nature portfolio

Peer Review File

IFN-γ-driven iNOS induction in macrophages mediates CAR T cell resistance in B cell lymphoma

Corresponding Author: Dr Marco Davila

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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

Version 0:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
This is a review of the resubmission of “CAR T cell-driven induction of iNOS in tumor-associated macrophages promotes CAR T cell resistance in B cell lymphoma” by Lee, Kang et al from the group of Dr. Marco Davila.
Although I had very few comments on the previous version, I have now re-read the entire resubmitted manuscript. This remains an excellent and interesting body of work.

MINOR COMMENTS
1. The abstract may now be over-detailed with respect to mechanism, but this is more a stylistic comment than anything else.
2. Again in the abstract, I wonder whether it is even deleterious to the overall message to end with the section on blocking IFNy secretion from CART cells. This seems more like a detail to me (albeit one with potential clinical utility) that could be left in the Discussion section. Particularly since there are no in vivo data showing that blockade of IFNy secretion from CART cells improves survival of mice.
3. P.6 Lines 28-29: Thank you for agreeing to change the macrophage nomenclature. Perhaps to avoid confusion by non-macrophage cognoscenti (who will interpret “activated macrophages” as anti-tumor polarized) consider using the term “polarized” instead i.e. … “have termed these polarized macrophages as…”.
4. P.7 line 1 – While Fig 2C does indeed show reduced nucleotide incorporation which is indicative of reduced DNA replication, I think for the sake of minimizing confusion the authors might consider just referring to this as proliferation?

Reviewer #4

(Remarks to the Author)
In this manuscript, Lee and colleagues describe a mechanism of resistance to CART cell therapy. Here, authors found that iNOS-expressing immunosuppressive macrophages led to increased CART apoptosis and senescence, reduced CART function, and changes in CART metabolism. Nonresponders to CART cell therapy had higher levels of immunosuppressive TAMs within the tumor site and higher iNOS+ monocytes found in leukapheresis products. iNOS inhibition enhanced CART efficacy in preclinical models. Authors conclude that blocking IFNg secretion by CART cells would reduce iNOS production by TAMs and thereby improve clinical outcomes.

Innovation and significance are strong, as iNOS has not been extensively studied in the context of causing CART cell inhibition—previous studies have shown that it is correlated with CRS or with enhanced CART activity. Experimental methods are robust and include deep sequencing data. However, the manuscript has several weaknesses, including the near total lack of testing of human CART/macrophage interactions, the omission of any M1 macrophage assessment, small patient sample size, little data on in vivo CART efficacy after iNOS blockade, and no data on in vivo CART efficacy after IFNg blockade. Additional clarification, discussion, and expansion of the existing data would strengthen the authors’ conclusions.

Major comments:

1. The authors used all murine CART cells and macrophages in experiments throughout the manuscript except for some patient sample data shown in Fig. 1 and 8I. The choice of this all murine system for in vitro models is unclear, as human CART/macrophage interactions would seem more clinically relevant. Additionally, the strengths of a syngeneic model—an intact immune system and the interplay between endogenous immune cells and CART cells—was largely reduced by using Rag1 knockout mice (B6 mice were shown in the extended data—this should be expanded upon). Authors should repeat CART cell efficacy experiments with human CART and human macrophages to assess if these observations are applicable across species.
2. Authors should also reconcile their observations that CART cells secreted very little IFNg after imMac coculture (Fig. 2H) and that L-NIL preserved CART secretion of IFNg in cocultures with imMacs (Fig. 4C) with their hypothesis that IFNg neutralization would reduce immunosuppressive macrophage activity.
3. Authors should assess the role of iNOS in M1 macrophages with respect to CART efficacy. Fig. 5 shows that IFNg neutralization universally reduced iNOS on unMac and imMac, suggesting that it would also reduce iNOS on M1…authors should test this. Authors state in the discussion that iNOS is a marker for antitumor macrophages and has direct tumoricidal effects. Authors need to include M1 macrophages in their experiments alongside unMac and imMac (presumably M0 and M2 macrophages) to assess if inhibiting iNOS can also be detrimental to CART functions by abrogating the antitumor effects of M1 macrophages.
4. Authors should assess if IFNg neutralization enhances CART activity and reduces iNOS expression in mouse models. Fig. 8 only shows treatment with L-NIL, but this should also be assessed via the IFNg mechanism proposed by the authors. Additional data showing tumor growth curves, CART cell expansion in the peripheral blood, and serum cytokine profiles should be shown in Fig. 8. Only a survival curve is shown (Fig. 8G). The supplementary data in B6 mice shows only modest antitumor efficacy in L-NIL-treated mice (extended data Fig. 8); here, it appears that the CART cells barely have an antitumor effect at all, regardless of L-NIL treatment. Total flux appears low as well (only 1 x 105 p/s). Authors should assess different tumor models to address these issues and to prove applicability across different tumor types.
5. Authors draw a direct link between CART-secreted IFNg and macrophage-secreted iNOS. However, they should explore intermediate cytokines and iNOS production. Giavridis et al shows that IFNg secreted by CART cells induces IL6 and IL1b from macrophages, which in turn induce iNOS from macrophages. Authors should test IL6 or IL1b blockade alongside IFNg blockade to determine which step in this pathway is most efficacious in reducing iNOS and preserving CART efficacy.
Minor comments:
1. Authors should define NDR (non-durable response) more precisely—if durable responders were defined as in remission at least six months following axi-cel, were NDR defined as relapsing any time before six months? Or does NDR include initial responders who relapsed after six months?
2. What is the parent population of % macrophage as shown in Fig. 1B?
3. Authors should explain potential mechanisms behind reduced CAR expression on CART cells cocultured with imMacs (Fig. 2E-F).
4. Fig. 2, 4, 5 show CART expansion over 48 hours, which is a relatively short timeframe. Authors should repeat the assay over longer timeframes (5 or 7 days).
5. The authors switch from DR/NDR to CR/NR in Fig. 8I. CR are defined as patients in complete remission at 3 months, rather than DR defined as patients in remission at 6 months as used in Fig. 1. Authors should explain this change in definition/parameters used with the patient samples.

Version 1:

Reviewer comments:

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(Remarks to the Author)
The authors have extensively revised the manuscript and adequately addressed my questions and comments.

REVIEWER #3: *This is a review of the resubmission of "CAR T cell-driven induction of iNOS in tumor-associated macrophages promotes CAR T cell resistance in B cell lymphoma" by Lee, Kang et al from the group of Dr. Marco Davila.*

Although I had very few comments on the previous version, I have now re-read the entire resubmitted manuscript. This remains an excellent and interesting body of work.

Minor Comments

Comment #1. *The abstract may now be over-detailed with respect to mechanism, but this is more a stylistic comment than anything else.*

Response. We appreciate the suggestion from the Reviewer. -We are agreeable to making stylistic changes to the abstract and will follow the guidance of the *Nature Communications* editorial team regarding their preferred level of detail. Our changes to the abstract are as follows:

> "Chimeric antigen receptor (CAR) T cell therapies have revolutionized B cell malignancy treatment, but subsets of patients with large B cell lymphoma (LBCL) experience primary resistance or relapse after CAR T cell treatment. To uncover tumor microenvironment (TME)-induced resistance mechanisms, we examined patients' intratumoral immune infiltrates and observed that elevated levels of immunoregulatory macrophages in pre-infusion tumor biopsies are correlated with poor clinical responses. In murine models, CAR T cell-produced interferon-gamma (IFN-γ) promotes the expression of inducible nitric oxide synthase (iNOS, NOS2) in immunoregulatory macrophages, impairing CAR T cell function. Mechanistically, proteomics analysis of CAR T cells revealed that iNOS-expressing macrophages promote the upregulation of genes mediating apoptosis and cell cycle arrest, while downregulating ribosome biogenesis and protein synthesis. Furthermore, CAR T cell metabolism is compromised by the depletion of glycolytic intermediates and rewiring of the TCA cycle. Pharmacological inhibition of iNOS enhances the CAR T cell treatment efficacy in B cell tumor-bearing mice. Notably, elevated levels of iNOS+CD14+ monocytes were observed in leukaphereses of patients with non-durable response to CAR T cell therapy. These findings suggest that mitigating iNOS in tumor-associated macrophages (TAMs), potentially by modulating IFN-γ expression in CAR T cells, could improve outcomes for LBCL patients.

