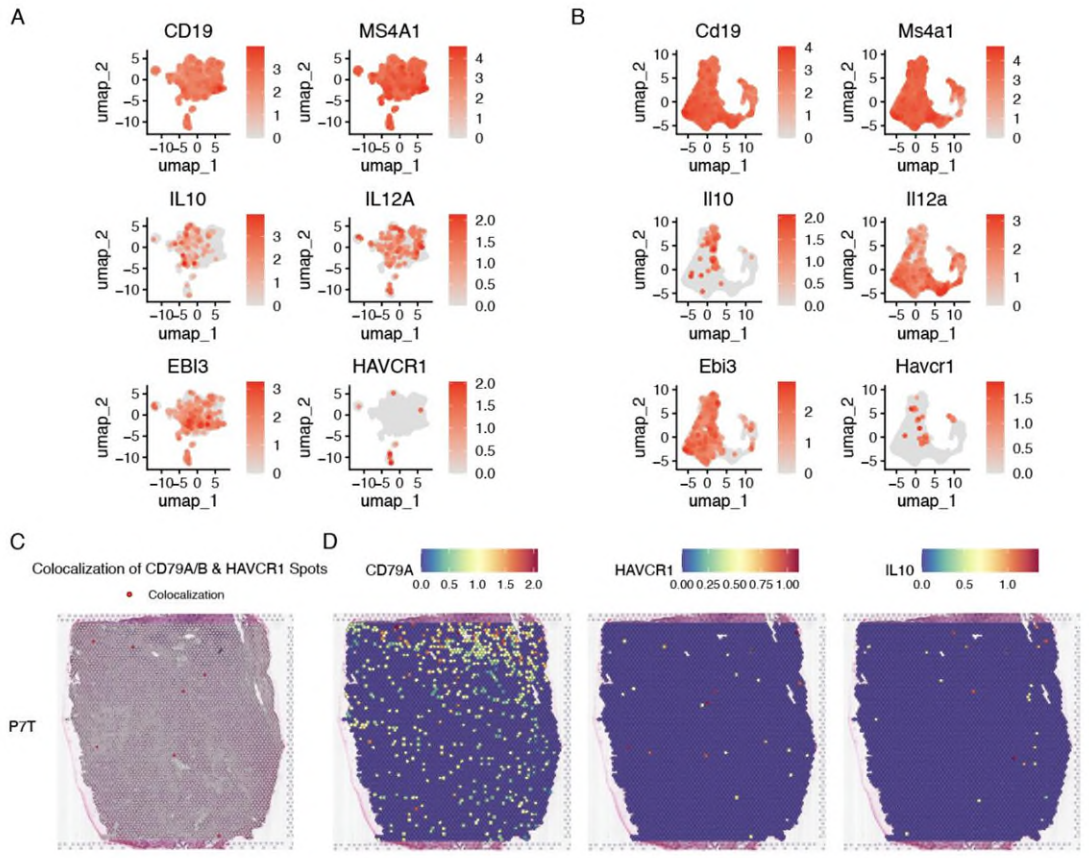

Fig. R2: Transcriptomic analysis of B-cell markers and associated genes.
(A) UMAP plot of published human HCC scRNA-seq data depicting B cells expressing IL10, IL12A, EBI3 and HAVCR1. (B) UMAP plot of published murine scRNA-seq data depicting B cells expressing Il10, Il12a, Ebi3 and Havcr1. (C) The colocalization of CD79A/B and HAVCR1 spots. Spots with colocalization are marked as red. (D) Spatial transcriptomic spots showing the expression level of CD79A, HAVCR1 and IL10. Representative data from the tumor of P7, who has response to the anti-PD-1 treatment.

3. B220 can be used as a B cell marker. However, B220 is also used as a marker to identify mouse plasmacytoid dendritic cells. It's better to use CD19 and B220 to define B cells in Fig. 6E.

Response: We thank the reviewer for this suggestion. We have now included both CD19 and B220 to more precisely define B cells in Fig. 6E. The updated analysis yielded results consistent with our previous findings. Figures 6E-J have been revised accordingly in the current version of the manuscript.

4. B cells have multiple subsets depending on their stage of development and function. The effect of B cells on tumor immunity depends on the balance of various B cell subtypes. Many studies have shown that B cells can promote tumor progression in mouse tumor models whereas human studies have shown that B cell infiltration is associated with better prognosis and response to immunotherapy. The translational relevance of findings from B cell depletion are unclear.

Response: We thank the reviewer for emphasizing the complexity of B cell biology and the important distinctions between murine and human tumor contexts. We fully agree that B cells represent a heterogeneous population whose functions vary with developmental stage, activation status, and tumor microenvironment, and our results also indicate the existence of heterogeneous B cell subpopulations in the tumor microenvironment after treatment (**Supplementary Fig. 1a, b**). In our study, we initially examined the overall B cell population, which expands following treatment with STING agonists or immune checkpoint blockade in mice. Depletion of either total B cells or the Tim-1^{+} B cell subset significantly improved therapeutic efficacy, indicating the immunosuppressive role for these cells in shaping anti-tumor immunity.

While these findings are derived from murine models, they are consistent with emerging evidence that regulatory B cells can contribute to immune evasion across multiple tumor types[15, 16, 17, 18, 19]. At the same time, we acknowledge that the translational implications of B-cell depletion strategies remain uncertain in human patients, and our results should not be interpreted as a recommendation for B-cell depletion in the clinical setting. To begin bridging this gap, we analyzed publicly available datasets from HCC patients treated with neoadjuvant anti-PD-1 therapy[11]. These data revealed that both T cells and B cells were significantly enriched in responders (**Fig. R3**), consistent with a role for these populations in modulating the balance between immune activation and suppression. However, given the limited sample size and the relatively low frequency of B cells in these human samples, the precise contribution of distinct B-cell subsets to therapeutic outcome remains unresolved.

Accordingly, we have expanded the results and discussion in the revised manuscript (Page 12, Line 414-423 and Page 15, Line 513-518) to highlight both the potential relevance and the limitations of extrapolating murine B cell data to human immunotherapy. We also highlight that future studies involving human samples, particularly with single-cell resolution, will be critical to delineate the roles of distinct B cell subsets in mediating clinical responses.

