

Leveraging glucan-induced trained immunity for the epigenetic and metabolic rewiring of macrophages to enhance colorectal cancer vaccine response

Corresponding Author: Professor Vincenzo Cerullo

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 entitled, "Leveraging trained immunity for the epigenetic and metabolic rewiring of macrophages to enhance colorectal cancer vaccination by inducing an NK-cDC1 axis" reported that combined trained immunity and tumor vaccine led to significant tumor killing using both mouse colon cancer models and human colon cancer organoids. The authors showed that the combined trained immunity with vaccine therapeutic efficacy appears to be dependent on a robust macrophage-NK cell-cDC axis. While these results have clear clinical translational potential, there are some concerns in this study.

1. The role of cDC1 is unclear in this study. While the authors showed that cDC1 were accumulated in the tumor-bearing mice treated with trained immunity plus PC vaccine and its correlation with enhanced T cell activity (Figure 2), the direct evidence to show that cDC1 accumulation is due to trained macrophages is lacking. In addition, cDC1 in mice normally do not express CD11b yet Figure 4 scRNAseq data showed cDC1 cluster using sorted CD11b+ cells from tumors.
2. The role of T cells. The authors depleted macrophages and NK cells to demonstrate that trained immunity+PC therapeutic efficacy was dependent on macrophages and NK cells (Figure 6). The authors should also deplete CD4/CD8 T cells to determine the relative contribution of innate antitumor immunity and T cell antitumor immunity in animal tumor models.
3. Figure 1A-E. These publicly available datasets are from mouse pancreas but not from spleen. The author should use these data in caution as some of myeloid cell subsets may be tissue specific.
4. Figure 2. The authors analyzed DCs and T cells from draining lymph nodes. The authors should also provide these data from tumors. In addition, it is unclear whether these myeloid cells have "trained immunity" phenotype.
5. Figure 3. It is nice to show metabolomics data. The authors may want to validate some of these pathways. For example, performing Seahorse assay to see whether indeed glycolysis pathway is enhanced in trained myeloid cells.
6. NK cells. The authors need to examine the functionality of NK cells, such as granzyme and perforin secretion.
7. Figure 8. The authors may use trained monocytes cultured with PDOs to see whether trained myeloid cells also have direct cancer killing activity.

Reviewer #2

(Remarks to the Author)

This study found that β -glucan-induced "trained immunity" enhances CRC vaccine efficacy by epigenetically and metabolically reprogramming macrophages. Mechanistically, trained immunity shifts CD11b+ myeloid cells toward aerobic glycolysis, while creatine metabolism provides the additional ATP required for sustained cytokine and chemokine production. This reprogramming converts immunoregulatory macrophages into anti-tumor effectors that recruit NK cells via CXCL9 and CXCL10, thereby enhancing cDC1 function. Finally, using human PBMC and patient-derived organoid co-culture models, they found that trained immunity enhance antigen-specific responses and tumor cell killing. This study presents a promising approach to overcoming the limitations of conventional immunotherapies in CRC by leveraging β -glucan-induced trained immunity to enhance vaccine efficacy. The mechanistic explanation is interesting but many statements/hypotheses are short of experimental evidence support. Specifically, several issues remain unresolved:

1. Figure 2B showed slightly enhanced anti-tumor efficacy of vaccination after β -glucan training, while Figure 2M is the result of a combination therapy approach. But for the same "Mock" group, the tumor growth is faster in therapeutic setting than the prophylactic setting. Did the mice receive the same amount of CT26 cells in both settings?

2. In Extended Figure 4, the authors showed tumor growth curves for 8 mice, but for the statistical analysis of dendritic cell phenotype in draining lymph nodes, they selected only 3 mice per group (Figure 2C). It's unclear whether these results come from three independent experiments. The same issue appears in Figures 2M-N. The authors need to describe their experimental methods in detail.
3. Figure 2D, the authors need to provide statistical results. From the presented data, the cell numbers in each group appear inconsistent.
4. Figure 3, the authors show broad alterations in cellular metabolism caused by different treatments, but these data contain speculative interpretations without solid evidence. For example: Lines 250-252: ECAR and OCR analysis would be desired to support their claims. Lines 277-279: Could the authors use isotope-labeled glutamate to validate the glutamate pathway as a metabolic hub? Although previous studies have shown glutamine is important for trained immunity, glucose metabolism is more widely recognized as the metabolic foundation of trained immunity (PMID: 27866838, PMID: 25258083). The authors need direct evidence to support their hypothesis.
5. Line 347 appears to describe results from Figure 4H, not 3H.
6. Similar to Figure 3, Figure 4 seems more descriptive and speculative without substantial experimental evidence. For example, why didn't the authors use CXCL9/10 blocking antibodies to confirm that trained macrophages recruit NK cells via CXCL9/10 (lines 347-349)? Is there direct evidence that NK cells recruit cDC1 cells by secreting CCL5/4 (lines 354-358)? Although Figures 4A-C show increased NK and DC cell numbers in the Trained+PeptiCrad group, these results appear to reflect overall changes in the immune cell landscape. Why did the authors consider NK and DC cells particularly important in this process? Although Figure 6B shows that depletion of macrophages and NK cells significantly weakens the anti-tumor effects of Trained+PC, would depletion of either macrophages or NK cells alone also promote tumor progression?
7. The authors consider trained macrophages as key immune cells in the NK-DC axis for anti-tumor effects, why does macrophage depletion alone show much weaker anti-tumor effects compared to simultaneous depletion of both macrophages and NK cells (Figure 6B)? Extended Figure 10 shows effective macrophage depletion. The authors need to clarify whether macrophages and NK cells have an upstream-downstream regulatory relationship or a parallel relationship. Trained neutrophils also have anti-tumor functions (PMID: 33125892). Did the authors consider this in their study?
8. Figure 4C, it's unclear whether this represents quantitative results from Figure 4B, but the proportion of cluster 20 in Figure 4C seems inconsistent with Figure 4B. The color for cluster "0" is almost same as cluster "20". Please modify the color to better differentiate them.
9. In Figure 5, the authors propose potential mechanisms regulating trained immunity, but I think the experimental design is confusing. The authors suggest creatine regulates trained immunity, but the author add rapamycin (mTOR/glycolysis inhibitor), ascorbic acid (HIF-1 α inhibitor), and 3-guanidinopropionic acid (GPA; creatine uptake inhibitor) during rest and secondary adenovirus stimulation rather than during WPG training (Figure 5A). Classical trained immunity inhibition is typically administered during training (Such as: PMID: 25258083, PMID: 29328908). The authors' experimental design can only show that rapamycin, ascorbic acid and GPA inhibit effector functions during secondary stimulation, not the priming stage when the immune memory undergoes induction during primary stimulation. The results in Figure 5I seem to confirm my suspicion, since 3-GPA didn't inhibit H3K4me3 modification levels, indicating that 3-GPA likely doesn't inhibit trained immunity in myeloid cells but rather inhibits effector immune responses during PC treatment.
10. If the authors want to firmly prove their conclusion (lines 422-423), I suggest transplanting bone marrow cells or splenic monocytes/macrophages from mice treated with WPG +3-GPA into recipient mice to observe whether they can enhance the anti-tumor effects of PC.
11. As the authors describe, "trained immunity is driven by long-lasting epigenetic modifications." Previous studies also consider epigenetics as the core mechanism of innate immune memory (PMID: 25258085). In my opinion, the genome-wide H3K4me3 modification analysis lacks precision, and the result complemented with ChIP-seq or ChIP-qPCR validation of relevant gene H3K4me3 modifications would be more accurate.

Minor issues:

1. The manuscript contains several errors that need correction: For example: Line 85: "TAMS" should be "TAMs". Line 132: "SINFEKL" should be "SIINFEKL"; Line 866: "PBMcs" should be "PBMCs". The authors should carefully proofread the manuscript.
2. It is suggested that the authors provide some technical background information or brief introduction about the "PeptiCrad (PC)" vaccine platform.
3. The Figure layout appears unpolished, with inconsistent magnification scales and font sizes among subpanels within the same Figure. The authors should revise these elements to enhance readability.

Reviewer #3

(Remarks to the Author)

The article by Hamdan and colleagues reports that β -glucan-induced macrophage trained immunity activates downstream NK cells, DCs and T cells. They conducted studies using the CT26 CRC model and validated their findings in the MC38 CRC model. They have also tried to validate their study in the human CRC organoid system. The study is comprehensive and novel, and most of the experiments are rigorous. Below are some comments that should be addressed:

1. The authors do not experimentally demonstrate how NK cells act on DCs. FLT3L is mentioned as a factor that can be produced by NK cells to activate DCs in their system. However, NK cells may not be the major source of FLT3L. If the authors believe that FLT3L produced by NK cells plays a role in DC activation, they should compare its expression in NK cells with that in other cell types, such as stromal cells, endothelial cells, and T cells. Then, they should design experiments to prove the role of FLT3 produced by NK cells in activating DCs. If they propose that other molecules such as CCL5 produced by NK cells are involved, the same comments regarding FLT3L also applies.

2. How long does the trained immunity last?

3. Does trained immunity induce cell exhaustion of immune cells, such as NK cells?

4. In the section on human studies, NK cells are not included. This does not recapitulate the mouse studies and also does not align with the title, which refers to "...enhancing colorectal cancer vaccination by inducing an NK-cDC1 axis."

5. Figure 6 should include a tumor-only group without any treatment.

Reviewer #4

(Remarks to the Author)

The study by Hamdan et al. explores the potential of β -glucan-induced trained immunity to enhance colorectal cancer (CRC) vaccination by modulating macrophage metabolism and promoting an NK-cDC1 axis. While the work addresses an important gap in CRC immunotherapy, its impact is limited by concerns regarding novelty, mechanistic depth, and experimental rigor. Below is a detailed critique organized by section.

Major Critiques

1. Novelty and Overlap with Prior Studies

- **Trained Immunity in Cancer:** The use of trained immunity as a cancer vaccine adjuvant has been previously demonstrated, notably by Wattenberg et al. (2023, *Sci Immunol*), who reported synergistic effects of β -glucan with CD40/Dectin-1 agonists in pancreatic cancer. The current study does not sufficiently differentiate its findings from this prior work.

- **NK-cDC1 Axis:** The mechanistic role of NK cells in recruiting cDC1s via CCL5/XCL1 is well-established (Böttcher et al., 2018, *Cell*). The authors should clarify how their results extend beyond these known mechanisms.

- **Creatine Metabolism:** Although the link between creatine metabolism and immune cell function is an emerging area, Peng & Saito (2023, *Front Immunol*) recently reported similar findings in macrophages, raising questions about the originality of this aspect.

2. Mechanistic Gaps

- **Epigenetic vs. Metabolic Contributions:** The assertion that trained immunity is driven by "epigenetic and metabolic rewiring" lacks robust support. While *Chil3*⁺ *Ly6C*^{hi} macrophages are implicated in the NK-cDC1 axis, the specific molecular mechanisms (e.g., how H3K4me3 regulates CXCL9/10 or creatine metabolism) remain unclear. Functional validation (e.g., ChIP-seq, CRISPR-edited models) is needed to strengthen these claims.