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Response. We agree with the Reviewer that our original wording overstated the therapeutic benefit of IFN-γ blockade. Our data specifically showed that IFN-γ deletion in CAR T cells reduced iNOS expression in macrophages *in vivo*, and targeting iNOS directly improved survival in mice. We have updated the abstract to better reflect our findings (see response to Comment #1).

Comment #3. *P.6 Lines 28-29: Thank you for agreeing to change the macrophage nomenclature. Perhaps to avoid confusion by non-macrophage cognoscenti (who will interpret "activated macrophages" as anti-tumor polarized) consider using the term "polarized" instead i.e. … "have termed these polarized macrophages as…".*

Response. We have changed P.6 lines 28-29 to define imMac cells stimulated with IL-4 and IL-10 as "polarized" macrophages instead of "activated" macrophages.

Lines 116-120: *"The BMDMs were either unpolarized (unMac), polarized with IFN-γ and LPS for M1-like macrophages, or polarized with type 2 cytokines IL-4 and IL-10 to exhibit an M2-like phenotype. We have termed these M2-like polarized macrophages as 'imMac' to emphasize their distinctive immunoregulatory activity when cocultured with CAR T cells."*

Comment #4. P.*7 line 1 – While Fig 2C does indeed show reduced nucleotide incorporation which is indicative of reduced DNA replication, I think for the sake of minimizing confusion the authors might consider just referring to this as proliferation?*

Response. For clarity we have edited our description of Fig. 2C to read:

***Lines 120-124**: "CAR T cells cocultured with imMac showed increased cell death (**Fig. 2B**) and reduced proliferation (**Fig. 2C**) compared to CAR T cells cocultured with unMac, M1-like macrophages, or without macrophages, while diminished CAR T expansion (**Fig. 2D and Extended Data Fig. 1B**) was observed in both imMac and M1-like macrophage cocultures."*

REVIEWER #4: *In this manuscript, Lee and colleagues describe a mechanism of resistance to CART cell therapy. Here, authors found that iNOS-expressing immunosuppressive macrophages led to increased CART apoptosis and senescence, reduced CART function, and changes in CART metabolism. Nonresponders to CART cell therapy had higher levels of immunosuppressive TAMs within the tumor site and higher iNOS+ monocytes found in leukapheresis products. iNOS inhibition enhanced CART efficacy in preclinical models. Authors conclude that blocking IFNg secretion by CART cells would reduce iNOS production by TAMs and thereby improve clinical outcomes.*

Innovation and significance are strong, as iNOS has not been extensively studied in the context of causing CART cell inhibition—previous studies have shown that it is correlated with CRS or with enhanced CART activity. Experimental methods are robust and include deep sequencing data. However, the manuscript has several weaknesses, including the near total lack of testing of human CART/macrophage interactions, the omission of any M1 macrophage assessment, small patient sample size, little data on in vivo CART efficacy after iNOS blockade, and no data on in vivo CART efficacy after IFNg blockade. Additional clarification, discussion, and expansion of the existing data would strengthen the authors' conclusions.

Major Comments

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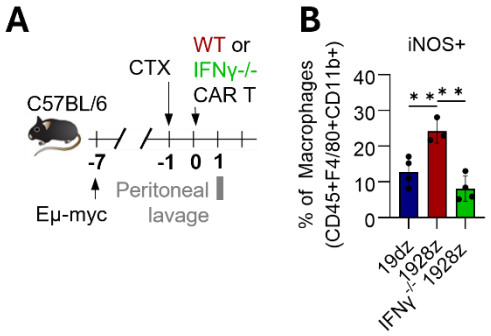
Response. We appreciate the Reviewer's insightful perspective on maximizing the clinical relevance of our findings. We agree that validating preclinical mechanisms that implicate iNOS-producing macrophages in inhibiting CAR T cell function in human systems is critical. To that end, we incorporated several lines of human data to confirm that the iNOS–CAR T suppression axis is conserved across species. First, our clinical correlative data demonstrates that the abundance of M2-like macrophages in tumor biopsies (**Fig. 1**) and elevated iNOS expression in monocytes in pre-lymphodepletion leukapheresis samples (**Fig. 8H**) are both associated with worse clinical outcomes following axi-cel treatment. Furthermore, we have already performed *in vitro* studies using human CAR T cells, cancer cells, and macrophages to validate our murine findings. Human macrophages conditioned with serum from non-responding patients significantly inhibited human CD19 CAR T cell expansion compared to unpolarized macrophages or macrophages conditioned with serum from responders, recapitulating the suppression seen in our murine imMac models (**Fig. 8I**). While we agree that additional human *in vitro* studies could provide valuable insights, they represent another model system with inherent limitations and do not always predict clinical outcomes. Ultimately, we believe the most definitive test of this hypothesis will be its translation to the clinic, which remains the primary goal of our research. Because we have established this mechanistic conservation *in vitro* and validated its clinical significance in patient cohorts, we respectfully submit that the translational relevance of this axis is well-supported, and that extensive duplication of experiments in additional human *in vitro* systems falls outside the scope of this manuscript. We have emphasized our human findings in the revised manuscript here;

Lines 322-334: *"A recent clinical study reported that iNOS in pretreatment LBCL tumors is negatively associated with clinical outcomes following axi-cel treatment [51]. These intratumoral iNOS+ cells may originate from the peripheral circulation; consistent with this, we observed that NDR patients have increased iNOS^{+} circulating CD11b^{+}CD14^{+} monocytes within pre-lymphodepletion leukaphereses (**Fig. 8H**). Upon trafficking to the tumor, these monocytes can differentiate into TAMs within the TME. The immune-suppressive landscape of the TME is likely shaped by systemic host factors, including baseline inflammation and lymphoma biology. Consistently, we found that pre-lymphodepletion serum from DLBCL patients directly modulated the immunoregulatory activity of macrophages. Specifically, conditioning macrophages with serum from non-responsive (NR) patients enhanced their suppressive capacity, dampening human CD19 CAR T cell expansion to levels comparable to our imMac model. In contrast, macrophages conditioned with serum from complete response (CR) patients resulted in CAR T cell expansion at levels similar to unMac (**Fig. 8I**)."*

We also strongly agree with the Reviewer's comment regarding the importance of immunocompetent models for understanding the interplay between endogenous immune cells and CAR T cells. Therefore, we expanded our *in vivo* data by evaluating the impact of CAR T cells with or without IFNγ on macrophages in immunocompetent C57BL/6 mice. Following intraperitoneal (i.p.) injection of Eμ-myc cells (Day -7) and lymphodepletion with 150 mg/kg cyclophosphamide (Day -1), mice received either wildtype (WT) or IFN-γ$^{-/-}$ CAR T cells i.p. (**Response Fig. 1A, Revised Manuscript Extended Data Fig. 8A**). At one day post-CAR T cell administration, WT CAR T cells significantly induced iNOS expression on endogenous peritoneal macrophages, while IFN-γ$^{-/-}$ CAR T cells did not (**Response Fig. 1B, Revised Manuscript Extended Data Fig. 8G**). These results support our model that CAR T-derived IFN-γ induces iNOS expression in macrophages, even in the presence of an intact immune system. We believe these new data provide valuable additional insights into the mechanisms at play in a physiologically relevant context.