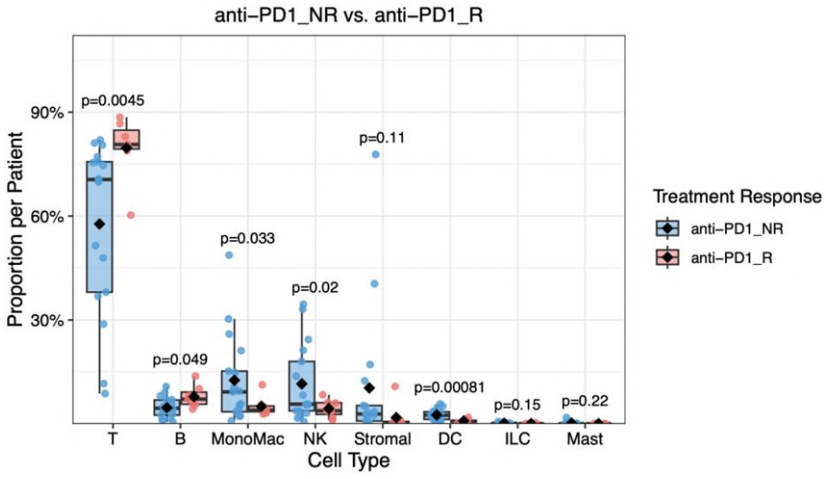

Fig. R3: The proportion of different immune cells in hepatocellular carcinoma patients treated by neoadjuvant anti-PD1 therapy based on single-cell RNA sequencing data. Anti-PD1_NR: non-response to anti-PD-1 therapy; anti-PD1_R: response to anti-PD-1 therapy.

Reviewer #2 (Remarks to the Author): with expertise in cancer immunology and HCC

The manuscript investigates the role of B cells, particularly TIM1+ B cells, in the context of PD-1 blockade and STING agonist therapy in murine hepatocellular carcinoma (HCC) models with underlying liver fibrosis. The authors reported that B cell depletion enhances the efficacy of immune checkpoint blockade (ICB) and STING agonism, while TIM1 blockade synergizes with STING agonists to improve antitumor responses. Additionally, they observed that STING agonism exhibits pronounced anti-metastatic activity, unlike ICB. While the study addresses an underexplored aspect of B cell biology in HCC immunotherapy, the depth could be further enhanced for publication.

Response: We are grateful for the careful evaluation of our work and the suggestions made by the expert reviewer. In response to the concerns raised, we have made appropriate revisions to the manuscript, as detailed below.

Suggestions:

The current study reports that B-cell depletion enhances the efficacy of combined ICB and STING agonist therapy. While the authors observe increased B-cell aggregates and TLS-like structures in STING agonist-treated mice, any further analysis to define B-cell subsets (e.g., regulatory vs. effector B cells) or TLS maturity?

Response: We thank the reviewer for this excellent suggestion. To further characterize the TLS-like structures observed in STING agonist–treated mice, we performed additional staining for CD21, peripheral node addressin (PNAd), and IL-10 on murine RIL-175 HCC tissue sections. Our analysis identified $B220^{-}CD21^{+}$ cells, suggesting the presence of follicular dendritic cells, but only minimal expression of PNAd and IL-10 in these areas (**Fig. R4**). These results suggest that the TLS-like aggregates are likely in an immature or intermediate state, and their transition toward maturity—and consequent impact on the balance between pro-tumor and anti-tumor immunity—remains to be fully defined. We have incorporated these new findings into the Results section of the revised manuscript (Page 7, Line 222-228).

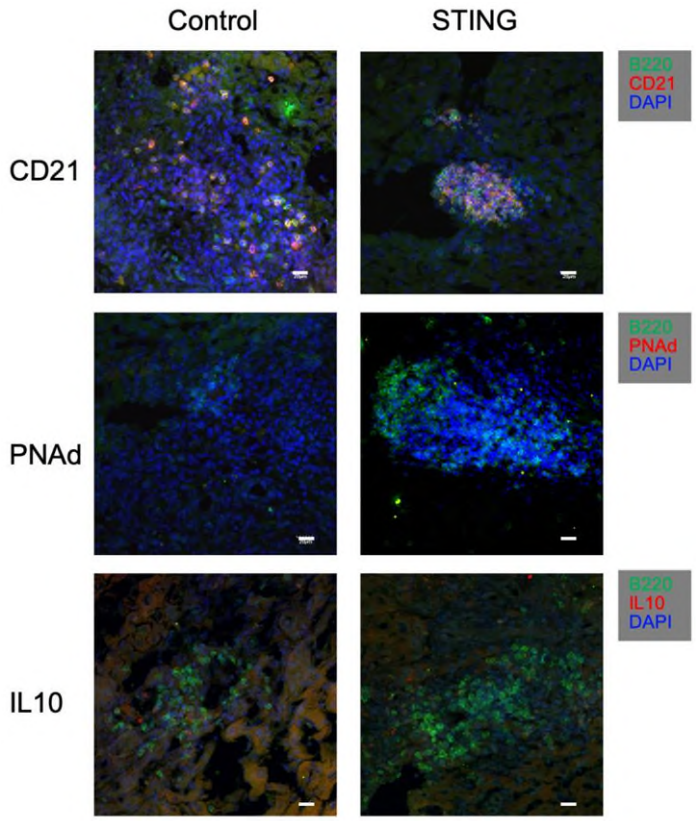

Fig. R4: Representative IF results of B220, CD21, PNAd and IL10 for tumor tissues collected from control and STING-agonism-treated mice (scale bar, 20μm).

1. The study lacks specificity of TIM1 blockade effects. TIM1 is expressed on multiple immune and non-immune cells (e.g., T cells, B cells). The authors use anti-TIM1 antibody therapy but fail to demonstrate TIM1 expression patterns in their model (e.g., flow cytometry or IHC to show TIM1+ cell distribution). They did not exclude off-target effects on non-B cells (e.g., does TIM1 blockade also affect TIM1+ T cells?).

Response: We thank the reviewer for this insightful comment. To address this concern, we have now included co-staining of TIM-1 and B220 in our murine HCC model to examine the distribution of TIM-1$^+$ B cells (**Fig. R5**). Consistent with our flow cytometry results, only a limited number of TIM-1$^+$B220$^+$ cells were detected in the tissues overall. Notably, TIM-1$^+$B220$^+$ cells were more frequent in the peritumor region of STING agonist-treated mice compared to controls (p=0.081), whereas no differences were observed within TLS-like structures or intratumor areas. These findings suggest that after STING agonist treatment, TIM-1$^+$ B cell infiltration may preferentially increase in the peri-tumoral areas and not within TLS-like structures or the tumor core.

In our previous study, we showed that the anti-tumor efficacy of anti-TIM-1 was abolished in *Cd19*$^{Cre/+}$ X *Havcr1*$^{fl/fl}$ mice, in which TIM-1 is selectively deleted in B cells, indicating that TIM-1 expression on B cells is required for therapeutic benefit. By contrast, in *Cd4*$^{Cre/+}$ X *Havcr1*$^{fl/fl}$ mice, in which TIM-1 is specifically deleted in all T cells, TIM-1 deficiency had no impact on tumor progression in the same model[9]. These findings, together with independent studies reporting that TIM-1 is predominantly expressed on B cells rather than T cells in mice[1, 3], suggest that off-target effects on non-B cells are likely minimal.

Furthermore, in our experiments, we did not observe any adverse effects following anti-TIM-1 treatment, supporting the specificity and tolerability of this approach. Taken together, we proposed that TIM-1 expression on B cells is a critical mediator of the observed therapeutic effects. To further clarify these points, we have incorporated the relevant results and discussion into the revised manuscript (Page 12, Line 396-402 and Page 14, Line 493-505).