- **Causality in the NK-cDC1 Axis:** Depletion experiments (Fig. 6) do not conclusively determine whether NK cells or macrophages are the primary drivers of cDC1 recruitment. Transwell assays (Fig. 6I) demonstrate macrophage-NK chemotaxis but fail to directly link this to cDC1 priming.

- **Metabolomic Data Interpretation:** The observed upregulation of creatine metabolites (Fig. 3C) is correlative. Isotope tracing or genetic knockout experiments are needed to establish a causal relationship between creatine metabolism and cytokine production. Additionally, does creatine supplementation rescue the effects of β -GPA inhibition in vivo?

3. Experimental Design and Rigor

- **Mouse Model Limitations:** The use of immunogenic models (CT26, MC38) may not fully recapitulate the immunosuppressive tumor microenvironment (TME) of human CRC. Including syngeneic models with low mutational burden (e.g., APC-mutant) would improve translational relevance. Furthermore, subcutaneous tumors do not adequately model metastatic CRC; orthotopic models would be more informative.

- **Inducer Specificity:** Relying solely on β -glucan (WPG) as a trained immunity inducer limits generalizability. Comparisons with other inducers (e.g., BCG, LPS) would strengthen the conclusions.

- **Human Data Limitations:** The PDO experiments (n=3) are underpowered, and PBMC data (Fig. 8D-E) lack sufficient donor diversity. Expanding to additional patient-derived samples (e.g., PDX models) and quantifying antigen-specific T-cell responses would enhance translational relevance.

Minor Critiques

4. Single-Cell RNA Sequencing

- The reanalysis of Geller et al. (2022, *Nat Commun*) (Fig. 1A-E) does not clearly advance beyond the original study. The authors should clarify its novel contributions.

- Cluster annotation (Fig. 4A-B) requires protein-level validation (e.g., *Chil3*⁺*Ly6C*^{hi} macrophages).

5. Metabolomics

- Pathway impact analysis (Fig. 3E-F) does not adjust for multiple testing, increasing the risk of false positives.

6. Methods Concerns

- Fig. 1F-I: The SIINFEKL model is not CRC-relevant; justification for using ovalbumin-derived peptides is lacking.
- Fig. 2L-N: A comparison with checkpoint inhibitors (e.g., anti-PD-1) is absent, despite their clinical relevance.
- Fig. 7 (MC38 Model): Data on tumor-infiltrating T cells and checkpoint markers (e.g., PD-L1) are missing.
- WPG Dosing: The β -glucan dose (1 mg/mouse) lacks human-equivalent translation, raising translational concerns.

7. Figure Clarity

- Tumor growth curves (Figs. 2B, 2M) should include individual data points to illustrate variability.
- Immunofluorescence images (Figs. 5I, 8G) need scale bars and higher resolution.
- The scRNA-seq methods (e.g., batch correction, cluster resolution) require more detail for reproducibility.

Reviewer #5

(Remarks to the Author)

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

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have made efforts to satisfactorily address the concerns raised in the previous review.

Reviewer #2

(Remarks to the Author)

The authors have carefully and sufficiently addressed my questions, and I would recommend acceptance of the manuscript for publication.

Reviewer #3

(Remarks to the Author)

I do not think the authors have adequately addressed my previous concerns.

For example, last time I questioned whether FLT3 is a mediator between NK cells and DCs. Now the authors have changed FLT3 to CCL5, but provide little or no new supporting evidence. CCL5 may be less specific because: 1) NK cells are not a major producer of it in this environment, and 2) CCL5 can recruit many types of immune cells, not just DCs.

Another example: I questioned, "How long does trained immunity last?" The authors mentioned "several weeks" but did not cite any figure or specify the exact duration. Moreover, claiming "several weeks" may not be sufficient to support trained immunity.

Additionally, when I asked, "Does trained immunity induce exhaustion in immune cells, such as NK cells?" the authors only reanalyzed their scRNA-seq dataset. Why did they not assess this by flow cytometry? The authors stated they did not want to present additional data in the manuscript, but the reason for this decision is unclear.

Regarding my concern #5, I still think the tumor-only group is important. But they authors do not provide the data.

Reviewer #4

(Remarks to the Author)

The authors performed new experiments, such as adoptive transfers, sequential migration assays, CD8+ T cell depletion, Seahorse assays, ChIP-qPCR, and validation in a more relevant tumor model. The manuscript now presents a compelling and well-supported narrative.

Reviewer #5

(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.

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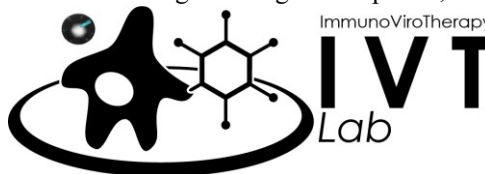
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Helsinki, October 3, 2025

Dear Reviewers,

We would like to sincerely thank all of you for the evaluation of our manuscript and for providing constructive and insightful comments. The feedback has significantly strengthened our work, guiding us to improve both the experimental rigor and the clarity of presentation. The revisions and additional experiments performed in response to these comments have elevated the manuscript to a higher level, and we are deeply grateful for the time, effort, and expertise invested in this review process.

Best regards,
Vincenzo Cerullo

Reviewer #1 (Remarks to the Author): with expertise in trained immunity, cancer immunology

The manuscript entitled, "Leveraging trained immunity for the epigenetic and metabolic rewiring of macrophages to enhance colorectal cancer vaccination by inducing an NK-cDC1 axis" reported that combined trained immunity and tumor vaccine led to significant tumor killing using both mouse colon cancer models and human colon cancer organoids. The authors showed that the combined trained immunity with vaccine therapeutic efficacy appears to be dependent on a robust macrophage-NK cell-

cDC axis. While these results have clear clinical translational potential, there are some concerns in this study.

Response: We thank the reviewer for their thoughtful evaluation and for recognizing the translational potential of our study. We acknowledge the concerns raised and have included additional experiments and clarifications in the revised manuscript to address them, further strengthening our conclusions on the role of the macrophage–NK cell–cDC1 axis in enhancing colorectal cancer vaccination.

1. The role of cDC1 is unclear in this study. While the authors showed that cDC1 were accumulated in the tumor-bearing mice treated with trained immunity plus PC vaccine and its correlation with enhanced T cell activity (Figure 2), the direct evidence to show that cDC1 accumulation is due to trained macrophages is lacking. In addition, cDC1 in mice normally do not express CD11b yet Figure 4 scRNAseq data showed cDC1 cluster using sorted CD11b⁺ cells from tumors.

Response: We thank the reviewer for raising this important point. To address it, we performed several new experiments that clarify the mechanistic link between trained macrophages, NK cells, and cDC1 accumulation:

1. **Adoptive transfer experiments:** We isolated macrophages and NK cells, treated them with PBS or WPG, and adoptively transferred them into CT26-bearing mice that were subsequently vaccinated with PC. Only mice that received *trained macrophages* (WPG treated) showed tumor control, whereas mice receiving trained NK cells did not. Importantly, mice given trained macrophages exhibited increased infiltration of macrophages, NK cells, and cDC1 in the TME, accompanied by enhanced effector memory CD8⁺ T cells and higher IFN- γ /TNF- α production. Furthermore, when splenocytes from mice given *trained macrophages* and treated with PC were stimulated with Syl peptide (epitope used in PC vaccination) an increase in IFN- γ /TNF- α production from CD8⁺ T cells were observed compared to other groups. This was also accompanied with higher cDC1 populations in the draining lymph nodes. This demonstrates that trained macrophages are the initiating population required for cDC1 accumulation and vaccine efficacy. Results for such experiments can be found in Figure 6 F-I and in Extended Figures 15 and 16
2. **Macrophage–NK axis:** Using transwell assays, we showed that macrophages from Trained+PC mice recruit NK cells via the CXCL9/10–CXCR3 axis. Blocking CXCR3 significantly reduced NK migration. Mechanistically, ChIP-qPCR revealed H3K4me3 enrichment at CXCL9 and CXCL10 loci in trained macrophages, demonstrating that these chemokines are epigenetically upregulated. Thus, trained macrophages are directly responsible for NK recruitment. Results for such experiments can be found in Figure 6 J&K and Extended Figure 17
3. **NK–cDC1 axis:** NK cells isolated from the TME of Trained+PC mice (but not freshly WPG-treated NK cells) induced migration of dendritic cells in transwell assays, an effect dependent on CCL5. This shows that NK cells are not epigenetically rewired by WPG directly but are functionally “educated” in the trained TME by macrophages to secrete CCL5, which in turn recruits cDC1. RT-qPCR confirmed upregulation of CCL5 only in NK cells isolated from the TME of Trained+PC mice, but not in freshly WPG-stimulated NK cells. Results for such experiments can be found in Figure 6 L&M and Extended Figure 17.
4. **Regarding CD11b sorting:** We acknowledge the reviewer’s point that cDC1 are typically CD11b[−]. Cells were enriched using magnetic sorting; hence contaminant cell populations could have been captured. Moreover, prior reports have noted that CD11b-based sorting in the TME can

capture minor cDC1 subsets due to overlapping myeloid marker expression (PMID: 39506629, PMID: 28611041)

5. . This could also explain why a cDC1 cluster was detected in our CD11b⁺ scRNA-seq dataset. Both these reasons could explain the low number of DCs found in the scRNA-seq dataset.

In summary, these new data demonstrate that trained macrophages initiate a cascade: they recruit NK cells via CXCL9/10, NK cells in turn secrete CCL5 to recruit cDC1, and cDC1 accumulation is essential for bridging trained immunity to adaptive T cell responses. Thus, the role of cDC1 is clarified as downstream of trained macrophages, with macrophages acting as the critical initiating population.

2. The role of T cells. The authors depleted macrophages and NK cells to demonstrate that trained immunity+PC therapeutic efficacy was dependent on macrophages and NK cells (Figure 6). The authors should also deplete CD4/CD8 T cells to determine the relative contribution of innate antitumor immunity and T cell antitumor immunity in animal tumor models.

Response: We thank the reviewer for this important point. To directly assess the role of CD8⁺ T cells in mediating the therapeutic efficacy of trained immunity combined with PC vaccination, we performed depletion experiments in CT26-bearing mice. Mice were injected with a 500 µg bolus of anti-CD8 antibody prior to training, followed by 100 µg maintenance doses every three days. Flow cytometry confirmed efficient and sustained CD8⁺ T cell depletion, as no circulating CD8⁺ T cells were detected. In the absence of CD8⁺ T cells, the Trained+PC treatment failed to control tumor growth, in stark contrast to CD8⁺-competent mice where tumor control was robust. These results demonstrate that the anti-tumor effects of Trained+PC are critically dependent on CD8⁺ T cell responses. Results for such experiments can be found in Extended Figure 14

We did not perform CD4⁺ T cell depletion experiments, as the peptides used in the PC vaccine are MHC-I-restricted and designed to specifically activate CD8⁺ T cells. Thus, CD8⁺ T cells represent the primary adaptive population mediating vaccine-induced tumor control in this model.