Response Figure 1. Impact of CAR T IFNγ secretion on iNOS expression in macrophages *in vivo*. **(A)** C57BL/6 mice received intraperitoneal (i.p.) injection of 3x10^{6} Eμ-myc cells on Day -7. On Day -1, mice were lymphodepleted with 150mg/kg cyclophosphamide (CTX). On day 0, mice were i.p. injected with WT or IFN-γ-/- 1928z CAR T cells, or 19dz control CAR T cells. One day later, mice were sacrificed for peritoneal lavage analysis (n=3-4 per group). **B)** iNOS expression on peritoneal macrophages (CD45+CD11b+F4/80+) was measured by flow cytometry. Significance was determined by one-way ANOVA with Tukey's multiple comparisons correction.

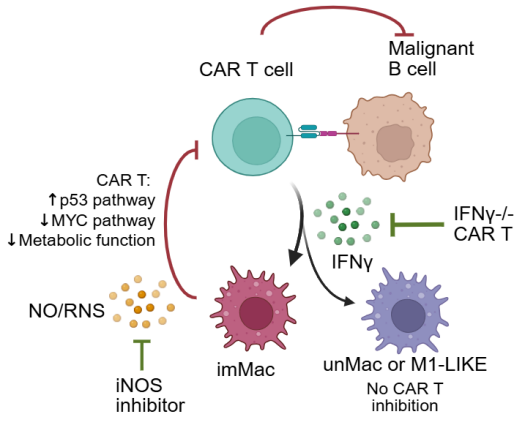
***Lines 302-310:** "To investigate the in vivo impact of therapeutic IFN-γ neutralization on CAR T cell efficacy and iNOS expression, we evaluated both IFN-γ deletion in CAR T cells or systemic IFN-γ blockade in immunocompetent C57BL/6 mice bearing Eμ-myc tumors. As expected, 1928z CAR T cells significantly inhibited tumor growth and extended survival compared to 19dz CAR T cells across all settings (**Extended Data Fig. 8A-F**). Notably, neither genetic ablation of IFN-γ in CAR T cells nor systemic anti-IFNγ treatment significantly compromised the overall anti-tumor efficacy of CAR T cell therapy. Mechanistically, IFN-γ deletion in CAR T cells but not anti-IFN-γ effectively reduced iNOS expression in macrophages (**Extended Data Fig. 8G**)."*

***Lines 401-412:** "Deletion of IFN-γ from CAR T cells, but not systemic IFN-γ blockade, inhibited iNOS induction in macrophages in vivo, suggesting that IFN-γ$^{-/-}$ CAR T cells may represent the most viable clinical strategy. However, optimization of CAR T therapy for B cell malignancies will likely require combination strategies to overcome additional immunosuppressive mechanisms present in the TME that may still limit efficacy. The clinical impact of IFN-γ-mitigating strategies will likely depend on host-specific factors such as inflammatory status and*

TME composition, as well as variables related to CAR T product and IFN-γ blockade dosing and timing. Our work supports the evaluation of targeting IFN-γ to improve clinical responses to CAR T therapy, but strategic patient selection should be guided by information derived from transcriptomic, genomic, or inflammatory status,. as we have reported 16,20,70,71. Translational efforts targeting IFN-γ to improve clinical outcomes in patients treated with CAR T cells are under clinical investigation (NCT06550141) and hold immense promise."

Comment #2. *Authors should also reconcile their observations that CART cells secreted very little IFNγ after imMac coculture (Fig. 2H) and that L-NIL preserved CART secretion of IFNγ in cocultures with imMacs (Fig. 4C) with their hypothesis that IFNg neutralization would reduce immunosuppressive macrophage activity.*

Response. We thank the Reviewer for highlighting this important point, which allows us to clarify the temporal dynamics of the mechanism we propose. The Reviewer correctly notes an apparent paradox: if neutralizing IFN-γ is protective, why do L-NIL-rescued CAR T cells secrete more IFN-γ? This is reconciled by considering the distinct temporal phases of our coculture model. Upon initial antigen encounter, CAR T cells secrete an early burst of IFN-γ that drives the upregulation of iNOS in the macrophages, converting them into a highly suppressive phenotype (**Fig. 5A-B, Fig. 8A-B**). Once induced, these iNOS+ imMac release NO, which subsequently dampens CAR T cell effector functions (**Fig. 4**) and drives the profound proteomic (**Fig. 6**) and metabolomic (**Fig. 7**) dysregulation we observed. When CAR T cells are restimulated after a 48-hour incubation with tumor cells and imMac (**Fig. 2H**), they are already dysfunctional from this negative feedback loop, and they secrete very little IFN-γ. Conversely, L-NIL treatment blocks NO-mediated suppression, maintaining CAR T cell function and allowing normal IFN-γ secretion (**Fig. 4C**). Therefore, our therapeutic strategy of neutralizing or deleting IFN-γ in CAR T cells works by interrupting this feedback loop at its initiation, preventing iNOS induction in imMac during early activation, thereby blocking downstream suppression. To improve clarity for the readers, we have added a discussion of this temporal mechanism to the revised text and included a new graphical abstract detailing this circuit (**Response Fig. 2; Revised Manuscript Extended Data Fig. 9**).

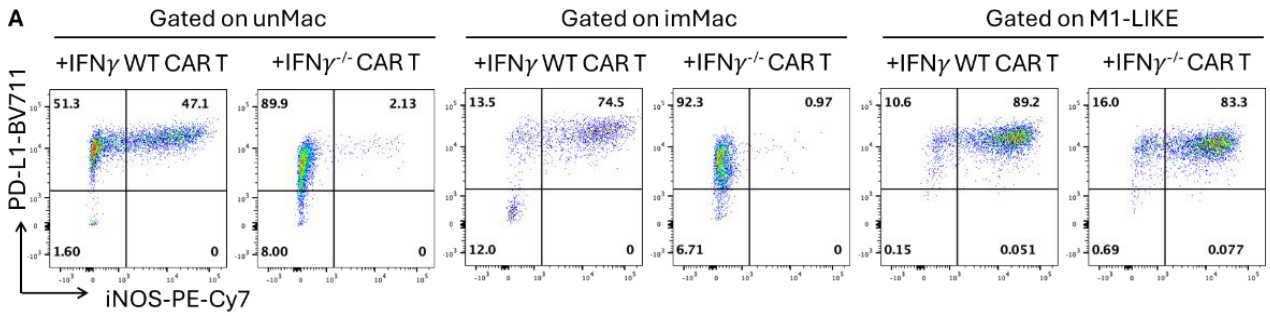


Response Figure 2. Graphical abstract. Schematic model depicting how early IFN-γ secretion by CAR T cells induces iNOS expression in immunoregulatory macrophages (imMac), leading to nitric oxide (NO) and reduced CAR T activation and metabolic dysfunction. Deletion of IFN-γ in CAR T cells disrupts this feedback loop by preventing iNOS induction in imMac, while sparing iNOS expression in pre-existing M1-like macrophages, thereby maintaining CAR T effector function. [Editorial note: Created in BioRender. Stone, M. (2026) https://BioRender.com/i9tiil0]