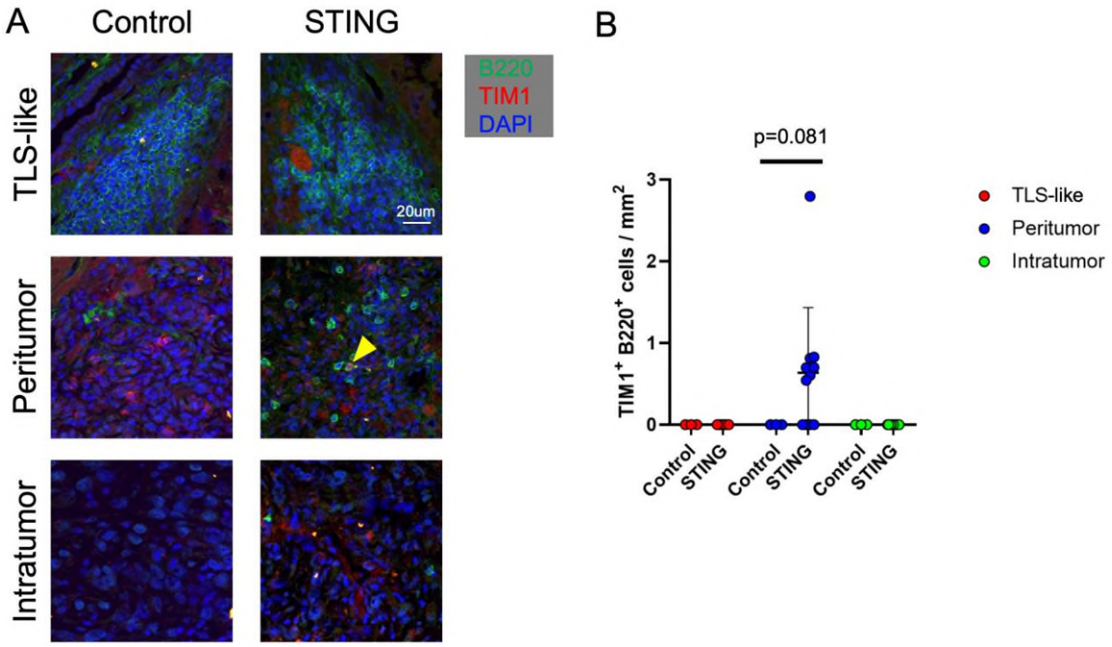

Fig. R5: Representative IF results of co-staining of B220 and TIM-1 for tumor tissues collected from control and STING-agonism-treated mice.
(A) Representative IF images of B220 and TIM-1 for RIL-175 murine HCC tumor tissues. (B) Statistical comparison of TIM-1$^+$B220$^+$ cell density (cell/mm^2) between STING and control groups (STING: n=11/group; Control: n=3/group).

2. Given that STING agonists can directly activate dendritic cells and reshape the TIME, the observed effects may not be B cell dependent. The authors should validate their findings in B cell-deficient models.

Response: We thank the reviewer for this valuable suggestion. In our current study, we performed B-cell depletion using a combination of anti-CD19 and anti-B220 antibodies. The efficacy of depletion was confirmed by flow cytometry, showing that after five doses, B cells were nearly undetectable in peripheral blood mononuclear cells, indicating effective and robust depletion (**Figure S8e**). Furthermore, our flow cytometry panel included CD45, CD11c, and MHC II markers, and we observed no significant differences in dendritic cells between STING-treated and control groups (**Fig. R1**).

We agree that genetic B cell-deficient models would provide additional, orthogonal evidence. However, classic models such as the μMT mice, while lacking mature B cells, are known to have compensatory changes such as alterations in T cell subsets, myeloid cell populations, and elevated baseline type I interferon or other cytokine signatures, which could independently influence STING pathway activation or tumor immune microenvironment. To avoid these confounding developmental effects, we chose an antibody-mediated depletion strategy, which preserves normal B cell development until depletion, and thus better isolates the role of B cells

in the adult TME under STING activation. Due to constraints of cost, availability, and our desire to avoid disrupting tumor development dynamics in the existing model, we were unable to include a genetic B-cell-knockout arm in this current study.

3. Are the expanded B-cell populations identical between PD-1 blockade and STING agonism? What is the proportion of TIM1+ B cells?

Response: We appreciate the reviewer's insightful question. To investigate whether the expanded B-cell populations differ between PD-1 blockade and STING agonism, we performed deconvolution analysis using bulk RNA-seq data to estimate B-cell subtype proportions. The results showed no significant differences in the predicted enrichment scores of B-cell subtypes between the two treatment groups in both the HCA-1 and RIL-175 murine HCC models (**Fig. R6A**). In addition, the expression of representative marker genes for these B-cell subsets were also comparable between the two groups (**Fig. R6B**).

Flow cytometry analysis further revealed that TIM-1^{+} B cells constituted approximately 1–3% of the total B-cell population following either PD-1 blockade or STING agonist treatment. Although no significant differences were observed in our current dataset, we think that higher-resolution approaches such as single-cell RNA sequencing may uncover more subtle differences, particularly given the distinct mechanisms of action of the two treatments. To clarify this point, we have added the data to the Results section of the manuscript (Page 5-6, Line 160-167).

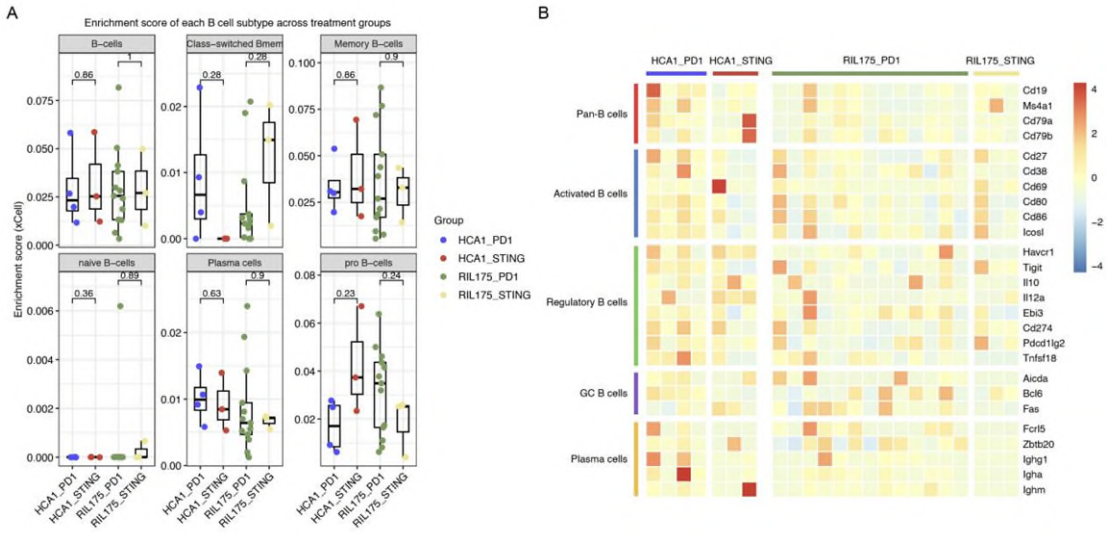

Fig. R6: Analysis of B cell subtypes in murine HCC models treated by PD-1 blockade and STING agonism.