3. Figure 1A-E. These publicly available datasets are from mouse pancreas but not from spleen. The author should use these data in caution as some of myeloid cell subsets may be tissue specific.

Response: We thank the reviewer for this comment and realizing our mistake. We have now corrected this in the text and added some comments about the tissue specific populations that should be taken into consideration.

4. Figure 2. The authors analyzed DCs and T cells from draining lymph nodes. The authors should also provide these data from tumors. In addition, it is unclear whether these myeloid cells have "trained immunity" phenotype.

Response: We thank the reviewer for this insightful comment. To address this, we analyzed immune populations directly within the tumor microenvironment (TME) in our adoptive transfer experiments. In CT26-bearing mice that received trained macrophages followed by PC vaccination, we observed a clear increase in effector memory CD8⁺ T cells as well as enhanced infiltration of cDC1 within the TME. Thus, beyond the systemic effects we reported in the spleen (expanded effector memory CD8⁺ T cells) and draining lymph nodes (increased cDC1), these populations also accumulated in the tumor site, highlighting

their direct contribution to anti-tumor immunity. Results for such experiments can be found in Extended Figure 16 D-G

Regarding the identity of "trained" cells, our scRNA-seq analysis (seen in Figure 4D) indicated that macrophages, monocytes, and NK cells exhibited signatures consistent with trained immunity, with macrophages emerging as the dominant population driving this effect. Importantly, our functional assays confirmed that β -glucan treatment directly reprograms macrophages, while NK cells were not intrinsically trained by β -glucan in vitro but rather were secondarily activated within the trained TME.

5. Figure 3. It is nice to show metabolomics data. The authors may want to validate some of these pathways. For example, performing Seahorse assay to see whether indeed glycolysis pathway is enhanced in trained myeloid cells.

Response: We thank the reviewer for this suggestion. To validate our metabolomics data, we performed Seahorse extracellular flux assays on macrophages isolated from the spleens of mock-treated mice and stimulated ex vivo with PBS or WPG for 18 hours. Cells were plated at two different densities (40,000 and 80,000 cells/well) to confirm reproducibility across cell numbers.

Trained macrophages consistently exhibited higher oxygen consumption rates (OCR) compared to PBS controls at both densities. However, most of this increase was attributable to non-mitochondrial oxygen consumption, as confirmed by inhibitor analysis, indicating that trained macrophages are not enhancing oxidative phosphorylation.

In contrast, extracellular acidification rates (ECAR) were strongly elevated in trained macrophages, both at basal levels and after stimulation, demonstrating increased glycolytic activity. This elevation in ECAR also signifies higher lactate production, consistent with a Warburg-like metabolic shift. Notably, when the experiment was repeated with only 2 hours of WPG stimulation, OCR remained unchanged between groups, yet ECAR was already significantly higher in trained macrophages. This rapid onset of glycolytic reprogramming highlights that β -glucan training quickly biases macrophages toward aerobic glycolysis observed in activated macrophages.

Together, these findings validate our metabolomic profiling and show that WPG-trained macrophages undergo robust metabolic rewiring, characterized by increased reliance on aerobic glycolysis rather than oxidative phosphorylation. Results for such experiments can be found in Figure 3 H-L and Extended Figure 8.

6. NK cells. The authors need to examine the functionality of NK cells, such as granzyme and perforin secretion.

Response: We thank the reviewer for this important suggestion. To assess NK cell functionality, we examined NK cells in the tumor microenvironment of CT26-bearing mice from our adoptive transfer experiments. In mice given trained macrophages and subsequently treated with PC, tumor-infiltrating NK cells displayed increased perforin expression, indicative of enhanced cytotoxic potential. Results for such experiments can be found Extended Figure 16 C

Furthermore, RT-qPCR analysis of NK cells isolated from these tumors revealed elevated CCL5 expression, consistent with an activated phenotype and their role in recruiting cDC1 (further seen in our migration studies). These findings align with our scRNA-seq data, which suggested enhanced NK cell

activity in the trained + PC condition, and collectively confirm that NK cells acquire functional effector properties within the trained tumor microenvironment. Results for such experiments can be Extended Figure 17 B&C

7. Figure 8. The authors may use trained monocytes cultured with PDOs to see whether trained myeloid cells also direct cancer have killing activity.

Response: We thank the reviewer for this important suggestion. We have performed such experiment with three new CRC PDOs. When trained macrophages/monocytes were added we did not see any difference in tumor killing compared to when PBS treated macrophages/monocytes were added. Moreover, levels of tumor killing were very minimal (>5%). Results for such experiments can be Extended Figure 22 B

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

This study found that β -glucan-induced "trained immunity" enhances CRC vaccine efficacy by epigenetically and metabolically reprogramming macrophages. Mechanistically, trained immunity shifts CD11b+ myeloid cells toward aerobic glycolysis, while creatine metabolism provides the additional ATP required for sustained cytokine and chemokine production. This reprogramming converts immunoregulatory macrophages into anti-tumor effectors that recruit NK cells via CXCL9 and CXCL10, thereby enhancing cDC1 function. Finally, using human PBMC and patient-derived organoid co-culture models, they found that trained immunity enhance antigen-specific responses and tumor cell killing. This study presents a promising approach to overcoming the limitations of conventional immunotherapies in CRC by leveraging β -glucan-induced trained immunity to enhance vaccine efficacy. The mechanistic explanation is interesting, but many statements/hypotheses are short of experimental evidence support. Specifically, several issues remain unresolved:

Response: We sincerely thank the reviewer for their thoughtful summary and constructive feedback on our study. We appreciate the recognition of the novelty of combining β -glucan-induced trained immunity with CRC vaccination, and we agree that mechanistic clarity is crucial for strengthening our conclusions.

In response to the reviewer's concern that several mechanistic statements required further experimental evidence, we have performed additional experiments and refined our analyses to directly address these gaps.

1. Figure 2B showed slightly enhanced anti-tumor efficacy of vaccination after β -glucan training, while Figure 2M is the result of a combination therapy approach. But for the same "Mock" group, the tumor growth is faster in therapeutic setting than the prophylactic setting. Did the mice receive the same amount of CT26 cells in both settings?

Response: We thank the reviewer for this comment. The tumor growth among mock groups in the experiments have different tumor growth kinetics. The number of cells that were injected (500,000 CT26 cells per mouse) were the same in both experiments. The difference in tumor growth could have been due to different cell batches. Moreover, it could be also due to human error of the amount of injection (we administer 50µl in insulin syringes) or the cell viability of cells when injected. Hence, why we have a mock group (or reference group) in each set of experiments to validate the tumor growth kinetics among groups within one set of experiments

2. In Extended Figure 4, the authors showed tumor growth curves for 8 mice, but for the statistical analysis of dendritic cell phenotype in draining lymph nodes, they selected only 3 mice per group (Figure 2C). It's unclear whether these results come from three independent experiments. The same issue appears in Figures 2M-N. The authors need to describe their experimental methods in detail.

Response: We thank the reviewer for pointing this out. We are sorry for not clearly indicating the appropriated experimental method. We have pooled the lymph nodes of all mice in a treatment group and did three technical replicates. The reason we did this was to have an appropriate number of DCs to perform phenotypical analysis. This has now been updated in the manuscript.

3. Figure 2D, the authors need to provide statistical results. From the presented data, the cell numbers in each group appear inconsistent.

Response: We thank the reviewer for this comment. In Figure 2C we have provided actual percentages of DCs populations in the lymph nodes along with statistical data. However, we have now removed the tSNE graphs in 2D since we believe this does not add any value since Figure 2C provides such information.

4. Figure 3, the authors show broad alterations in cellular metabolism caused by different treatments, but these data contain speculative interpretations without solid evidence. For example: Lines 250-252: ECAR and OCR analysis would be desired to support their claims. Lines 277-279: Could the authors use isotope-labeled glutamate to validate the glutamate pathway as a metabolic hub? Although previous studies have shown glutamine is important for trained immunity, glucose metabolism is more widely recognized as the metabolic foundation of trained immunity (PMID: 27866838, PMID: 25258083). The authors need direct evidence to support their hypothesis.

Response: We thank the reviewer for this valuable suggestion. We agree that our data does not show that glutamate is a metabolomic hub for trained immunity. Furthermore, we also believe that glucose metabolism is the main metabolomic function in trained immunity. Our metabolomics data also further demonstrate that. We have now fixed our assumptions in our results section. Moreover, to further validate our metabolomics data and investigate the key role of glucose, we performed a Seahorse extracellular flux assays on macrophages isolated from the spleens of mock-treated mice and stimulated ex vivo with PBS or WPG for 18 hours. Cells were plated at two different densities (40,000 and 80,000 cells/well) to confirm reproducibility across cell numbers.

Trained macrophages consistently exhibited higher oxygen consumption rates (OCR) compared to PBS controls at both densities. However, most of this increase was attributable to non-mitochondrial oxygen consumption, as confirmed by inhibitor analysis, indicating that trained macrophages are not enhancing oxidative phosphorylation.

In contrast, extracellular acidification rates (ECAR) were strongly elevated in trained macrophages, both at basal levels and after stimulation, demonstrating increased glycolytic activity. This elevation in ECAR also signifies higher lactate production, consistent with a Warburg-like metabolic shift. Notably, when the experiment was repeated with only 2 hours of WPG stimulation, OCR remained unchanged between groups, yet ECAR was already significantly higher in trained macrophages. This rapid onset of glycolytic reprogramming highlights that β -glucan training quickly biases macrophages toward aerobic glycolysis.

Together, these findings validate our metabolomic profiling and show that WPG-trained macrophages undergo robust metabolic rewiring, characterized by increased reliance on aerobic glycolysis rather than oxidative phosphorylation. Results for such experiments can be found in Figure 3 H-L and Extended Figure 8

5. Line 347 appears to describe results from Figure 4H, not 3H.

Response: We thank the reviewer for pointing this out. We have now fixed that in the manuscript.

6. Similar to Figure 3, Figure 4 seems more descriptive and speculative without substantial experimental evidence. For example, why didn't the authors use CXCL9/10 blocking antibodies to confirm that trained macrophages recruit NK cells via CXCL9/10 (347-349)? Is there direct evidence that NK cells recruit cDC1 cells by secreting CCL5/4 (lines 354-358)? Although Figures 4A-C show increased NK and DC cell numbers in the Trained+PeptiCrad group, these results appear to reflect overall changes in the immune cell landscape. Why did the authors consider NK and DC cells particularly important in this process? Although Figure 6B shows that depletion of macrophages and NK cells significantly weakens the anti-tumor effects of Trained+PC, would depletion of either macrophages or NK cells alone also promote tumor progression?