Lines 395-401: *In our model (**Extended Data Fig. 9**), we posit that an initial burst of IFN-γ secretion from CAR T cells drives iNOS expression in imMac, which then dampen further CAR T cell effector functions and promote proteomic and metabolomic dysfunction. Therapeutic intervention targeting IFN-γ interrupts this feedback circuit by preventing iNOS induction in imMac during the initial CAR T cell activation phase while preserving iNOS expression in pre-existing M1-like macrophages, thereby protecting the therapeutic efficacy of CAR T cell therapy.*

Comment #3. *Authors should assess the role of iNOS in M1 macrophages with respect to CART efficacy. Fig. 5 shows that IFNγ neutralization universally reduced iNOS on unMac and imMac, suggesting that it would also reduce iNOS on M1…authors should test this. Authors state in the discussion that iNOS is a marker for antitumor macrophages and has direct tumoricidal effects. Authors need to include M1 macrophages in their experiments alongside unMac and imMac (presumably M0 and M2 macrophages) to assess if inhibiting iNOS can also be detrimental to CART functions by abrogating the antitumor effects of M1 macrophages.*

Response. We thank the Reviewer for raising this critical point regarding the dual nature of iNOS and the potential unintended consequences of abrogating the antitumor effects of M1 macrophages. To address whether targeting CAR T cell-derived IFN-γ would detrimentally impact the M1 compartment, we evaluated iNOS expression across all three macrophage subsets (unMac, imMac, and M1-like macrophages) following coculture with Eμ-myc tumor cells and either wild-type (WT) or IFN-γ$^{-/-}$ CAR T cells. As anticipated, all three subtypes of macrophages expressed high levels of iNOS after coculture with WT CAR T cells (**Response Fig. 3, Revised Manuscript Extended Data Fig. 4B**). However, when cocultured with IFN-γ$^{-/-}$ CAR T cells, *de novo* iNOS induction was abrogated in unMac and imMac. In contrast, M1-like macrophages, which were pre-polarized with IFN-γ and LPS, maintained robust iNOS expression even in the absence of CAR T cell-derived IFN-γ. These new data reveal a key mechanistic distinction; deletion of CAR T cell-derived IFN-γ prevents the *de novo* induction of iNOS in naive or immunosuppressive macrophages (unMac/imMac), but it does not extinguish pre-existing iNOS expression in M1-like macrophages. Therefore, our proposed IFN-γ targeting strategy specifically mitigates TME-driven immunosuppression without compromising the baseline tumoricidal capacity of the M1 compartment.

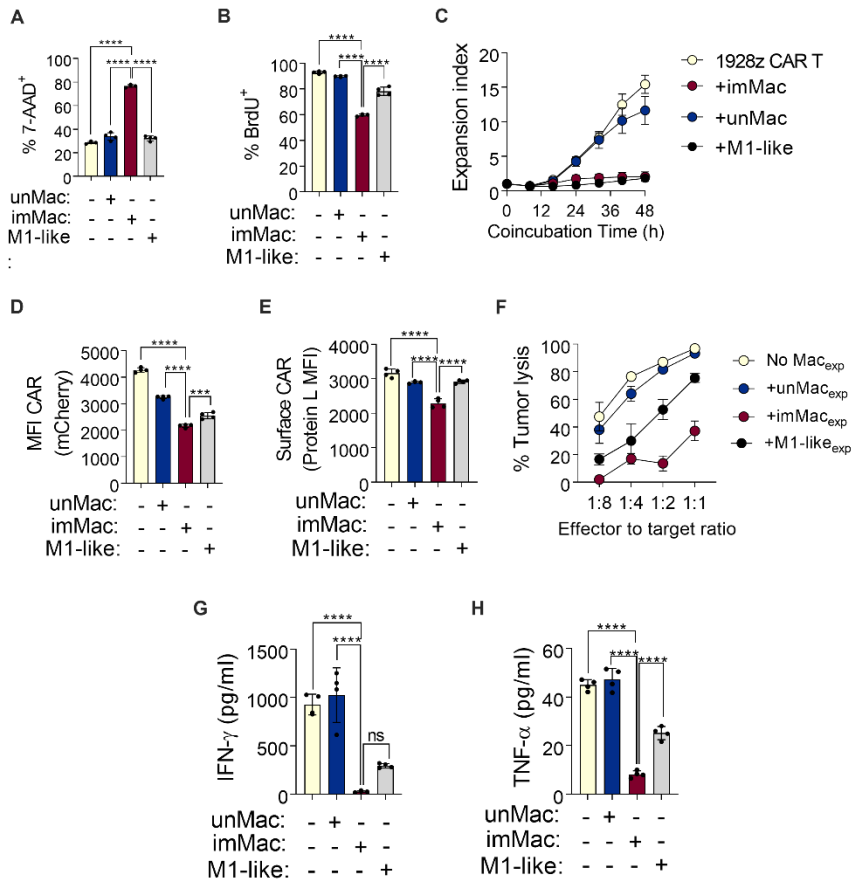


Response Figure 3. Impact of CAR T IFNγ secretion on iNOS expression in macrophage populations. (A) Wildtype (WT) or IFNγ-/- CAR T cells were cocultured with Eμ-myc tumor cells and either unMac, imMac, or M1-like macrophages. After 24 hours, iNOS expression on macrophages was measured by flow cytometry. Representative flow plots are shown.

Lines 207-210: *"Consistently, CAR T cells deficient in IFN-γ (IFN-γ$^{-/-}$ CAR T cells) neither induced iNOS expression in unMac and imMac nor induced NO production in cocultures, while iNOS expression in previously polarized M1-like macrophages was preserved (**Extended Data Fig. 4A-C**).*

Lines 215-217: *"These findings demonstrate that blocking IFN-γ production by CAR T cells mitigates the counter-regulatory, iNOS-driven inhibitory effects of imMac, while sparing the iNOS expression associated with the anti-tumor potential of M1-like macrophages."*

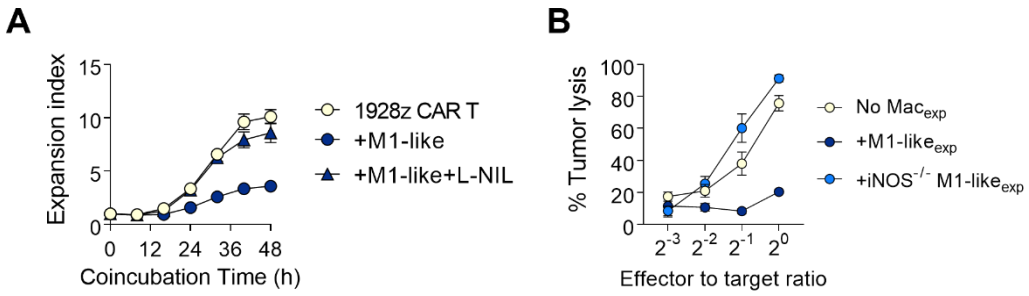
Secondly, we appreciate the Reviewer's suggestion to include iNOS+ M1-like macrophages in our experiments assessing CAR T cell function. In response, we have added M1-like macrophage data in our primary in vitro functional assays (**Response Fig. 4, Revised Manuscript Fig.2**). While M1-like macrophages did not induce CAR T cell death, inhibit proliferation, or downregulate CAR expression, they still exerted a net inhibitory effect on CAR T cell function, significantly reducing CAR T cell expansion, tumor lysis, and IFN-γ secretion. This suggests a complex but generally inhibitory interaction between M1-like macrophages and CAR T cells.