(A) The enrichment score of B cell and its subtypes in the HCA-1 and RIL-175 tumor samples from STING agonist and anti-PD-1 groups was calculated by xCell, p values were calculated by Wilcoxon test. (B) Expression levels of representative markers of B cell subtypes.

4. Does adding VEGFR2 inhibition to PD-1 blockade alter B-cell abundance or polarization?

Response: To address whether VEGFR2 inhibition alters B-cell abundance or polarization when combined with PD-1 blockade, we performed additional flow cytometry experiments on tumor tissues collected from RIL-175 murine HCC models. Mice were assigned to four different treatment groups: (1) IgG control, (2) anti-VEGFR2, (3) anti-PD-1, and (4) combination of anti-

VEGFR2 and anti-PD-1. All treatments were administered once every three days, and tumors were harvested on day 10 after treatment initiation.

Our results showed that the addition of anti-VEGFR2 did not affect overall B cell abundance (**Fig. R7A**). TIM-1$^+$ B cells were increased only in the anti-PD-1-containing groups (**Fig. R7B**). Notably, B cells from the dual anti-PD-1 and anti-VEGFR2 group exhibited elevated IL-10 and PD-L1 expression (**Fig. R7C, D**). These findings suggest that while VEGFR2 blockade does not change B-cell numbers, its combination with PD-1 blockade may enhance B-cell polarization toward a more immunosuppressive phenotype. We have now added the data to the Results section of the manuscript (Page 9, Line 298-310).

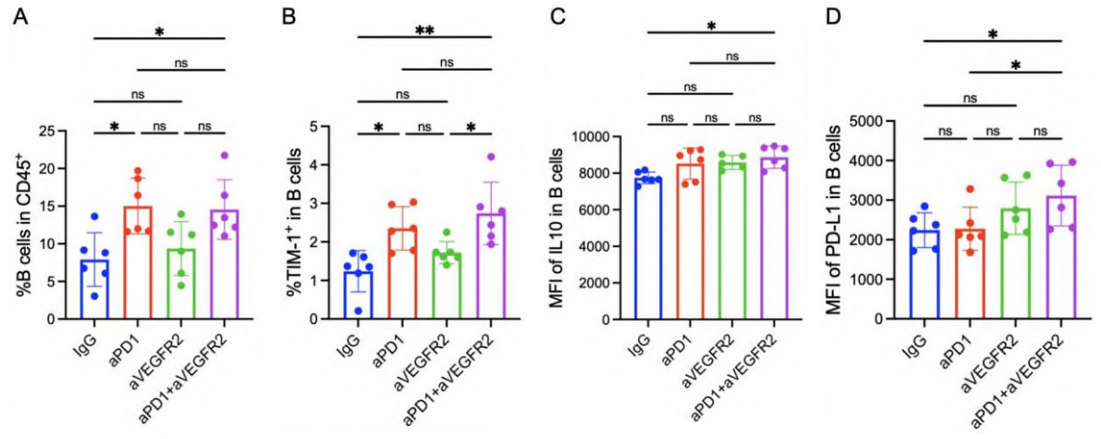

Fig. R7: Flow cytometry analysis of time-matched RIL-175 tumor tissues after treatment. (A) Proportion of CD19$^+$B220$^+$ B cells in CD45$^+$ cells. (B) Proportion of TIM1$^+$ B cells in total B cells. (C) MFI of IL10 in CD19$^+$B220$^+$ B cells. (D) MFI of PD-L1 in CD19$^+$B220$^+$ B cells. P values were calculated by one-way ANOVA with Tukey's test.

5. Do STING agonists/TIM1 blockade show similar efficacy in spontaneous models (e.g., Myc; Trp53 KO or DEN-induced HCC)?

Response: We thank the reviewer for raising this important question. To explore this, we evaluated the same STING agonist in the MST spontaneous HCC model, in which CCl4 is administered for 12 weeks after tail-vein injection of Cre-expressing adenovirus (adeno-Cre) in *Stk4*$^{-/-}$*Stk3*$^{F/-}$ (also known as *Mst1*$^{-/-}$*Mst2*$^{F/-}$) mice, leading to spontaneous HCC development concomitant with liver cirrhosis[20]. We observed that a single dose of STING agonist produced a non-significant tumor growth delay, suggesting treatment resistance in this model (**Fig. R8A, B**). Immunofluorescence staining of tumor tissues from STING agonist-treated mice showed a trend toward increased B-cell infiltration compared with controls (**Fig. R8C, D**), warranting future studies of TIM-1 and B-cell targeting in this model. We have now added the data to the Results section of the manuscript (Page 7, Line 205-211).

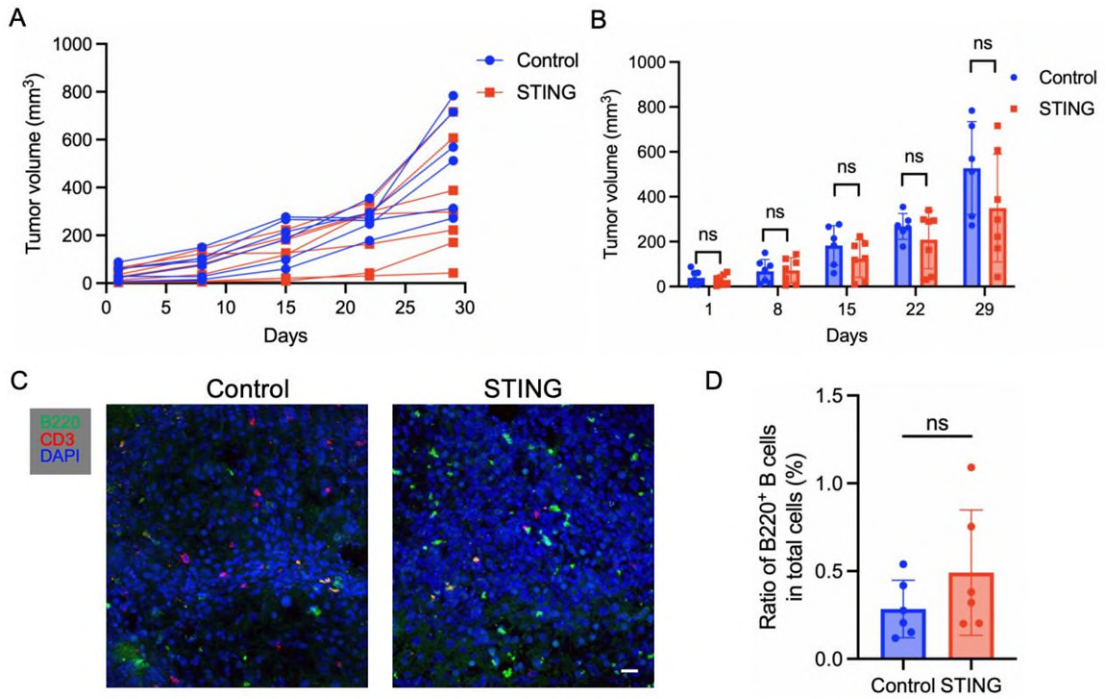

Fig. R8: The efficacy of STING agonist in MST spontaneous HCC model.
(A, B) Tumor volume of STING and control groups in MST model. (C) Representative IF results of B and T cells for MST tumor tissues collected from control and STING-agonism-treated mice. (D) Statistical comparison of B220⁺ cell ratio between STING and control groups (n=6/group).