Response:

We thank the reviewer for these constructive comments. To directly address the mechanisms, we performed a series of transwell migration assays. First, we tested whether trained macrophages could recruit NK cells. Macrophages isolated from the TME of Trained+PC mice induced robust NK cell migration compared to controls, and this effect was abrogated when NK cells were pretreated with CXCR3 blocking antibodies, confirming that CXCL9/10 secreted by macrophages mediates NK cell recruitment. Next, we tested whether NK cells could recruit DCs. NK cells isolated from the TME of Trained+PC mice induced DC migration, which was blocked by CCL5 neutralization. However, freshly isolated NK cells treated with WPG in vitro did not induce DC migration, indicating that NK cells require conditioning within the trained TME to acquire this function. Results for such experiments can be found in Figure 6 L&M. Moreover, we have also performed ChIP-qPCRs and RT-qPCR to demonstrate that Macrophages upregulate CXCL9 and 10 (via H3K4me3) and NK cells upregulate CCL5. These experiments are described in the reviewer's concern #11.

The rationale for focusing on NK cells and DCs stemmed from integrative analysis of our metabolomics and scRNA-seq datasets (Figure 4J), where NK cell activation emerged as one of the most enriched pathways. In addition, chemokines CCL4 and CCL5—well-known cDC1 chemoattractants—were among the most strongly upregulated transcripts. These findings guided us to explore the macrophage–NK–DC axis in greater depth.

To further validate these observations in vivo, we performed adoptive transfer experiments with trained macrophages or NK cells in CT26-bearing mice treated with PC. Only mice receiving trained macrophages demonstrated tumor control, which was accompanied by increased infiltration of NK cells and subsequent accumulation of cDC1. This strongly supports that macrophages act upstream, conditioning NK cells to

promote cDC1 recruitment. However, details of such experiment are further highlighted in the reviewer's concern (7).

Finally, regarding tumor growth, previous reports (PMID: 30198904; PMID: 33511454; PMID: 29136508) have demonstrated that depletion of NK cells or macrophages alone does not enhance tumor progression in CT26-bearing mice. Consistent with these studies, we observed that depletion of either population partially impaired, but did not abolish, tumor control. In contrast, simultaneous depletion of both cell types of completely abrogated vaccine efficacy. To provide a cleaner dissection of their relative contributions, we conducted adoptive transfers of trained macrophages or NK cells, which confirmed that trained macrophages are indispensable drivers of the NK–cDC1 axis and downstream T cell responses.

7. The authors consider trained macrophages as key immune cells in the NK-DC axis for anti-tumor effects, why does macrophage depletion alone show much weaker anti-tumor effects compared to simultaneous depletion of both macrophages and NK cells (Figure 6B)? Extended Figure 10 shows effective macrophage depletion. The authors need to clarify whether macrophages and NK cells have an upstream-downstream regulatory relationship or a parallel relationship. Trained neutrophils also have anti-tumor functions (PMID: 33125892). Did the authors consider this in their study?

Response: We thank the reviewer for this important point. To clarify the relationship between macrophages and NK cells, we performed adoptive transfer experiments where we isolated macrophages and NK cells from PBS- or WPG-treated mice and transferred them into CT26-bearing mice that were subsequently vaccinated with PC. We found that only mice receiving trained macrophages together with PC exhibited tumor control, accompanied by increased infiltration of macrophages, NK cells, and cDC1 within the TME. In contrast, adoptive transfer of trained NK cells did not result in tumor control, nor did it promote cDC1 accumulation. Importantly, mice given trained macrophages also showed a significant increase in effector memory (EM) CD8⁺ T cells and antigen-specific T cell responses against the Syl peptide used for vaccination, further demonstrating that trained macrophages act as the primary upstream drivers of adaptive immunity. Results for such experiments can be found in Figure 6 F-I and also in Extended Figures 15 and 16

As for the reviewer's observation that macrophage depletion alone still permitted some tumor control, we believe this may be explained by residual monocytes or other CD11b⁺ subsets capable of secreting CXCL9/10, albeit at lower levels, which may continue to recruit NK or T cells. Similarly, NK cell depletion alone did not completely abolish tumor control, likely because T cells are still recruited directly by macrophages through chemokine signaling. However, when both macrophages and NK cells were depleted, these compensatory mechanisms were lost, leading to complete abrogation of tumor control.

Finally, regarding neutrophils, we carefully examined our scRNA-seq dataset and did not observe significant upregulation of trained-immunity-associated or activation signatures in neutrophil clusters. Moreover, their presence in the TME did not change among Trained+PC groups and Mock (Extended Figure 6A). Given this, and consistent with the limited neutrophil involvement we observed in our TME analyses, we did not pursue them further in this study. We agree that neutrophils can have anti-tumor functions (PMID: 33125892), but in our system, macrophages emerged as the dominant trained population orchestrating the NK–cDC1 axis and enabling robust T cell immunity.

8. Figure 4C, it's unclear whether this represents quantitative results from Figure 4B, but the proportion of

cluster 20 in Figure 4C seems inconsistent with Figure 4B. The color for cluster “0” is almost same as cluster “20”. Please modify the color to better differentiate them.

Response: We thank the reviewer for pointing this out. We agree that the color similarity is confusing, and it has been changed.

9. In Figure 5, the authors propose potential mechanisms regulating trained immunity, but I think the experimental design is confusing. The authors suggest creatine regulates trained immunity, but the author add rapamycin (mTOR/glycolysis inhibitor), ascorbic acid (HIF-1 α inhibitor), and 3-guanidinopropionic acid (GPA; creatine uptake inhibitor) during rest and secondary adenovirus stimulation rather than during WPG training (Figure 5A). Classical trained immunity inhibition is typically administered during training (Such as: PMID: 25258083, PMID: 29328908). The authors' experimental design can only show that rapamycin, ascorbic acid and GPA inhibit effector functions during secondary stimulation, not the priming stage when the immune memory undergoes induction during primary stimulation. The results in Figure 5I seem to confirm my suspicion, since 3-GPA didn't inhibit H3K4me3 modification levels, indicating that 3-GPA likely doesn't inhibit trained immunity in myeloid cells but rather inhibits effector immune responses during PC treatment.

Response: We thank the reviewer for raising this important point and agree that our initial description may have caused confusion. As correctly noted, the original experiments in Figure 5 were designed to test the impact of metabolic inhibitors during secondary stimulation, thereby demonstrating that rapamycin, ascorbic acid, and 3-GPA suppress effector cytokine and chemokine production rather than the establishment of trained immunity itself. This was intentional, as one of our goals was to show that creatine metabolism regulates effector functions but does not influence the acquisition of epigenetic marks associated with trained immunity. Moreover, 3-GPA did not alter H3K4me3 levels, indicating that creatine does not affect the induction of trained immunity.

To directly address the reviewer's concern, we repeated these experiments by adding metabolic inhibitors during the WPG training phase rather than only at the secondary stimulation. In these experiments, CD11b⁺ cells treated with WPG alone upregulated IL-6, TNF- α , CXCL9, and CXCL10 upon adenovirus stimulation, consistent with our previous findings. However, when rapamycin, ascorbic acid, or 3-GPA were added during the training phase, this heightened cytokine and chemokine production was markedly reduced. Immunofluorescence staining of H3K4me3 further confirmed that 3-GPA treatment did not diminish this histone modification, reinforcing that creatine uptake is not required for the epigenetic reprogramming of trained macrophages but rather for sustaining cytokine production during restimulation. Hence, it seems that creatine metabolism, while dispensable for epigenetic imprinting, is required for the enhanced effector function during secondary stimulation. Results for such experiments can be found Extended Figures 10

10. If the authors want to firmly prove their conclusion (lines 422-423), I suggest transplanting bone marrow cells or splenic monocytes/macrophages from mice treated with WPG +3-GPA into recipient mice to observe whether they can enhance the anti-tumor effects of PC.

Response: We thank the reviewer for raising this important point and agree that such experiment would help our hypothesis. In our adoptive transfer experiments, CT26 bearing mice given trained macrophages and treated with PC and β -GPA (daily) had a loss of tumor control. Hence it demonstrated the reliance of macrophages with β -GPA. Results for such experiments can be found Extended Figures 18

Moreover, when we treated CD11b⁺ cells with β -GPA, washed and then supplemented with creatine we managed to rescue the expression of CXCL9 and CXCL10. Hence, it seems that macrophages rely on creatine for CXCL9 and CXCL10 expression and inducing an anti-tumor response *in vivo*. Results for such experiments can be found Extended Figures 10 D&E

11. As the authors describe, "trained immunity is driven by long-lasting epigenetic modifications." Previous studies also consider epigenetics as the core mechanism of innate immune memory (PMID: 25258085). In my opinion, the genome-wide H3K4me3 modification analysis lacks precision, and the result complemented with ChIP-seq or ChIP-qPCR validation of relevant gene H3K4me3 modifications would be more accurate.

Response: We thank the reviewer for this valuable comment. We fully agree that genome-wide H3K4me3 analysis offers only a broad perspective and requires gene-specific validation to directly link epigenetic modifications to functional outcomes. To address this, we complemented our immunofluorescence analysis with targeted ChIP-qPCR experiments.

In tumor-derived CD11b⁺F4/80⁺ macrophages, ChIP-qPCR revealed a clear increase in H3K4me3 enrichment at the promoters of CXCL9 and CXCL10, two chemokines that were strongly upregulated in our scRNA-seq analysis and are critical for NK cell recruitment. These findings confirm that WPG-induced trained immunity directly rewires macrophages at the epigenetic level to promote expression of chemokines central to the macrophage–NK–cDC1 axis. Results for such experiments can be found Extended Figures 17A By contrast, analysis of the creatine transporter gene (Slc6a8) showed no enrichment for H3K4me3, even though mRNA expression was increased, indicating that creatine metabolism supports effector functions during secondary stimulation but is not under conventional epigenetic regulation of trained immunity. Results for such experiments can be found Extended Figures 18 C&D

We also extended our analysis to NK cells. ChIP-qPCR of NK cells isolated from PBS- or WPG-treated mice did not reveal enrichment of H3K4me3 at loci such as CCL5, CCL4, or Flt3, even in NK cells recovered from the TME of Trained+PC mice. However, RT-qPCR analysis of whole-cell lysates demonstrated that NK cells from the TME of Trained+PC mice did upregulate CCL5 expression. These data suggest that NK cells are not epigenetically rewired by WPG but instead acquire their activated state within the trained tumor microenvironment, likely through cues provided by trained macrophages. Results for such experiments can be found Extended Figures 17B&C

Together, these ChIP-qPCR experiments strengthen our conclusions by showing that trained macrophages undergo targeted epigenetic rewiring at chemokine loci that drive downstream immune recruitment, and NK cells, in contrast, are not epigenetically modified but are functionally activated within the trained TME. This highlights the distinct but complementary roles of epigenetic regulation in macrophages and environmental licensing in NK cells.