Response Figure 4. Impact of M1 macrophages on CAR T activity. CAR T cells and Eμ-myc cells were cocultured with unMac, imMac, M1-like macrophages, or without macrophages. **(A)** CAR T cell death at 48 h was assessed by 7-aminoactinomycin D (7-AAD) incorporation via flow cytometry (n=4). **(B)** DNA replication of CAR T cells at 42 h was measured by bromodeoxyuridine (BrdU) incorporation via flow cytometry (n=4). **(C)** CAR T cell expansion was measured over 48 h by a live cell analysis system (n=4). **(D)** Total CAR expression levels in CAR T cells at 48 h were assessed via flow cytometry (n=4). (**E**) Surface CAR expression levels in CAR T cells at 48 h were detected by staining single-chain variable fragments (scFv) of CAR with protein L and analyzed via flow cytometry (n=4). (**F**) Lysis of Eμ-myc cells by CAR T cells at 36 h was assessed using a bioluminescence assay (n=4). (**G and H**) Levels of IFN-γ and TNF-α in coculture supernatants of CAR T cells and Eμ-myc cells at 36 h were analyzed by an automated enzyme-linked immunosorbent assay (ELISA) (n=4).

***Lines 116-140**: "The BMDMs were either unpolarized (unMac), polarized with IFN-γ and LPS for M1-like macrophages, or polarized with type 2 cytokines IL-4 and IL-10 to exhibit an M2-like phenotype. We have termed these M2-like polarized macrophages as 'imMac' to emphasize their distinctive immunoregulatory activity when cocultured with CAR T cells. CAR T cells cocultured with imMac showed increased cell death (**Fig. 2B**), reduced proliferation (**Fig. 2C**), and diminished expansion (**Fig. 2D** and **Extended Data Fig. 1B**) compared to CAR T cells cocultured with unMac, M1-like macrophages, or without macrophages. 48 hours was chosen as a timepoint to measure CAR expansion because nutrient depletion limits CAR T cell survival over a longer timeframe (**Extended Data Fig. 1C**), while refreshing the media could disrupt CAR T-macrophage interactions. Moreover, CAR T cells cocultured with imMac showed lower total CAR expression (**Fig. 2E**) as well as reduced surface CAR expression (**Fig. 2F**) compared to CAR T cells cocultured with unMac, M1-like macrophages, or without macrophages. We next explored the impact of imMac on CAR T cell effector function. To exclude the direct contribution of macrophage effector activities in these functional assays, we first cocultured CAR T cells, Eμ-myc cells, and macrophages for 48 hours (**Fig. 2G**). Next, CAR T cells isolated from the cocultures were evaluated for their effector function against fresh Eμ-myc cells. CAR T cells derived from cocultures with imMac exhibited impaired production of effector cytokines IFN-γ and tumor necrosis factor-alpha (TNF-α) (**Fig. 2H and I**) and demonstrated decreased ability to lyse target tumor cells (**Fig. 2J**). In contrast, exposure to unMac did not impact tumor lytic capacity or cytokine secretion, while exposure to M1-like macrophages had a more modest inhibitory effect than imMac (**Fig. 2H-J**). Collectively, these results show that macrophages polarized towards an M2-like phenotype exert immunoregulatory actions that impair multiple aspects of CAR T cell biology, including survival, expansion, and CAR-dependent effector functions."*

We hypothesized that iNOS inhibition would mitigate these suppressive effects of M1-like macrophages on CAR T cell function. As suggested by the Reviewer, we tested the impact of pharmacological inhibition (L-NIL) and genetic deletion of iNOS on CAR T cell function during co-culture with M1-like macrophages. Coculture with M1-like macrophages suppressed CAR T cell expansion, an effect was fully reversed by L-NIL treatment (**Response Fig. 5A, Revised Manuscript Extended Data Fig. 3D)**. Similarly, M1-like macrophages decreased CAR T cell-mediated tumor cell lysis, but genetic deletion of iNOS in these macrophages restored the efficacy (**Response Fig. 5B, Revised Manuscript Extended Data Fig. 3D**). Together, these new data demonstrate that rather than being detrimental, targeting macrophage-derived iNOS comprehensively protects CAR T cells from M1-mediated suppression, underscoring iNOS as a highly promising therapeutic target to enhance CAR T cell function.



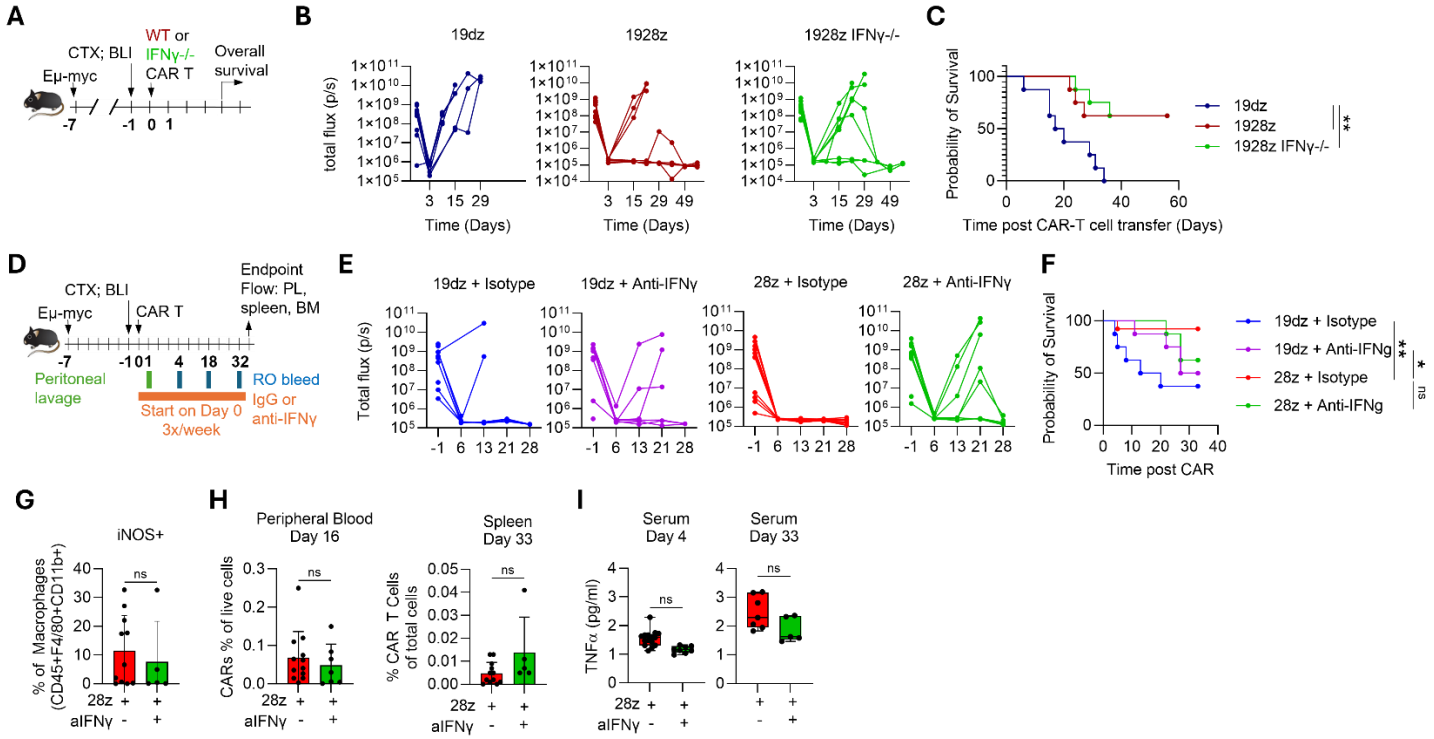
Response Figure 5. Inhibition of iNOS during coculture with macrophages rescues CAR T activity. (A) CAR T cells and Eμ-myc cells were cocultured with imMac, M1-like macrophages, or without macrophages with or without L-NIL (50mM). CAR T cell expansion was measured over 48 h by a live cell analysis system. **(B)** CAR T cells and Eμ-myc cells were cocultured with WT or INOS-/- imMac, WT or INOS-/- M1-like macrophages, or without macrophages. Lysis of Eμ-myc cells by CAR T cells at 36 h was assessed using a bioluminescence assay.