6. Human HCC data (e.g., TIM1+ B cell correlates from public datasets) would strengthen the translational impact.

Response: We appreciate the reviewer's helpful suggestion. We analyzed the relationship between *HAVCR1* expression and the Breg score in tumor-infiltrating B cells from a public human HCC single-cell RNA-seq dataset[11]. While the correlation was statistically significant, the effect size was modest (R=0.02; **Fig. R9**). In addition, we examined cell-cell interaction and spatial transcriptomic data from human HCC, as detailed in Fig. R10 below and described in our response to the following comment. These analyses provide supportive evidence from human datasets that strengthen the translational relevance of our findings.

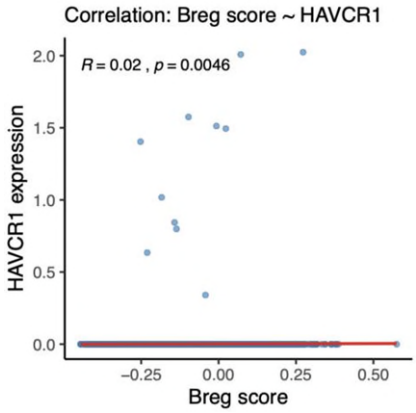

Fig. R9: Relationship between HAVCR1 expression and Breg score in tumor-infiltrating B cells.

7. The work could be improved by adding data related to single-cell RNA-seq or high-dimensional flow cytometry to dissect B cell subsets and genetic models (e.g., conditional TIM1 KO) to validate B cell-specific roles, or the spatial profiling of TLS and immune crosstalk in the TIME.

Response: We appreciate the reviewer's valuable suggestion. To address these points, we analyzed publicly available datasets. First, we examined immune cell composition in hepatocellular carcinoma patients who either responded or did not respond to neoadjuvant anti-PD-1 therapy[11]. In this dataset, responders showed a significant increase in both T cells and B cells (**Fig. R3**), suggesting that these populations contribute to balancing immune activation and suppression. Further analysis of cell-cell interactions revealed that anti-PD-1 non-responders had greater overall intercellular communication, with enrichment of immunosuppressive interactions such as CLEC2C-KLRB1 and HLA-E-CD94:NKG2A, particularly involving B cells with NK cells and other immune subsets (**Fig. R10A, B**).

We also investigated a spatial transcriptomic dataset from HCC patients treated with neoadjuvant anti-PD-1 therapy[10]. In these data, we observed colocalization of B-cell markers (CD79A, CD79B) with HAVCR1 (encoding TIM-1), particularly in responders to anti-PD-1 therapy (**Fig. R10C-G**). This colocalization was significantly higher in responders compared with non-responders, supporting our finding that immune-activating therapies such as STING agonists or PD-1 blockade can induce immunosuppressive TIM-1$^+$ B cells, which mediate acquired resistance to immunotherapy.

Together, these results support our conclusion that TIM-1 marks an immunosuppressive subset within the heterogeneous B-cell compartment. Targeting TIM-1$^+$ B cells, in combination with other approaches, may therefore represent a promising strategy to enhance the efficacy of immunotherapy. We have expanded the Results section of the revised manuscript (Page 12, Line 414-423) to highlight these findings and acknowledge the importance of future studies employing high-dimensional technologies and genetic models to further define the roles of B-cell subsets in the tumor immune microenvironment.

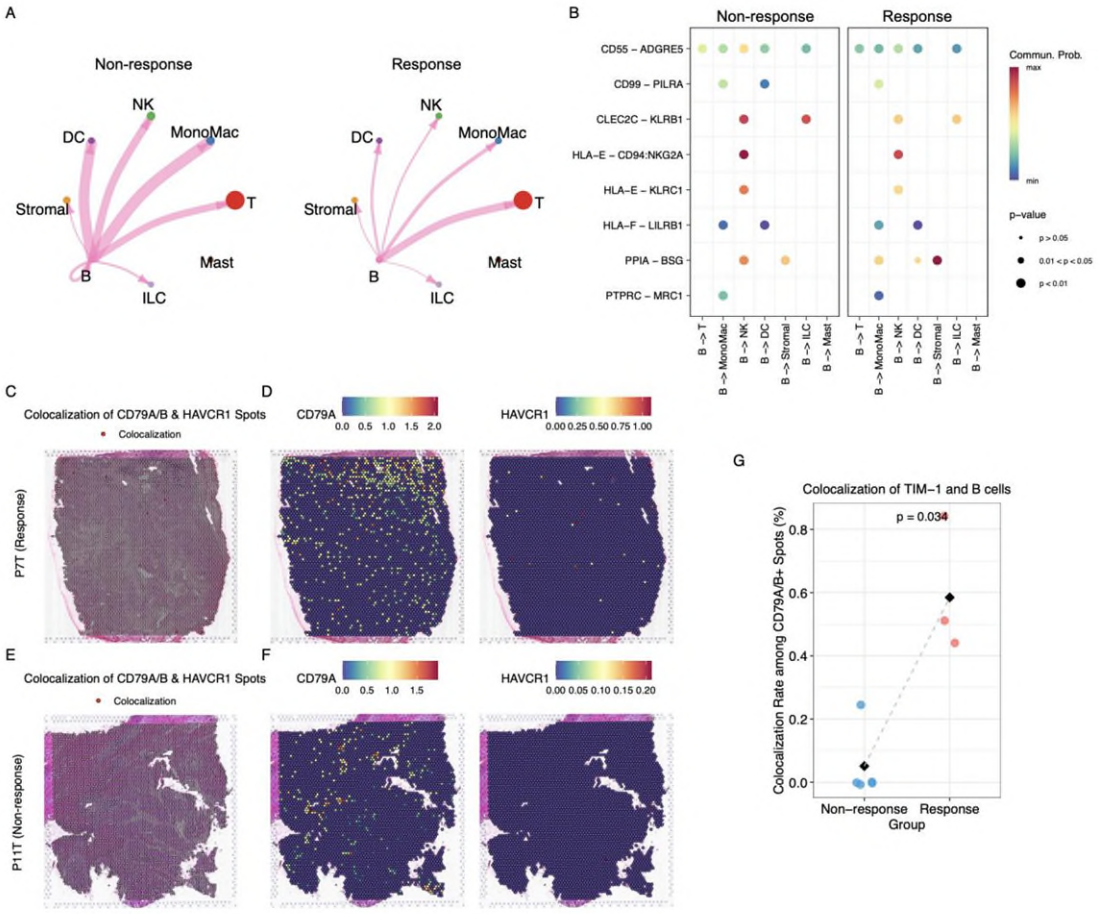

Fig. R10: Transcriptomic differences between anti-PD-1 responders and non-responders. (A) Anti-PD-1 non-responders had greater intercellular interactions between B cells and other cell types than responders. (B) Immunosuppressive ligand-receptor pairs were increased in anti-PD-1 non-responders than responders. (C, E) The colocalization of CD79A/B and HAVCR1 spots. Spots with colocalization are marked as red. (D, F) Spatial transcriptomic spots showing the expression level of CD79A and HAVCR1. (G) Colocalization rate of TIM-1 and CD79A/B was higher in anti-PD-1 responders than non-responders.