Minor

issues:

1. The manuscript contains several errors that need correction: For example: Line 85: "TAMS" should be "TAMs". Line 132: "SINFEKL" should be "SIINFEKL"; Line 866: "PBMcs" should be "PBMCs". The authors should carefully proofread the manuscript.

Response: We thank the reviewer for pointing such mistakes, we have corrected them and gone through the manuscript carefully.

2. It is suggested that the authors provide some technical background information or brief introduction about the "PeptiCrad (PC)" vaccine platform.

Response: We agree with the reviewer that an introduction to PC will help readers understand the vaccine platform. We have added a short description of PC in the introduction.

3.The Figure layout appears unpolished, with inconsistent magnification scales and font sizes among subpanels within the same Figure. The authors should revise these elements to enhance readability.

Response: We agree with the reviewer and have now enhanced the readability of the figures.

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

The article by Hamdan and colleagues reports that β -glucan-induced macrophage trained immunity activates downstream NK cells, DCs and T cells. They conducted studies using the CT26 CRC model and validated their findings in the MC38 CRC model. They have also tried to validate their study in the human CRC organoid system. The study is comprehensive and novel, and most of the experiments are rigorous. Below are some comments that should be addressed:

Response: We thank the reviewer for their positive assessment of our work and for recognizing the novelty and rigor of our study. We also appreciate the reviewer's constructive comments and agree that addressing these points will further strengthen the manuscript.

1.The authors do not experimentally demonstrate how NK cells act on DCs. FLT3L is mentioned as a factor that can be produced by NK cells to activate DCs in their system. However, NK cells may not be the major source of FLT3L. If the authors believe that FLT3L produced by NK cells plays a role in DC activation, they should compare its expression in NK cells with that in other cell types, such as stromal cells, endothelial cells, and T cells. Then, they should design experiments to prove the role of FLT3 produced by NK cells in activating DCs. If they propose that other molecules such as CCL5 produced by NK cells are involved, the same comments regarding FLT3L also applies.

Response:

We thank the reviewer for this insightful comment. We agree that NK cells are not the only potential source of DC-activating factors such as FLT3L, and that it was important to experimentally assess their contribution. To address this, we performed RT-qPCR analyses on NK cells isolated from the TME of CT26-bearing mice treated with Trained+PC. We found that CCL5 was the most highly expressed chemokine, while FLT3L and CCL4 were expressed at lower levels. Results for such experiments can be found Extended Figure 17 B&C

To functionally test this, we performed transwell migration assays using NK cells isolated from the TME of Trained+PC mice. These NK cells significantly enhanced DC migration compared to NK cells from mock-treated mice. Importantly, blocking CCL5 with neutralizing antibodies abrogated this migration, demonstrating that NK cells promote DC recruitment primarily through the CCL5–CCR5 axis in our model. Results for such experiments can be found in Figure 6L&M

Together, these findings provide direct evidence that NK cells in the Trained+PC context acquire the ability to recruit DCs, with CCL5 playing a central role.

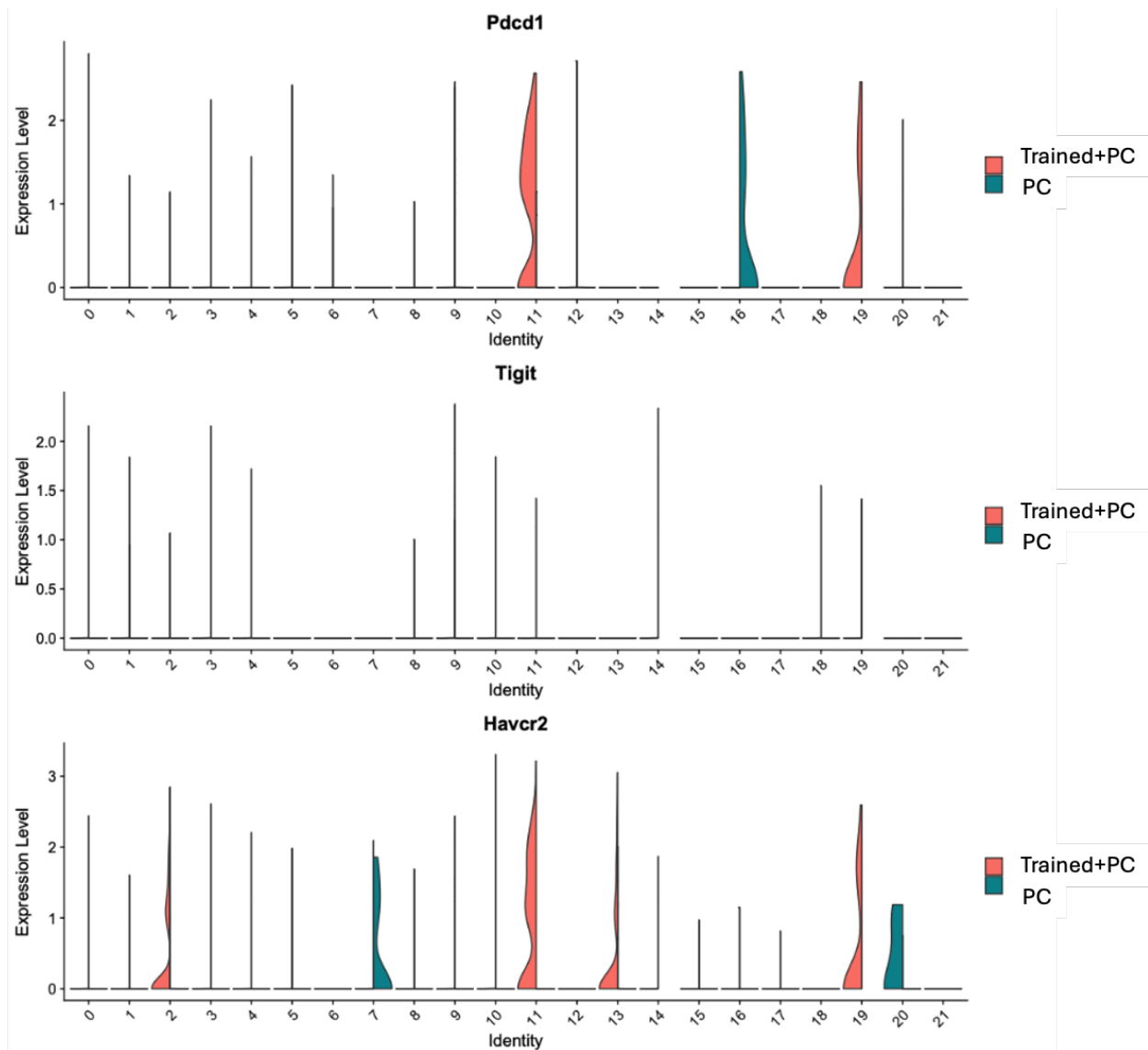
2.How long does the trained immunity last?

We thank the reviewer for raising this important point. The duration of trained immunity can vary depending on the inducer and the cell type involved. Prior studies have shown that β -glucan-induced training in humans can persist for several months in circulating monocytes (PMID: 25258085; PMID: 29328908), while in mice trained immunity signatures have been observed up to 9 weeks post-training (PMID: 30166439). In our study, we focused primarily on mechanistic dissection of the pathways involved rather than the long-term persistence. Nonetheless, in our tumor models we observed functional effects of trained immunity **several weeks after initial β -glucan exposure (as seen in our animal experiments)**, suggesting that training persists long enough to shape adaptive immune responses.

We agree that future work should include longitudinal experiments to define the exact durability of trained immunity in the tumor setting, including rechallenge studies or late tumor inoculation models. We have also briefly discussed this in our “Discussion” section.

3.Does trained immunity induce cell exhaustion of immune cells, such as NK cells?

Response: We thank the reviewer for raising this important point. To address it, we reanalyzed our scRNA-seq dataset and examined canonical exhaustion markers, including *Pdcd1* (PD-1), *Tigit* (TIGIT), and *Havcr2* (TIM-3). No detectable expression or differential expression of these markers was observed in NK cells (cluster 1) between Trained+PC and PC groups. In contrast, increased PD-1 and TIM-3 expression was noted in clusters 11 and 19 (NKT cells) in Trained+PC mice compared to PC, suggesting that trained immunity may promote some degree of exhaustion in NKT cells but not in NK cells. These findings are included in Response to Reviewers Figure 1. We have not added this analysis to the main manuscript but will do so if the reviewer feels it is essential. Consistent with this, NK cells in Trained+PC mice showed an activated phenotype, with high levels of *CCL5* (Supplementary Figure 17 B&C) and perforin expression (Extended Figure 16 C).



Response to reviewers Figure 1: Expression levels of PD1 (Pdc1), TIGIT(Tigit) and TIM-3 (Havcr2) across all clusters between Trained+PC and PC treated mice.

4. In the section on human studies, NK cells are not included. This does not recapitulate the mouse studies and also does not align with the title, which refers to “...enhancing colorectal cancer vaccination by inducing an NK-cDC1 axis.”

Response: We thank the reviewer for this valuable comment. In response, we have now expanded our human experiments to include both macrophages and NK cells in the PBMC/CRC organoid system. Specifically, we demonstrate that WPG-trained macrophages not only undergo epigenetic remodeling (CXCL9/CXCL10), but also drive NK cell recruitment and activation, thereby mirroring the NK-cDC1

axis observed in mice. These new results strengthen the translational link between our mouse and human findings and align with the title of the manuscript. Results for such experiments can be found in Supplementary Figure 22.

5. Figure 6 should include a tumor-only group without any treatment

Response: We thank the reviewer for this point. In our earlier experiments, we included mock (tumor-only) groups as baseline references. However, in Figure 6 our goal was different: we specifically wanted to test whether depleting or adding back immune populations could recapitulate the tumor control observed with Trained+PC. For this reason, Trained+PC was used as the reference group rather than mock, allowing us to directly assess the functional contribution of each immune subset. This design follows the 3Rs principle implemented by our animal facility to minimize the number of mice used, while still providing robust scientific comparisons. We have clarified this in the revised figure legend and methods.

Reviewer #4 (Remarks to the Author): with expertise in innate immunity

The study by Hamdan et al. explores the potential of β -glucan-induced trained immunity to enhance colorectal cancer (CRC) vaccination by modulating macrophage metabolism and promoting an NK-cDC1 axis. While the work addresses an important gap in CRC immunotherapy, its impact is limited by concerns regarding novelty, mechanistic depth, and experimental rigor. Below is a detailed critique organized by section.

Response: We thank the reviewer for their thoughtful evaluation of our study and for highlighting both its strengths and areas for improvement. We appreciate the acknowledgment that our work addresses an important gap in CRC immunotherapy by leveraging β -glucan-induced trained immunity to modulate macrophage metabolism and promote the NK-cDC1 axis.