Lines 178-180: *Importantly, neither pharmacologic inhibition nor genetic ablation of iNOS in M1-like macrophages impaired CAR T cell expansion or tumor-killing capacity (**Extended Data Fig. 3D**).*

Comment #4. *Authors should assess if IFNg neutralization enhances CART activity and reduces iNOS expression in mouse models. Fig. 8 only shows treatment with L-NIL, but this should also be assessed via the IFNg mechanism proposed by the authors. Additional data showing tumor growth curves, CART cell expansion in the peripheral blood, and serum cytokine profiles should be shown in Fig. 8. Only a survival curve is shown (Fig. 8G). The supplementary data in B6 mice shows only modest antitumor efficacy in L-NIL-treated mice (extended data Fig. 8); here, it appears that the CART cells barely have an antitumor effect at all, regardless of L-NIL treatment. Total flux appears low as well (only 1 x 105 p/s). Authors should assess different tumor models to address these issues and to prove applicability across different tumor types.*

Response. We thank the Reviewer for this feedback. To address the impact of IFN-γ neutralization on CAR T cell efficacy and iNOS expression, we performed two *in vivo* experiments in immunocompetent C57BL/6 mice bearing Eμ-myc tumors: one testing genetic IFN-γ deletion in CAR T cells and one testing systemic IFN-γ blockade. We observed that in both settings, as expected, 1928z CAR T cells significantly inhibited tumor growth and extended survival compared to control 19dz CAR T cells (**Response Fig. 6A-F; Revised Manuscript Extended Data Fig. 8A-F**). Neither strategy of targeting IFN-γ compromised the anti-tumor efficacy of CAR T cell therapy. This aligns with existing literature (Bailey et al, *Blood Cancer Discovery* 2022) demonstrating that IFN-γ is not strictly essential for CAR T cell antitumor activity. To provide the detailed in vivo profiling the Reviewer requested, we have included tumor growth curves, CAR T cell frequencies in the peripheral blood and spleen, and serum TNFα profiles in the revised manuscript (**Response Fig. 6H-I; Revised Manuscript Extended Data Fig. 8H-I**). Mechanistically, genetic deletion of IFN-γ effectively reduced *in vivo* iNOS expression in macrophages, demonstrating successful disruption of the IFN-γ–iNOS axis within the TME (**Response Fig. 1; Revised Manuscript Extended Data Fig.8G**). This reduction was not observed with the anti-IFN-γ (**Response Fig. 6G; Revised Manuscript Extended Data Fig.8G**), likely reflecting the pharmacokinetic challenges of achieving sufficient neutralizing antibody concentrations within the dense tumor microenvironment. Additionally, anti-IFN-γ did not negatively impact CAR T cell frequency in the peripheral blood or spleen, or circulating TNFα levels (**Response Fig. 6H-I**). Therefore, we posit that therapeutically disrupting the IFN-γ/iNOS axis via genetic

deletion (IFN-γ-/- CAR T cells) represents the most viable clinical strategy. Our data support the clinical investigation of blocking IFN-γ to improve CAR T cell clinical outcomes, which is in development by us and already initiated by others (ClinicalTrials.Gov ID NCT06550141). The lack of a synergistic therapeutic response with targeting IFN-γ suggests that redundant immunosuppressive mechanisms remain at play in our immune competent models. Tumor intrinsic factors and other suppressive TME populations, such as regulatory T cells or myeloid derived suppressive cells, may present additional barriers. Future optimization of CAR T therapy in B cell malignancies may therefore require combinatorial strategies, for example, armoring CAR T cells with stimulatory cytokines, to fully overcome the TME.



Response Figure 6. Impact of targeting IFNγ on iNOS expression in macrophages and CAR T efficacy *in vivo*. **(A)** C57BL/6 mice received intraperitoneal (i.p.) injection of 3x10^6 Eμ-myc cells on Day -7. On Day -1, mice were lymphodepleted with 150mg/kg cyclophosphamide (CTX). On day 0, mice were i.p. injected with WT or IFNγ-/- 1928z CAR T cells, or dz control CAR T cells. **B)** Survival of the mice. Significance was determined by Mantel-Cox test. **C)** Tumor growth was monitored by weekly BLI. **(D)** Schematic for E-I. C57BL/6 mice received intraperitoneal (i.p.) injection of 3 x10^6 Eμ-myc cells on Day -7. Anti-IFNγ or control IgG (250μg/mouse, i.p.) began the same day and continued 3x/week. On Day -1, mice were lymphodepleted with 300mg/kg cyclophosphamide (CTX). On day 0, mice were i.p. injected with 5x10^6 1928z CAR T cells or dz control CAR T cells. One day later, mice were sacrificed for peritoneal lavage analysis (n=4 per group). **E)** Tumor growth was monitored by weekly BLI. **F)** Survival of the mice. Significance was determined by Mantel-Cox test. **G)** iNOS expression on peritoneal macrophages (CD45+CD11b+F4/80+) was measured by flow cytometry. Significance was determined by one-way ANOVA with Tukey's multiple comparisons correction**. H)** CAR T cells in the peripheral blood on Day 16 or spleen at endpoint (Day 33) were quantified by flow cytometric detection. **I)** Serum levels of TNFα at day 4 and day 33 were analyzed by Ella assay.

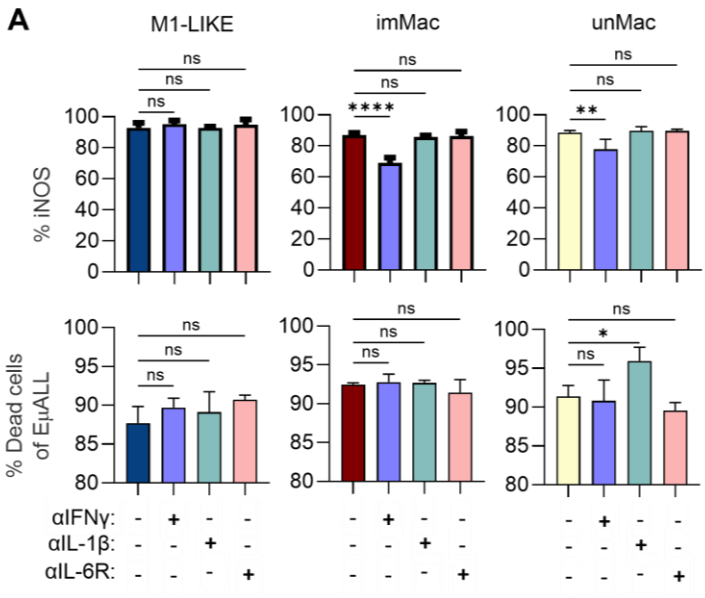
Lines 302-314: *"To investigate the in vivo impact of therapeutic IFN-γ neutralization on CAR T cell efficacy and iNOS expression, we evaluated both systemic IFN-γ blockade and IFN-γ-/- in CAR T cells or in immunocompetent C57BL/6 mice bearing Eμ-myc tumors. As expected, 1928z CAR T cells significantly inhibited tumor growth and extended survival compared to 19dz CAR T cells across all settings (**Extended Data Fig. 8A-F**). Notably, neither systemic anti-IFN-γ treatment nor genetic ablation of IFN-γ in CAR T cells significantly compromised the overall anti-tumor efficacy of CAR T cell therapy. Importantly, IFN-γ deletion in CAR T cells but not anti-IFN-γ effectively reduced iNOS expression in macrophages (**Extended Data Fig. 8G**). The failure of the blocking antibody to reduce iNOS likely reflects the pharmacokinetic challenges of achieving sufficient neutralizing concentrations within the local tumor microenvironment. Consistent with the lack of local effect, systemic anti-IFN-γ treatment did not alter CAR T cell frequencies in the peripheral blood or spleen, nor did it impact systemic TNFα levels (**Extended Data Fig. 8H-I**)."*