Reviewer #3 (Remarks to the Author): with expertise in cancer immunology and B cells

In this manuscript, Liu et al. explore the role of B cells in modulating the efficacy of immunotherapy in hepatocellular carcinoma (HCC). Using mouse models, they show that treatment with the STING agonist BMS-986301 promotes the expansion of a tumor-supportive B cell subset and TLS-like structures in an immune checkpoint inhibitor (ICI)-sensitive model. B cell depletion enhanced the response of both ICI-resistant and ICI-sensitive tumors to STING agonist treatment and/or checkpoint blockade. Additionally, combining the STING agonist with TIM-1 blockade resulted in synergistic tumor suppression. While the manuscript is generally well written and the data are presented clearly, there are significant limitations in data interpretation and mechanistic insight that should be addressed.

Response: We thank the reviewer for the expert evaluation of our manuscript. We appreciated the insightful suggestions and modified the manuscript accordingly.

Major points.

- The experiments in Figure 4 lack a single-arm B cell depletion control, which is necessary to demonstrate whether the efficacy of the STING and checkpoint inhibitor combination is dependent on B cells.

Response: We thank the reviewer for this valuable suggestion. To address this, we performed an additional cohort using the RIL-175 model in which mice were treated with either control IgG or anti-CD19/anti-B220 therapy. The results showed that B-cell depletion by dual anti-CD19/anti-B220 did not affect tumor growth or overall survival (**Fig. R11**). These findings indicate that while B-cell depletion alone has no therapeutic effect, its combination with STING agonist and dual anti-PD-1/VEGFR2 therapy further improves survival. We have incorporated these data into the revised Results section (Page 9-10, Line 311-325).

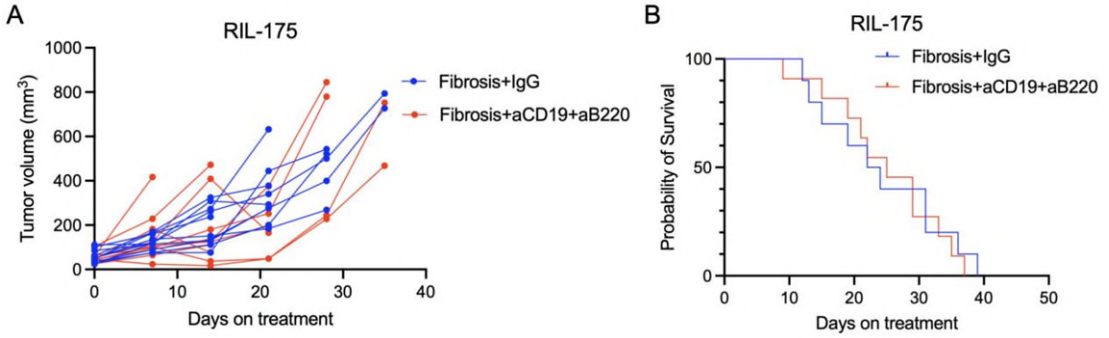

Fig. R11: B cell depletion alone has no effect on the tumor growth and overall survival in the RIL-175 murine HCC model.
(A) Tumor growth curve after treatment of IgG control or dual anti-CD19/anti-B220 therapy. (B) Overall survival of RIL-175 murine HCC-bearing mice after the treatment of IgG control or dual anti-CD19/anti-B220 therapy (n=10-11/group).

- In Figure 6, the experiments should be repeated using a B cell-deficient background or under conditions of conditional STING and/or TIM-1 deletion specifically in B cells to establish a mechanistic link. This is particularly important given that TIM-1$^+$ cells represent only ~1% of the

B cell population. What accounts for their disproportionate effect? Are these cells antigen-specific?

Response: We appreciate the reviewer's insightful comment. Although TIM-1$^+$ B cells represent a relatively small fraction (~1%) of the total B-cell population in HCC tumors, we consistently observed their expansion following STING agonist treatment across multiple murine HCC models. Importantly, selective depletion of TIM-1$^+$ B cells further enhanced the efficacy of immunotherapy, indicating that even rare subsets can exert a significant functional impact. This observation is consistent with prior studies showing that immunoregulatory B-cell subsets can disproportionately shape the tumor immune microenvironment despite their low abundance[15, 16, 17, 18, 19] (See also response to Reviewer #1).

TIM-1$^+$ B cells likely represent one immunosuppressive subset within the heterogeneous B-cell population. To further explore their functional characteristics, we analyzed scRNA-seq data from human HCC[11]. We found that antigen-presentation markers were expressed at higher levels in TIM-1-negative B cells compared with TIM-1-positive B cells (**Fig. R12**), suggesting that TIM-1$^+$ B cells are less specialized for antigen presentation but may instead contribute to immune suppression. These results reinforce the concept that TIM-1$^+$ B cells, while infrequent, can mediate therapeutic benefits. We have incorporated these new data into the Results section of the revised manuscript (Page 13, Line 433-437).

We agree with the reviewer that validation in B-cell-deficient or conditional knockout models would further strengthen the mechanistic link. While such experiments were not feasible in the current study due to the small proportion of these cells, and because assays to measure tumor-specific B cells are challenging. We recognize their importance and plan to incorporate these genetic models in future work.