We also recognize the reviewer's concerns regarding novelty, mechanistic depth, and experimental rigor. To address these points, we have conducted several additional experiments in response to the comments and have also discussed in detail the areas where our study provides novel insights.

Major

Critiques

1. Novelty and Overlap with Prior Studies
 - Trained Immunity in Cancer: The use of trained immunity as a cancer vaccine adjuvant has been previously demonstrated, notably by Wattenberg et al. (2023, Sci Immunol), who reported synergistic effects of β -glucan with CD40/Dectin-1 agonists in pancreatic cancer. The current study does not sufficiently differentiate its findings from this prior work.
 - NK-cDC1 Axis: The mechanistic role of NK cells in recruiting cDC1s via CCL5/XCL1 is well-established (Böttcher et al., 2018, Cell). The authors should clarify how their results extend beyond these known mechanisms.
 - Creatine Metabolism: Although the link between creatine metabolism and immune cell function is an

emerging area, Peng & Saito (2023, Front Immunol) recently reported similar findings in macrophages, raising questions about the originality of this aspect.

Response: We thank the reviewer for pointing this out. We believe that our study provides novel mechanistic insights that do not overlap with previous publications. Starting with Wattenberg et al. (Sci Immunol, 2023), they tested β -glucan in combination with a CD40L agonist as a therapy. In contrast, we used β -glucan-induced trained immunity in the context of a cancer vaccination, which employs a completely different arm of immunotherapy, namely the induction of antigen-specific T cell immunity. Moreover, no one has tested the use of trained immunity in the context of colorectal cancer (CRC). Most of the existing work has focused on pancreatic cancer, likely due to the tropism of β -glucan for the pancreas. Our study therefore uniquely demonstrates that trained immunity can overcome the immunosuppressive environment of CRC and significantly potentiate peptide-based cancer vaccines, a setting where immunotherapy has historically failed.

As for Böttcher et al. (Cell, 2018), our study differs in a fundamental way. While they described the NK–cDC1 axis in general, we show that epigenetic and metabolic rewiring of macrophages by WPG acts as the initiating event that establishes this NK–cDC1 axis in the tumor microenvironment. This has not been published before in the context of trained immunity. By integrating metabolomics, scRNA-seq, adoptive transfers, and transwell assays, we provide mechanistic evidence that trained macrophages produce CXCL9/CXCL10 through H3K4me3-driven regulation, recruit NK cells, and subsequently license NK cells to secrete CCL5/4, thereby promoting cDC1 infiltration. This work therefore highlights how WPG can reprogram an immunosuppressive TME into a pro-inflammatory network via the macrophage–NK–cDC1 axis.

As for Peng & Saito (Front Immunol, 2023), while they showed that macrophages utilize creatine, the underlying mechanism was not known. We demonstrate for the first time that creatine specifically supports CXCL9 and CXCL10 production in trained macrophages, which is critical for NK cell recruitment and the amplification of adaptive immunity. Importantly, our ChIP-qPCR showed that creatine metabolism itself is not epigenetically imprinted, further clarifying its mechanistic role. Moreover, in the field of metabolomics, most studies of trained immunity focus on glycolysis, whereas no study has previously dissected the creatine pathway in trained immunity.

We have cited all these papers in our revised manuscript and clarified how they support—but do not overlap with—our findings. We have further emphasized this in our manuscript. Our work therefore builds on these studies while providing several novel insights, including (i) the use of trained immunity to enhance CRC vaccination, (ii) the identification of macrophage-driven epigenetic and metabolic rewiring as the upstream trigger of the NK–cDC1 axis, and (iii) the demonstration of creatine metabolism as a critical but non-epigenetically imprinted regulator of cytokine production in trained macrophages.

2.	Mechanistic	Gaps
- Epigenetic vs. Metabolic Contributions: The assertion that trained immunity is driven by "epigenetic and metabolic rewiring" lacks robust support. While Chil3+ Ly6Chi macrophages are implicated in the NK–cDC1 axis, the specific molecular mechanisms (e.g., how H3K4me3 regulates CXCL9/10 or creatine metabolism) remain unclear. Functional validation (e.g., ChIP-seq, CRISPR-edited models) is needed to strengthen these claims.		

Review: We thank the reviewer for raising this important point. To address the mechanistic contribution of epigenetic versus metabolic rewiring, we performed targeted ChIP-qPCR analyses on tumor-derived macrophages and NK cells.

In macrophages, ChIP-qPCR for H3K4me3 revealed a significant enrichment at the CXCL9 and CXCL10 loci, demonstrating that β -glucan training directly imprints these chemokines through histone modifications. This supports our conclusion that epigenetic rewiring of macrophages regulates the NK–cDC1 axis. Results for such experiments can be found Extended Figures 17A. In contrast, analysis of the creatine transporter gene (Slc6a8) showed no H3K4me3 enrichment in trained macrophages, although RT-qPCR revealed increased transcriptional expression. Results for such experiments can be found Extended Figures 18 E&F. These results indicate that creatine metabolism is transcriptionally regulated but not epigenetically imprinted by canonical trained-immunity signatures, distinguishing the relative contributions of metabolic versus epigenetic mechanisms in trained immunity.

In NK cells, ChIP-qPCR did not reveal H3K4me3 enrichment at CCL5, CCL4, or Flt3 loci, suggesting that NK cells are not epigenetically reprogrammed by WPG. However, RT-qPCR of NK cells isolated from the TME of Trained+PC mice showed increased CCL5 expression, consistent with their functional activation within the trained tumor microenvironment. Results for such experiments can be found Extended Figures 17 B&C

Together, these experiments provide direct mechanistic evidence that macrophages are the epigenetically trained population driving CXCL9/10 production, while NK cells are transcriptionally activated downstream in the TME. Moreover, creatine metabolism plays a supportive metabolic role but is not part of the epigenetic program.

- Causality in the NK–cDC1 Axis: Depletion experiments (Fig. 6) do not conclusively determine whether NK cells or macrophages are the primary drivers of cDC1 recruitment. Transwell assays (Fig. 6I) demonstrate macrophage-NK chemotaxis but fail to directly link this to cDC1 priming.

Response: We agree with the reviewer that depletion studies alone cannot fully establish causality in the NK–cDC1 axis. To address this, we performed a series of migration assays that dissected the sequential interactions among macrophages, NK cells, and cDC1.

1. Macrophage- NK cell migration:
Using a transwell system, we showed that macrophages isolated from Trained+PC-treated mice induced robust NK cell migration, whereas macrophages from PBS controls did not. Importantly, NK migration was significantly reduced when CXCR3 was blocked, directly implicating the CXCL9/10–CXCR3 axis as the mechanism by which trained macrophages recruit NK cells. Results for such experiments can be found in Figure 6 J&K
2. NK cell - DC migration:
We then tested whether NK cells from the TME could in turn recruit DCs. NK cells from Trained+PC mice significantly enhanced DC migration, while NK cells from PBS-treated mice did not. Blocking CCL5 abrogated this effect, demonstrating that NK cells function as intermediaries, attracting DCs via CCL5-dependent signaling. Notably, freshly isolated NK cells treated with WPG in vitro did not induce DC migration, indicating that activation within the trained TME is required. Results for such experiments can be found Figure L&M
3. Epigenetic underpinning of macrophage function:
ChIP-qPCR further showed that trained macrophages acquire H3K4me3 enrichment at the CXCL9 and CXCL10 loci, confirming an epigenetic basis for their ability to recruit NK cells. Results for such experiments can be found in Extended Figure 17A. In contrast, NK cells did not show epigenetic modifications at CCL5/CCL4 loci, consistent with their transcriptional activation downstream of macrophages. Results for such experiments can be found Extended Figures 17B

Taken together, these sequential migration studies establish a causal cascade: trained macrophages (via CXCL9/10) recruit and activate NK cells, which subsequently secrete CCL5 to recruit cDC1. This mechanistic framework directly links macrophage training to cDC1 priming through an NK cell intermediary, thereby extending the findings beyond depletion experiments alone.

- Metabolomic Data Interpretation: The observed upregulation of creatine metabolites (Fig. 3C) is correlative. Isotope tracing or genetic knockout experiments are needed to establish a causal relationship between creatine metabolism and cytokine production. Additionally, does creatine supplementation rescue the effects of β -GPA inhibition in vivo?

Response: We thank the reviewer for this insightful comment. We agree that isotope tracing or genetic knockout would provide definitive mechanistic validation, but these approaches are beyond the scope of the current study. To directly address causality, we performed both in vitro metabolic blocking and rescue assays as well as in vivo adoptive transfer experiments.

In vitro, CD11b⁺ cells trained with WPG in the presence of the creatine uptake inhibitor β -GPA showed a marked reduction in CXCL9 and CXCL10 expression. Importantly, when creatine supplementation was added back following β -GPA treatment, cytokine levels were rescued to trained control levels, demonstrating that creatine metabolism is directly required for the production of these cytokines. Results for such experiments can be found Extended Figure 10 D&E

In vivo, we performed adoptive transfers of trained macrophages into CT26-bearing mice treated with PC. These mice normally exhibited robust tumor control, with increased infiltration of NK cells and cDC1. However, when the recipient mice were also treated daily with β -GPA, tumor control was significantly impaired, and this was accompanied by a loss of NK infiltration and reduced cDC1 accumulation in the TME. Notably, macrophage infiltration itself was not affected, supporting that creatine metabolism specifically regulates the effector functions of trained macrophages rather than their survival. Results for such experiments can be found in Extended Figure 18 A-D

Together, these results show that creatine metabolism is causally linked to cytokine release (CXCL9/10), NK recruitment, and downstream cDC1 accumulation. While genome-wide metabolic tracing would strengthen this, our blockade-and-rescue assays, combined with in vivo β -GPA treatment during adoptive transfer, provide strong functional evidence that creatine metabolism is a key driver of the trained immunity phenotype.

3. Experimental Design and Rigor

- Mouse Model Limitations: The use of immunogenic models (CT26, MC38) may not fully recapitulate the immunosuppressive tumor microenvironment (TME) of human CRC. Including syngeneic models with low mutational burden (e.g., APC-mutant) would improve translational relevance. Furthermore, subcutaneous tumors do not adequately model metastatic CRC; orthotopic models would be more informative.

Response: We agree with the reviewer that highly immunogenic models such as CT26 and MC38 have limitations in fully recapitulating the immunosuppressive TME of human CRC. To address this, we extended our study to the Colon-26 model, which is considered more clinically relevant due to its immunosuppressive TME, APC mutation phenotype, and lack of response to PD-1/PD-L1 blockade. Indeed, Colon-26 tumors grew very aggressively, and mock-treated mice had to be sacrificed by day 19 due to tumor size.