Lines 388-389: *"Despite the anti-tumor effects of IFN-γ, our data and work from others demonstrate that IFN-γ can be inhibited with preservation of CAR T function."*

Lines 401-412: *"Deletion of IFN-γ from CAR T cells, but not systemic IFN-γ blockade, inhibited iNOS induction in macrophages in vivo, suggesting that IFN-γ$^{-/-}$ CAR T cells may represent the most viable clinical strategy. However, optimization of CAR T therapy for B cell malignancies will likely require combination strategies to overcome additional immunosuppressive mechanisms present in the TME that may still limit efficacy. The clinical impact of IFN-γ-mitigating strategies will likely depend on host-specific factors such as inflammatory status and TME composition, as well as variables related to CAR T product and IFN-γ blockade dosing and timing. Our work supports the evaluation of targeting IFN-γ to improve clinical responses to CAR T therapy, but strategic patient selection should be guided by information derived from transcriptomic, genomic, or inflammatory status. as we have reported [16,20,71,72]. Translational efforts targeting IFN-γ to improve clinical outcomes in patients treated with CAR T cells are under clinical investigation (NCT06550141) and hold immense promise."*

Comment #5. *Authors draw a direct link between CART-secreted IFNg and macrophage-secreted iNOS. However, they should explore intermediate cytokines and iNOS production. Giavridis et al shows that IFNg secreted by CART cells induces IL6 and IL1b from macrophages, which in turn induce iNOS from macrophages. Authors should test IL6 or IL1b blockade alongside IFNg blockade to determine which step in this pathway is most efficacious in reducing iNOS and preserving CART efficacy.*

Response. We thank the Reviewer for raising this excellent point and highlighting the foundational work by Giavridis et al. regarding macrophage-mediated CAR T cell toxicities. While Giavridis et al. elegantly demonstrated that IL-6 and IL-1β serve as critical intermediaries between IFN-γ and iNOS induction during systemic Cytokine Release Syndrome (CRS), our study investigates whether this same signaling cascade drives localized immunosuppression within the tumor microenvironment (TME). We have expanded our Discussion to explicitly note this literature and the potential mechanistic overlap. To explore the Reviewer's suggestion of investigating intermediary cytokines, we performed the head-to-head comparison of IFNγ, IL-1 β, or IL-6R neutralizing antibodies in coculture with CAR T cells, tumor cells, and macrophages (unMacs, imMacs, M1-like, or none). After 24 hours, macrophage iNOS expression was measured via flow cytometry. Crucially, only anti-IFN-γ treatment significantly decreased the percentage of iNOS+ imMacs and unMacs; blockade of IL-6 or IL-1β failed to prevent iNOS induction (**Response Fig. 8; Revised Manuscript Extended Data Fig. 4E**). Furthermore, consistent with our previous data (**Response Fig. 3**), anti-IFN-γ did not significantly alter the pre-existing iNOS expression in M1-like macrophages. Together, these data definitively show that unlike the systemic CRS cascade, the localized induction of suppressive iNOS in TME-associated macrophages is directly driven by IFN-γ, independent of IL-6 and IL-1β intermediaries.



Response Figure 8. IFNγ blockade reduces iNOS expression on immunomodulatory macrophages without inhibiting CAR T cell efficacy. (A) Murine hematopoietic stem cells were differentiated into macrophage subtypes in a 7-day incubation with M-CSF alone (for unpolarized macrophages; "unMac"), with the addition of IFNγ and LPS (for M1-like macrophages), or with the addition of IL-4 and IL-10 (for M2-like, immunoregulatory macrophages; "imMac"). CAR T cells (5×10^5 cells/ml) and Eμ-myc-GFP-FFL cells (5×10^5 cells/ml) were cocultured with macrophages (1.25×10^5 cells/ml) for 24 hours. The cultures were prepared in either a 6-well plate with 2 ml of per cell type (CAR T cells, Eμ-myc cells, and macrophages), totaling 6 ml, or in a 10-cm dish with 12 ml of per cell type, totaling 36 ml. 24h post coculture, iNOS expression on adherent macrophages and dead tumor cells were quantified by flow cytometry.

Lines 211-215: *"Because IFN-γ signaling can upregulate multiple cytokines that can in turn induce iNOS in macrophages[25], we next investigated the impact of blocking IFN-γ, IL-1β, or IL-6R on iNOS expression in macrophages cocultured with CAR T cells and Eu-myc tumor cells. Only treatment with anti-IFN-γ reduced iNOS expression on imMac and unMac (**Extended Data Fig. 4E**)."*

To further contextualize the hierarchical relationship between IL-6 and IFN-γ *in vivo*, we evaluated the efficacy of anti-IL-6R versus anti-IFN-γ blockade in a murine model of CAR T cell-induced CRS and cytopenia (IL2Rα-/- mice infused with $2x10^6$ CAR T cells) (**Response Fig. 9A**). Following CAR T cell infusion, we observed a robust systemic induction of both IL-6 and IFN-γ. While anti-IL-6R treatment failed to inhibit the upregulation of either cytokine, anti-IFN-γ treatment successfully blocked the downstream upregulation of IL-6 (**Response Fig. 9B**). This definitively supports the paradigm that IL-6 acts as an intermediary cytokine downstream of IFN-γ. Furthermore, while both interventions improved survival compared to CAR T therapy alone, neutralizing the upstream IFN-γ proved significantly more effective. At 6 weeks, anti-IFN-γ treatment yielded 100% survival, compared to 60% with anti-IL-6R and only 20% in the untreated CAR T control group (**Response Fig. 9C**). These data confirm that targeting IFN-γ more effectively dampens the inflammatory cascade and improves overall outcomes compared to targeting IL-6 alone. A comprehensive detailing of this specific in vivo CRS/cytopenia model is part of a separate manuscript that we recently published, which we have now formally cited in the revised text (Goala et al, *J Clin Invest*, 2025). Given this, and because the central focus of the current manuscript is the direct relationship between CAR T cell-derived IFN-γ and localized iNOS induction in tumor-associated macrophages, we respectfully submit that extensive in vivo profiling of additional intermediary cytokines falls outside our current scope.