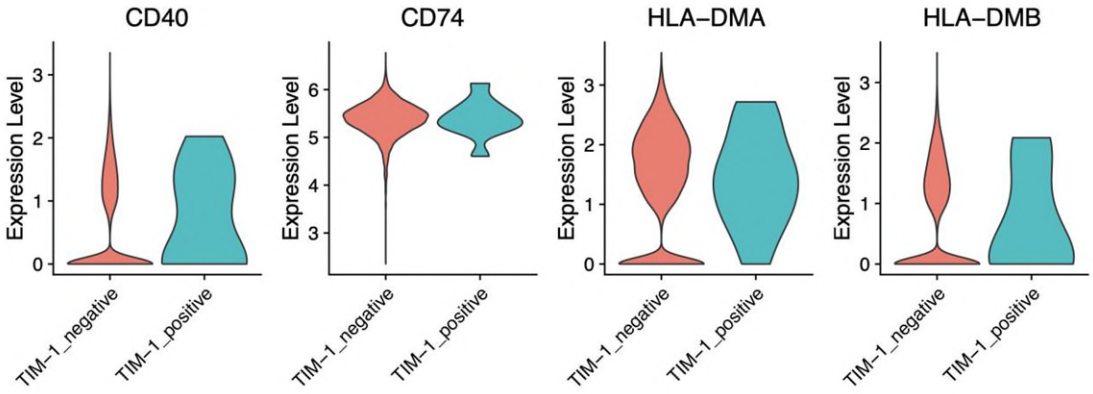

Fig. R12: Comparison of antigen presentation marker expressions between TIM-1-positive and TIM-1-negative B cells in human HCC tumors.

- Related to the above point, it remains unclear which immune cell populations are responding to STING agonist and/or anti-PD-1 treatment. Targeted depletion of T and/or B cells could help clarify the cellular contributors to the observed therapeutic effects.

Response: We appreciate the reviewer's insightful comment. We agree that dissecting the specific immune cell populations responding to STING agonist and anti-PD-1 therapy is important, though this is inherently complex given that both T and B cells increase following treatment, and multiple pathways are engaged. The role of T cells in mediating the efficacy of

STING and PD-1 blockade has been extensively documented in the literature, whereas the contribution of B cells has been less recognized.

To specifically examine the role of B cells, we performed co-staining of TIM-1 and B220 in our murine HCC model, which confirmed the distribution of TIM-1$^+$ B cells in STING agonist-treated versus control tumors (**Fig. R5**). In addition, our previous work demonstrated that the anti-tumor efficacy of anti-TIM-1 antibody treatment was abolished in *Cd19*$^{Cre/+}$ × *Havcr1*$^{fl/fl}$ mice, indicating that TIM-1 expression on B cells is required for therapeutic benefit. By contrast, TIM-1 deletion in T cells (*Cd4*$^{Cre/+}$ × *Havcr1*$^{fl/fl}$) had no impact on tumor progression in the same model[9]. Together with independent studies reporting that TIM-1 is predominantly expressed on B cells rather than T cells in mice[1, 3], these findings suggest that the observed effects are largely mediated by B cells and that off-target effects on T cells are minimal.

We have clarified these points in the revised Discussion section (Page 14, Line 493-505) to better delineate the immune populations contributing to the therapeutic effects.

- In Figure 3D, the potential impact of fibrosis on B cells has not been addressed; an additional control arm is needed. Furthermore, the metastasis data in Figures 3G and 3H are confounded by differences in primary tumor growth, which may influence the results.

Response: We appreciate the reviewer's insightful comment. To directly address the potential impact of fibrosis on B cells, we performed an additional cohort study in the HCA-1 murine HCC model with four groups: (1) non-fibrosis + IgG, (2) non-fibrosis + anti-CD19/anti-B220, (3) fibrosis + IgG, and (4) fibrosis + anti-CD19/anti-B220. The results showed that mice with a fibrotic background had worse survival outcomes, as those treated with IgG in the fibrosis group exhibited significantly shorter survival compared with the non-fibrosis group. However, within both the fibrosis and non-fibrosis settings, there were no survival differences between B-cell-depleted mice (anti-CD19/anti-B220) and IgG controls (**Fig. R13**), indicating that B-cell depletion alone does not affect survival. We have now added the data to the Results section of the manuscript (Page 9-10, Line 311-325).

Regarding the metastasis data in Figures 3G and 3H, we agree that primary tumor burden could influence metastatic outcomes. In our study, however, primary tumor size was controlled by the experimental endpoint defined in the animal protocol (tumor length ≥13 mm as measured by ultrasound). Thus, the primary tumors across treatment groups were comparable in size at endpoint, minimizing the potential for confounding. To clarify this point, we have now explicitly described the tumor size endpoint in the Methods section (Page 16, Line 570-572).

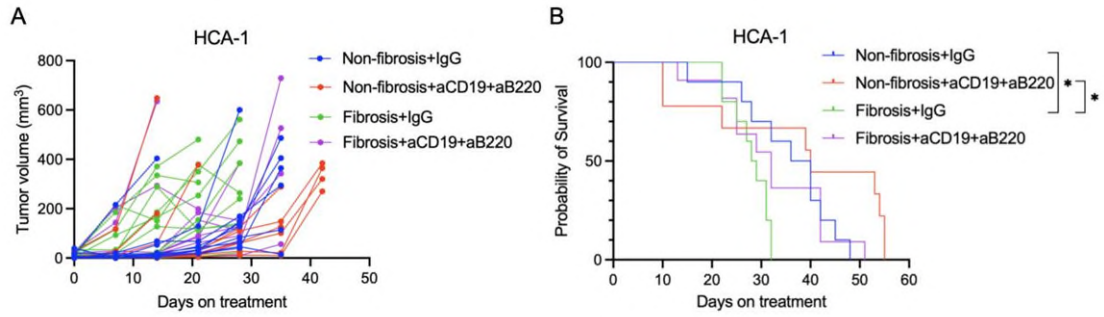

Fig. R13: B cell depletion has no effect on the tumor growth and overall survival in the HCA-1 murine HCC model.

(A) Tumor growth curve of mice with or without fibrosis after treatment of IgG control or dual anti-CD19/anti-B220 therapy. (B) Overall survival of HCA-1 murine HCC-bearing mice with or without fibrosis after the treatment of IgG control or dual anti-CD19/anti-B220 therapy (n=9-11/group).

- To strengthen the generalizability of the findings, the study should ideally include more than one STING agonist.

Response: We thank the reviewer for this insightful comment and fully agree that evaluating multiple STING agonists would strengthen the generalizability of our findings. However, due to restrictions under the Material Transfer Agreement between MGH and Bristol Myers Squibb, we were unable to compare BMS-986301 with other STING agonists in this study. Importantly, our results are not limited to STING agonism alone: we also observed consistent effects when combining B-cell modulation with anti-PD-1 therapy, a broadly used immune checkpoint inhibitor. These findings suggest that the mechanisms we describe are relevant not only to STING agonists but more generally to immune-activating therapies and highlight a potential strategy to enhance the efficacy of immunotherapy in HCC.

Minor points

- Numbers of mice and error bars need to be added for all the graphs. Some in vivo experiments have quite low n (3-4) and the sample size needs to be increased.

Response: We thank the reviewer for this helpful suggestion. We have now added the numbers of mice as well as error bars to all graphs. For the bulk RNA sequencing experiments, the sample size was limited to three mice per group due to the experimental design; however, for other in vivo experiments, larger sample sizes were used to ensure robustness of the results.

- B220 is not specific for B cells, CD19 or CD20 should be used (Figure 2E, F)

Response: We thank the reviewer for the helpful suggestion. We have now updated the staining to use both B220 and CD19 as B-cell markers (**Fig. R14**), and the results are consistent with our previous findings.