Despite this hostile environment, Trained Immunity + PC vaccination significantly reduced tumor growth compared to controls. Mechanistically, this was accompanied by:

- Enhanced macrophage and NK cell infiltration within the TME,
- Increased accumulation of cDC1 in the draining lymph nodes, and
- Potent CD8⁺ T cell responses, including elevated IFN- γ and IL-2 production upon peptide stimulation, together with an expanded effector memory phenotype.

These findings demonstrate that the trained immunity–driven NK–cDC1 axis is functional even in a highly immunosuppressive, APC-mutant CRC model, highlighting the translational robustness of our approach. Results for such experiments can be found in Extended Figure 20

- Inducer Specificity: Relying solely on β -glucan (WPG) as a trained immunity inducer limits generalizability. Comparisons with other inducers (e.g., BCG, LPS) would strengthen the conclusions.

Response: We appreciate the reviewer’s suggestion. We chose to focus on β -glucan (WPG) as the trained immunity inducer for several reasons:

Clinical Relevance: β -glucans, including WPG, are already being tested in multiple clinical trials as immune modulators in oncology, making them among the most clinically advanced trained immunity inducers. Focusing on WPG ensures that our preclinical findings have the highest translational potential.

Distinct Mechanisms Compared to BCG: Prior studies have shown that WPG and BCG induce different metabolomic and epigenetic rewiring programs. Exploring both in the same study would confound mechanistic insights. Our goal here was to dissect the specific macrophage–NK–cDC1 axis elicited by WPG, which had not previously been defined in the context of cancer vaccination.

Limitations of LPS: While LPS is a potent innate immune activator, it is well known to induce endotoxin tolerance upon repeated exposure, rather than sustained trained immunity. This makes it less suitable for vaccination strategies that rely on long-lasting memory-like innate responses.

Focus and Depth Over Breadth: To provide a comprehensive mechanistic characterization, we concentrated on WPG rather than diluting the study across multiple inducers. We aimed to go beyond descriptive findings and instead connect WPG-driven metabolic/epigenetic rewiring to functional outcomes in vaccination.

For these reasons, we believe that a focused, mechanistic investigation of WPG provides the most rigorous and translationally meaningful conclusions. Comparative studies with other inducers (e.g., BCG, LPS) remain of high interest but are better suited for future work once the WPG-specific mechanisms are fully established.

- Human Data Limitations: The PDO experiments (n=3) are underpowered, and PBMC data (Fig. 8D-E) lack sufficient donor diversity. Expanding to additional patient-derived samples (e.g., PDX models) and quantifying antigen-specific T-cell responses would enhance translational relevance.

Response: We thank the reviewer for this important comment. We have addressed both aspects as follows:

PDO Experiments: We expanded our PDO analysis from n=3 to n=6 colorectal cancer patients, which provided stronger statistical support for our conclusions. In these experiments, trained PBMCs consistently

enhanced CD8⁺ T cell-mediated killing of PDOs compared to non-trained PBMCs. Results for such experiments can be found in Figure 8 I Extended Figure 21A

PBMC Donor Functional Readouts: To strengthen our PBMC data, we performed additional assays using HCT116 CRC cells as targets. PBMCs from HLA-A2⁺ donors were expanded against CEA peptide with or without WPG training. We observed significantly enhanced tumor killing in the WPG-trained PBMCs. Importantly, when monocytes/macrophages were depleted during WPG training (but reintroduced after washing), this enhanced killing was lost, highlighting the pivotal role of macrophages in mediating the trained immunity effect. Results for such experiments can be found Extended Figure 21F

Mechanistic Validation in Human Macrophages: To further connect the human data to our mechanistic findings, we showed that WPG-trained human macrophages exhibited increased glucose uptake, upregulation of CXCL9/10 through H3K4me3 modifications (ChIP-qPCR), and the ability to attract NK cells in transwell assays. Together, these results demonstrate that macrophages are central mediators of WPG-induced vaccine enhancement in human systems. Results for such experiments can be found Extended Figure 21C-E

While larger PBMC donor cohorts and PDX models remain valuable for future studies, our expanded PDO cohort and mechanistic human PBMC/macrophage data substantially strengthen the translational relevance of our findings. However, we have also written in our discussion the limitation of donor diversity, as this should be studied in the future. CD8⁺ T cell CRC antigens of other HLA types have not been fully described in literature hence why performing such experiments would be laborious and out of this time frame.

Minor

Critiques

- | | | | |
|--|-------------|-----|------------|
| 4. | Single-Cell | RNA | Sequencing |
| - The reanalysis of Geller et al. (2022, Nat Commun) (Fig. 1A-E) does not clearly advance beyond the original study. | | | |

Response: We thank the reviewer for raising this point. Our novel contribution in the re-analysis of the Geller et al. dataset was to specifically investigate clusters 7, 11, and 12, which we identified as *Chil3*⁺ *Ly6C* macrophages and monocytes. We then performed differential expression analysis in these populations, which was not studied in the original work. Importantly, our analysis placed emphasis on antigen presentation pathways and functional reprogramming of these myeloid subsets, while the Geller study primarily focused on CCR2-driven influx of monocytes and macrophages. We have now clarified these aspects in the results section to highlight the novelty of our approach.

- | | |
|---|--------------|
| 5. | Metabolomics |
| - Pathway impact analysis (Fig. 3E-F) does not adjust for multiple testing, increasing the risk of false positives. | |

Response: We thank the reviewer for this important comment. In our pathway analysis, enrichment statistics were generated using MetaboAnalyst 6.0 with false discovery rate (FDR) correction applied to p-values, thereby reducing the risk of false positives in the enrichment component of the analysis. The pathway impact score, by contrast, is not a p-value but a topological metric based on metabolite centrality within a pathway. As such, it cannot be adjusted for multiple testing and should be interpreted as a descriptive measure of pathway relevance rather than as a formal test of significance. To address this concern, we have clarified this point in the revised manuscript and emphasized that our conclusions are based on both FDR-

adjusted enrichment statistics and independent functional validation experiments (e.g., Seahorse assays and creatine blockade/rescue experiments), which confirm the metabolic rewiring observed in the pathway analysis.

6. Methods Concerns
- Fig. 1F-I: The SIINFEKL model is not CRC-relevant; justification for using ovalbumin-derived peptides is lacking.

Response: We thank the reviewer for this point. We used the SIINFEKL/OVA system as a preliminary proof-of-concept to test whether trained immunity could enhance vaccine responses in a highly controlled setting. SIINFEKL is a well-characterized immunogenic epitope with established reagents (e.g., MHC tetramers, TCR-transgenic mice), which makes it an ideal candidate for mechanistic testing of vaccine enhancement. We agree that it is not CRC-specific, which is why all subsequent experiments were performed with CRC-relevant antigens and models. This clarification has been added to the manuscript.

- Fig. 2L-N: A comparison with checkpoint inhibitors (e.g., anti-PD-1) is absent, despite their clinical relevance.

Response: Our study was specifically designed to investigate whether trained immunity can enhance vaccination efficacy, and checkpoint inhibitors were outside of the scope. Nonetheless, we note that this has been investigated in prior studies (PMID: 35140221), which demonstrated interactions between β -glucan and PD-1/PD-L1 blockade. To avoid redundancy, we focused our efforts on defining the mechanistic contribution of trained immunity in vaccination.

- Fig. 7 (MC38 Model): Data on tumor-infiltrating T cells and checkpoint markers (e.g., PD-L1) are missing.

Response: We agree and have expanded our analysis of tumor-infiltrating lymphocytes. Specifically, we assessed CD8⁺ T cell infiltration and memory phenotypes in the TME. Trained+PC-treated mice showed both increased T cell infiltration and a higher proportion of effector memory CD8⁺ T cells compared to controls. Results for such experiments can be found in Extended Figure 16G.

- WPG Dosing: The β -glucan dose (1 mg/mouse) lacks human-equivalent translation, raising translational concerns.

Response: We appreciate this important translational concern. The 1 mg/mouse dose of whole β -glucan (WPG) was selected based on multiple prior in vivo studies where this dose was shown to be both safe and immunologically active in murine models of infection and cancer (e.g., PMID: 25258083, PMID: 29328908, PMID: 35140221). Importantly, β -glucans have been tested in humans at doses equivalent or higher on a mg/kg basis and have demonstrated safety and immunostimulatory activity in clinical trials. Therefore, while interspecies scaling must always be interpreted with caution, the 1 mg/mouse dose used here is consistent with the literature and compatible with translational use. We have added a note on this in the revised methods/discussion. In multiple human clinical trials, Imprime PGG (a soluble yeast β -glucan) has been administered at 4 mg/kg IV weekly (in e.g. Phase Ib/II studies) often in combination with other therapies (PMID: 26975418). In safety/dose-escalation studies in healthy volunteers, comprehensive dosing from 0.5 up to 6 mg/kg has also been explored, and weekly or multi-day regimens at 1-4 mg/kg were found to be well tolerated (PMID: 26865390).

7. Figure Clarity
- Tumor growth curves (Figs. 2B, 2M) should include individual data points to illustrate variability.

Response: We thank the reviewer for such comment. All tumor growth experiments have individual data points. For Figure 2B they can be found in Extended Figure 4, while for Figure 2M the individual data points are in Figure 2N.

- Immunofluorescence images (Figs. 5I, 8G) need scale bars and higher resolution.

Response: We thank the reviewer for pointing this out. We have added the scale bars and enhanced the resolution.

- The scRNA-seq methods (e.g., batch correction, cluster resolution) require more detail for reproducibility.

Response: We thank the reviewer for pointing this out, we have now corrected this and added more detail to our methods section. We have added the following:

- QC thresholds (mito %, ribo %, UMI, genes per cell).
- Batch correction, PC selection, clustering resolution values.
- Clarity on how annotation was done (manual + ImmGen).

Reviewer #5 (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.

Response: We would like to thank the reviewer for their help in reviewing this manuscript.



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Helsinki, December 6, 2025

Dear Reviewer3,

We would like to sincerely thank you for the evaluation of our manuscript and for providing constructive and insightful comments. We hope that our revisions and additional experiments performed have been adequate.

Best regards,

Vincenzo Cerullo

Reviewer #3 (Remarks to the Author)

I do not think the authors have adequately addressed my previous concerns.

Answer: We thank the reviewer for their continued engagement and careful re-evaluation of our manuscript. We are sorry to hear that our previous revisions did not fully address all concerns. In this resubmission, we have carefully revisited each of the reviewer's points and substantially expanded our experimental evidence and explanations to clarify the mechanistic and conceptual aspects in question. We have now included new data and clearer justifications in the revised manuscript and detailed responses below. We hope that these additions more thoroughly address the reviewer's concerns and demonstrate our commitment to strengthening the rigor and clarity of the study.

For example, last time I questioned whether FLT3 is a mediator between NK cells and DCs. Now the authors have changed FLT3 to CCL5 but provide little or no new supporting evidence. CCL5 may be less specific because: 1) NK cells are not a major producer of it in this environment, and 2) CCL5 can recruit many types of immune cells, not just DCs.