[editorial note: third party material redacted]

Lines 391-395: *"We previously determined that IFN-γ also induces cytopenias and its blockade can rescue mice from CRS and cytopenias[69], confirming pilot studies of IFN-γ blockade in patients with severe CRS[70]. Notably, blocking intermediary signaling through IL-6R fails to prevent toxicity in mice. Likewise, we now report that blocking IFN-γ, but not IL-6R or IL-1β, mitigates the suppressive induction of iNOS in imMac."*

MINOR COMMENTS

Comment #1. *Authors should define NDR (non-durable response) more precisely—if durable responders were defined as in remission at least six months following axi-cel, were NDR defined as relapsing any time before six months? Or does NDR include initial responders who relapsed after six months?*

Response. As the reviewer notes, responders were defined as patients in remission at least six months following axi-cel. Non-durable responders (NDR) were defined as relapsing any time before 6 months. In our revised manuscript we defined NDR more clearly.

Lines 422-426: "*Patients who achieved sustained remission for at least 3 months or 6 months following axi-cel infusion were classified as complete responders (CR) or durable responders (DR), respectively. Non-durable responders (NDR) were patients who either experienced lymphoma relapse or passed away due to recurrent disease within 6 months of treatment."*

Comment #2. *What is the parent population of % macrophage as shown in Fig. 1B?*

Response. The percentages shown in Fig. 1B represent the percentage of the macrophage population out of the total of all the 22 immune cell types deconvoluted by CIBERSORTx, as illustrated in Fig. 1A. We agree that this could be made clearer, and we revised the y-axis labeling accordingly to improve clarity.

Comment #3. *Authors should explain potential mechanisms behind reduced CAR expression on CART cells cocultured with imMacs (Fig. 2E-F).*

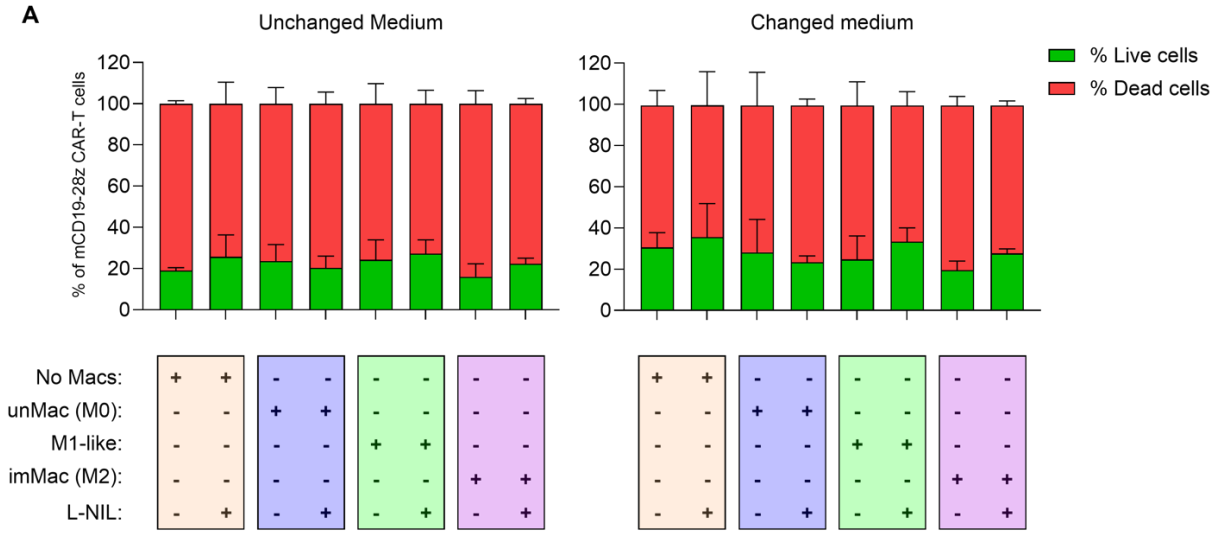
Response. We thank the Reviewer for this thoughtful question. Our proteomics data (**Fig. 6H**) provides a strong mechanistic rationale for this observation. MYC target proteins involved in ribosome biogenesis, protein translation, and amino acid transport are downregulated in CAR T cells cocultured with imMac compared to unMac or no macrophages. This downregulation may contribute to reduced CAR expression by impairing global protein synthesis machinery. We have included this hypothesis in the discussion section of our revised manuscript.

Lines 365-366: *"Furthermore, this impaired protein synthesis machinery may contribute to the observed reduction in CAR expression on CAR T cells cocultured with imMac."*

Comment #4. *Fig. 2, 4, 5 show CART expansion over 48 hours, which is a relatively short timeframe. Authors should repeat the assay over longer timeframes (5 or 7 days).*

Response. We thank the Reviewer for this practical suggestion, as evaluating long-term persistence is a critical aspect of CAR T cell biology. We deliberately focused our in vitro functional assays on the 48-hour timepoint due to the profound metabolic constraints inherent to this specific, dense coculture system. As demonstrated by our metabolomics data (**Fig. 3B-C**), these cultures rapidly experience severe nutrient depletion. While refreshing the media could extend the culture period, it would also risk physically perturbing the macrophage–CAR T cell–tumor cell interactions, potentially confounding the results. Furthermore, the inherent fragility of primary murine T cells makes prolonged in vitro culture highly susceptible to viability loss independent of the experimental variables. Therefore, we chose 48 hours as a consistent and biologically relevant timeframe to capture early functional responses while maintaining the integrity of the coculture system.

To address the Reviewer's concern, we repeated expansion experiments (Fig. 2D and Fig. 4A) out to Day 5. We evaluated the expansion of 1928z CAR T cells cultured alone (No Macs), with unMac, or with imMac, in the presence or absence of the iNOS inhibitor L-NIL. As anticipated, after 5 days, approximately 80% of CAR T cells were dead. Refreshing media after 48 hours only modestly improved survival (**Response Fig. 10**).



Response Figure 10. CAR T expansion over 5 days in coculture. (A) Anti-mCD19 CAR T cells incorporating a CD28 co-stimulatory domain were cocultured with Eμ-ALL tumor cells in the presence or absence of differentiated bone marrow–derived macrophages, including unpolarized macrophages (unMac, M0), M1-like macrophages, or immunosuppressive macrophages (imMac, M2-like), for 5 days. L-NIL was added to the culture medium at a final concentration of 50 μM in all experimental conditions. For one experimental plate, the culture medium was refreshed 48 h after initiation of the coculture, while the other plate was maintained without medium change. CAR T-cell viability was assessed by staining with Zombie NIR viability dye at the end of the coculture. Data are shown as mean ± SEM from three technical replicates per condition.

Lines 124-126: *"48 hours was chosen as a timepoint to measure CAR T cell expansion because nutrient depletion limits CAR survival over a longer timeframe (**Extended Data Fig. 1C**), while refreshing the media could disrupt CAR T-macrophage interactions."*

Comment #5. *The authors switch from DR/NDR to CR/NR in Fig. 8I. CR are defined as patients in complete remission at 3 months, rather than DR defined as patients in remission at 6 months as used in Fig. 1. Authors should explain this change in definition/parameters used with the patient samples.*

Response. Thank you for the comment. The change in response criteria comes from the patient samples being used. The initial studies (Fig. 1) using patient biopsies collected at the Moffitt Cancer Center included DR and NDR response criteria since we had long enough follow-up to make those clinical assessments. However, the addition of the new Fig. 8I included samples from Roswell Park where our follow-up was not long enough to classify all the patients in a DR or NDR so we relied on the shorter-term CR/NR response criteria. We thank the Reviewer for pointing this out and in the revision we made this clear in Methods.

Lines 422-426: "*Patients who achieved sustained remission for at least 3 months or 6 months following axi-cel infusion were classified as complete responders (CR) or durable responders (DR), respectively. Non-durable responders (NDR) were patients who either experienced lymphoma relapse or passed away due to recurrent disease within 6 months of treatment."*