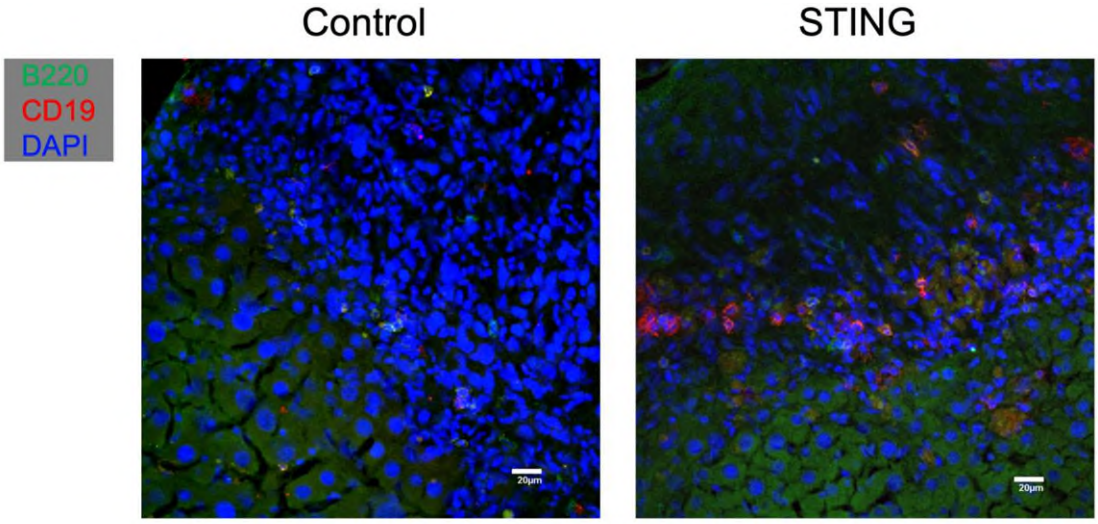

Fig. R14: Representative immunofluorescence (IF) for the B-cell markers B220 and CD19 among the control and STING-treated groups (scale bar, 20μm).

- Please specify which media and/or supplements were used for B cell culture.

Response: We thank the reviewer for this helpful suggestion. B cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin–streptomycin, 2 mM L-glutamine, 1.5 mM sodium pyruvate, 1× non-essential amino acids, 10 mM HEPES, and 0.05 mM 2-mercaptoethanol. We have now included this information in the Methods section (Page 15, Line 539-542).

- B cell depletion should also be confirmed within the tumor tissue, as intratumoral B cells are typically more resistant to depletion than those in peripheral compartments.

Response: We thank the reviewer's suggestion. We have added immunofluorescence staining of tumor tissues (now shown in **Fig. S8f** and **Fig. R15**), which confirms effective B-cell depletion within the tumor microenvironment.

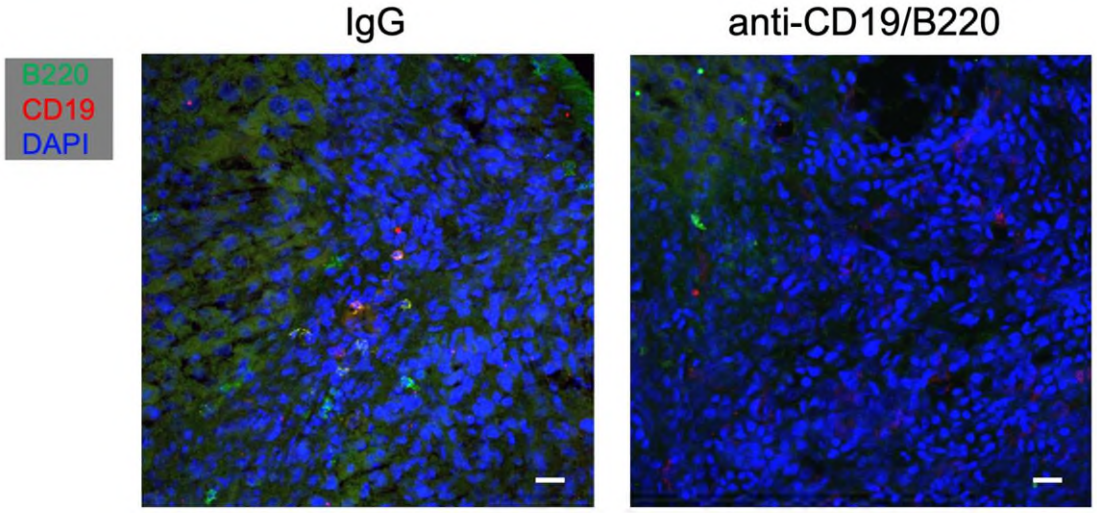

Fig. R15: Representative immunofluorescence (IF) for the B-cell markers B220 and CD19 among the IgG control and dual anti-CD19/B220 groups in HCA-1 murine HCC model (scale bar, 20μm).

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POINT-BY-POINT RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Authors have addressed my concerns.

Response: We thank the reviewer for their evaluation and suggestions for revising our manuscript.

Reviewer #2 (Remarks to the Author):

The authors have addressed all my concerns. Thank you very much for the detailed revision.

Response: We thank the reviewer for their evaluation and suggestions for revising our manuscript.

Reviewer #3 (Remarks to the Author):

Unfortunately, most of the pertinent points were not addressed experimentally, but rather referred to prior studies (which cannot address B cell/STING aspect) or provided transcriptomic based data, which is not sufficient to address the questions at hand.

Response: We thank the reviewer for raising the concern. We would like to respectfully emphasize that the mechanism under investigation—TIM-1–dependent B cell–mediated suppression of T cell responses—reflects a fundamental immunoregulatory interaction, not a tumor-type–specific phenomenon. This conclusion is supported by our current study and prior work from our group and others (including our publication in Nature, PMID: 37344597), which established that TIM-1$^+$ B cells exert immunosuppressive effects on T cells across tumor contexts.

Repeating these experiments using B–cell–specific TIM-1 knockout mice in our liver fibrosis/HCC model would require lengthy and costly experiments. The rederivation of a conditional knockout line is no longer feasible, as Dr. Lloyd Bod—who generated the mice—has left Dr. Kuchroo's Laboratory, and the MGH animal colony we used before was closed. Moreover, Dr. Duda recently transferred to a new institution, and the HCC models we developed are unique. Finally, since the TIM-1–B cell–T cell axis is tumor type–independent, we think that reproducing the full model in HCC would not yield significant additional mechanistic insights beyond what is already demonstrated.

Our revised manuscript already integrates extensive new data, including expanded flow cytometry, transcriptomic analyses, and validation in human HCC datasets. Collectively, these results provide robust, convergent evidence that TIM-1$^+$ B cells suppress T cell–mediated immunity and that TIM-1 blockade enhances STING agonist efficacy.

We have revised the Discussion to explicitly emphasize the reviewer's thoughtful points on the mechanistic nuances and the limits of current validation, ensuring his/her perspective is clearly reflected.