Answer: We appreciate the reviewer's continued attention to this mechanistic point. We would like to clarify that we did not replace FLT3L with CCL5; rather, in our initial submission, we identified **FLT3L, CCL4, and CCL5** as potential NK cell-derived chemokines involved in dendritic cell (DC) recruitment. Following requests from multiple reviewers during the first revision, we performed additional experiments to determine which of these mediators were functionally relevant. Based on these results we presented in our first re-submission; we respectfully disagree that our evidence for CCL5 involvement is limited.

Our data from our first resubmission directly compared FLT3L, CCL4 and CCL5 expression. **ChIP-qPCR analyses** revealed no H3K4me3 enrichment at the *CCL4*, *CCL5*, or *FLT3L* loci in NK cells (Extended Figure 17A), indicating that these genes were not epigenetically rewired by trained immunity. However, **RT-qPCR analyses** of NK cells isolated from the tumor microenvironment (TME) of **Trained+PC** mice showed a marked upregulation of *CCL5* transcripts, whereas *FLT3L* and *CCL4* remained unchanged (Extended Figure 17B–C) compared to NK cells taken from mock mice or treated *ex-vivo* with beta-glucan. This demonstrates that NK cells selectively increase *CCL5* expression within the trained TME rather than *FLT3L*.

To test the functional significance of this finding, we performed **transwell migration assays**. NK cells from **Trained+PC** tumors significantly enhanced DC migration compared to NK cells from mock-treated mice, and this effect was completely abolished by **CCL5 neutralization**, confirming the requirement of the **CCL5–CCR5 axis** in NK-mediated DC recruitment (Figure 6L–M).

Together with previous reports identifying NK cell–derived CCL5 as a selective recruiter of cDC1s (PMID: 33613552; PMID: 29429633; DOI: 10.1158/2159-8290.CD-RW2018-030) and demonstrating that activated NK cells strongly express CCL5 (PMID: 33613552; PMID: 19124744), our data provide robust mechanistic support that CCL5 is the key mediator driving NK–DC communication in our model. Moreover, FLT3/FLT3L has been more implicated in survival and differentiation of DCs.

We have repeated the transwell assays but rather than blocking CCL5 we have blocked FLT3L. Blocking FLT3L did not affect the migration of DCs by NK cells taken from the tumor microenvironment of **Trained+PC** mice. This data can be seen in Supplementary figure 18A.

Another example: I questioned, “How long does trained immunity last?” The authors mentioned “several weeks” but did not cite any figure or specify the exact duration. Moreover, claiming “several weeks” may not be sufficient to support trained immunity.

Answer: We thank the reviewer for this important and constructive comment and sincerely apologize for not specifying an exact timeframe and experimental evidence in our previous response. We have carefully revisited our data to provide a clearer explanation.

In our study, we observed that trained immunity–associated effects persisted up to day 23 post- β -glucan administration, as shown in Figure 2L. At this time point, mice treated with β -glucan (WPG) and PeptiCRAAd (PC) maintained robust tumor control, and immune profiling confirmed ongoing trained immunity–related phenotypes. Specifically, ChIP-qPCR analyses demonstrated sustained H3K4me3 enrichment at the promoters of IL6, TNF- α , CXCL9, and CXCL10 — hallmark genes of the trained immunity response. These modifications indicate continued epigenetic reprogramming well beyond the initial training phase.

In parallel, metabolomic analyses revealed a persistent Warburg-like metabolic state, consistent with enhanced glycolytic activity that has been widely described in β -glucan–trained macrophages (PMID: 30166439; 29328908). Together, these molecular findings provide strong evidence that the trained phenotype remains functionally active for 23–26 days after the initial β -glucan exposure.

We fully acknowledge, however, that our experiments were limited to a 23–26 day observation window due to ethical constraints related to tumor burden, leading to animal sacrifice. As such, we cannot definitively determine the full duration of trained immunity beyond this point. We agree with the reviewer that longitudinal experiments, such as tumor rechallenge or late vaccination models, are essential next steps to quantify the durability of β -glucan–induced training in vivo.

Can further clarify this point in the revised Discussion, emphasizing that while our mechanistic and metabolic data strongly support sustained trained immunity during the tumor experiments, future studies will be needed to determine how long these effects persist beyond the timeframe tested.

Additionally, when I asked, “Does trained immunity induce exhaustion in immune cells, such as NK cells?” the authors only reanalyzed their scRNA-seq dataset. Why did they not assess this by flow cytometry? The authors stated they did not want to present additional data in the manuscript, but the reason for this decision is unclear.

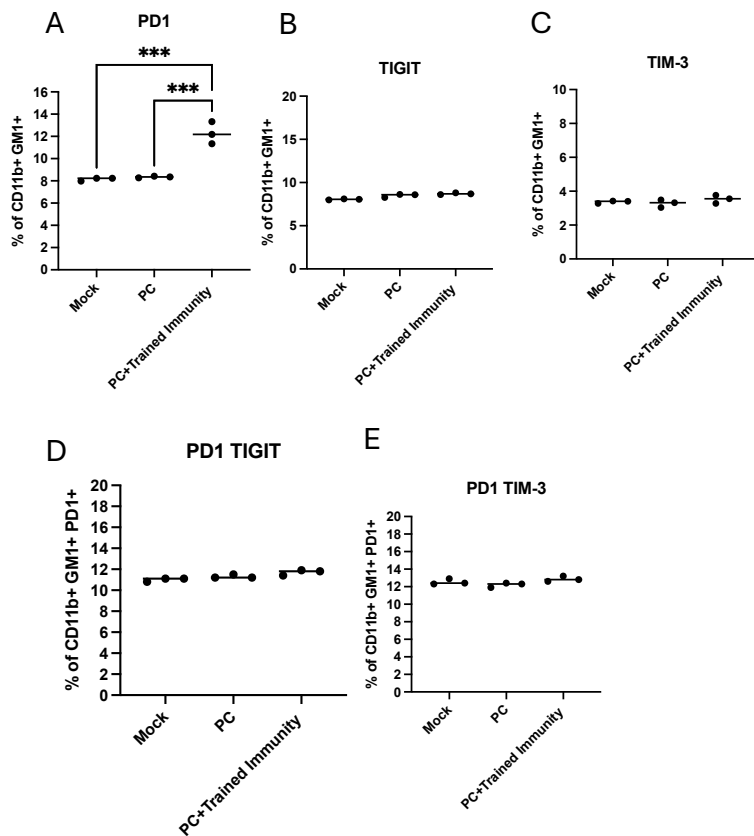
Answer: We thank the reviewer for this insightful comment and apologize for not sufficiently addressing it in our previous response. To further validate our scRNA-seq findings and directly assess whether trained immunity induces immune cell exhaustion, we performed flow cytometry analysis on tumors collected at day 23 from mice treated with PBS (Mock), PC, or Trained+PC (Figure 2L).

Flow cytometric assessment of NK cells from the tumor microenvironment (TME) revealed that PD-1 expression was significantly increased in Trained+PC mice compared to Mock and PC groups (Reviewer Figure 1A). However, there were no significant changes in TIGIT (Reviewer Figure 1B) or TIM-3 (Reviewer Figure 1C) across groups. Furthermore, within the PD-1⁺ NK-cell subset, no differences were detected in TIGIT (Reviewer Figure 1D) or TIM-3 (Reviewer Figure 1E) expression, confirming that these NK cells were not functionally exhausted.

This interpretation is consistent with our functional data: in adoptive-transfer experiments, NK cells from mice receiving trained macrophages plus PC displayed increased perforin expression (Supplementary Figure 16C), a hallmark of activated and non-exhausted NK cells. Additionally, these NK cells maintained high CCL5 expression and the ability to recruit dendritic cells (DCs) in transwell assays (Figure 6L–M), further supporting their active state.

Together, these results demonstrate that trained immunity does not induce NK-cell exhaustion. Instead, NK cells from Trained+PC mice remain metabolically and functionally active, characterized by enhanced perforin production, chemokine secretion, and DC recruitment capacity—all features consistent with an activated, non-exhausted phenotype.

We also do apologize if our previous wording gave the impression that we did not wish to present additional data. At the time, we decided to omit the analysis because it did not reveal any striking or differential findings that we felt would meaningfully strengthen the manuscript and the analysis was not fully complemented with other functional assays to fully support our claim. However, we have now complemented our previous scRNA-seq analysis with new flow cytometry data, which confirms that NK cells in the Trained+PC group are not exhausted but rather active. With this new data dataset, we are confident to conclude that trained immunity does not induce NK exhaustion, and we are happy to include these results in the main manuscript. Such data can be found in Supplementary Figure 10.



Reviewer Figure 1: Frozen tumors were taken from experiment Figure 2L and re-analyzed. NK cells (CD11b+ GM-1+) were analysed for PD-1 (A), TIGIT (B) and TIM-3 (C) using flow cytometry. NK cells which were positive for PD+1 were also analyzed for TIGIT (D) and TIM-3 (E) using flow cytometry

Regarding my concern #5, I still think the tumor-only group is important. But they authors do not provide the data.

Answer: We thank the reviewer for their comment and understand the concern regarding the absence of a tumor-only control group in Figure 6. In this experiment, our goal was not to assess overall tumor growth dynamics but rather to determine whether specific depletion of macrophages and/or NK cells would abolish the trained immunity-mediated enhancement of vaccination. Therefore, we intentionally used the Trained+PC group as the internal reference (mock) to compare the effects of immune subset depletion under the same experimental and immunological context. This allowed us to isolate the contribution of each immune population (macrophages and NK cells) to the vaccine-induced protection driven by trained immunity. We therefore believe that the Trained+PC group serves as an appropriate internal control for the depletion study, as the comparison directly tests whether the removal of specific immune subsets abrogates the protective effect of trained immunity. Importantly, the mock (tumor-only) group had already been included and analyzed in our previous in vivo vaccination experiments (Figures 2B), where it consistently demonstrated the expected baseline tumor progression. To minimize animal use and comply with the 3Rs principles of animal welfare, specifically the *Reduction* guideline required by our

institutional animal facility, we did not include additional tumor-only mice in this mechanistic depletion experiment. Hence, this is why we did not provide such information since we did not include a mock group to the depletion experiments.

Moreover, to further validate our findings, we performed new adoptive transfer experiments (Figure 6F) in which mice received either trained or untrained macrophages and NK cells, followed by PC vaccination. These experiments included proper mock controls (mice receiving untrained macrophages or NK cells) and independently demonstrated that trained macrophages serve as the initiating population, leading to NK cell recruitment, CCL5 production, and subsequent cDC1 activation. These findings confirm and expand on the depletion data, providing strong mechanistic evidence for the macrophage–NK–cDC1 axis.

Nonetheless, if the reviewer still feels that the inclusion of a tumor-only group is necessary for this figure, we would be willing to clarify this design choice more explicitly in the manuscript or, if preferred, omit the animal experiment and focus on the adoptive transfer data, which fully captures the mechanism.