Blockade or deletion of IFNγ reduces macrophage activation without compromising CAR-T function in hematologic malignancies

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Headline: IFNg is not required for CAR-T efficacy in heme malignancies

Summary: A significant toxicity of CAR T cell therapy is cytokine release syndrome. Production of IFNg by activated T cells has long been thought to be necessary for anti-tumor activity, but it is also implicated in initiating the CRS cascade and macrophage activation. Bailey et al have discovered that either blockade or genetic knockout of IFNg does not impact the anti-tumor activity of CAR T cells in hematologic malignancies. Instead, there is reduced expression of immune checkpoints on the CAR T cells and less activation of macrophages. Thus, it may be possible to separate the toxicity from the efficacy of CAR T cells, which could enable safer and yet effective use.

ABSTRACT

Chimeric antigen receptor T cells (CAR-T) induce impressive responses in patients with hematologic malignancies but can also trigger cytokine release syndrome (CRS), a systemic toxicity caused by activated CAR-T and innate immune cells. Although interferon-gamma (IFNγ) production serves as a potency assay for CAR T cells, its biologic role in conferring responses in hematologic malignancies is not established. Here we show that pharmacologic blockade or genetic knockout of IFNγ reduced immune checkpoint protein expression with no detrimental effect on anti-tumor efficacy against hematologic malignancies *in vitro* or *in vivo*. Furthermore, IFNγ blockade reduced macrophage activation to a greater extent than currently used cytokine antagonists in immune cells from healthy donors and serum from CAR-T treated lymphoma patients who developed CRS. Collectively, these data show that IFNγ is not required for CAR-T efficacy against hematologic malignancies, and blocking IFNγ could simultaneously mitigate cytokine-related toxicities while preserving persistence and anti-tumor efficacy.

STATEMENT OF SIGNIFICANCE

Blocking IFNγ in CAR T cells does not impair their cytotoxicity against hematologic tumor cells and paradoxically enhances their proliferation and reduces macrophage-mediated cytokines and chemokines associated with CRS. These findings suggest that IFNγ blockade may improve CAR T cell function while reducing treatment-related toxicity in hematologic malignancies.

INTRODUCTION

IFNγ is a cytokine produced primarily by T cells and NK cells and plays an important role in orchestrating immune responses in cancer and infectious disease. Specifically, IFNγ activates

innate immune cells (macrophages) (1,2), and upregulates both antigen-presentation pathways and immune checkpoint proteins (3-6) in immune cells and tumor cells (7). Because IFNγ production is easily measured and reliably triggered following CAR T cell engagement with antigen, measurements of IFNγ release have emerged as the de facto potency assay for CAR T cell therapeutic products, despite the lack of a clear role for IFNγ in mediating their anti-tumor efficacy in hematologic malignancies. In melanoma and other solid tumors, mutations in IFNγ receptor signaling have been identified as a mechanism of resistance to checkpoint blockade (8,9), and we have recently identified that IFNγ receptor signaling also confers relative resistance to CAR T cell mediated cytotoxicity only in solid tumors(10). In contrast, IFNγ produced by CAR T cells has a clear role in mediating cytokine release syndrome and macrophage activation syndrome in hematologic malignancies. Patients suffering from CAR-T-associated toxicities, such as CRS and immune-effector cell associated neurotoxicity syndrome (ICANS), have elevated levels of IFNγ (11-14), similar to patients with underlying rheumatologic or malignant disease that trigger macrophage activation syndrome, or genetic diseases that trigger hemophagocytic lymphohistiocytosis (HLH) (12,15,16). Pharmacologic blockade of IFNγ using emapalumab-lzsg has recently been approved for the management of primary (genetic) HLH but has not yet been systematically tested with CAR-T cell induced CRS or HLH, primarily due to concerns about potentially abrogating the anti-tumor effects; however, a recent case demonstrates that IFNγ antibody blockade may alleviate CRS without impacting anti-tumor efficacy(17).

We hypothesized that IFNγ is a by-product of CAR T cell activation in hematologic malignancies that does not have direct anti-tumor effects but contributes to macrophage

activation, which is associated with CAR T cell toxicities. We sought to test this hypothesis using pharmacologic and genetic approaches to block or delete IFNγ in CAR-T cells and probe the downstream effects in established and new models of both anti-tumor efficacy and toxicity, respectively. These models leveraged both xenograft models of hematologic malignancies and serum samples derived from patients who went on to develop clinically significant cytokine-related toxicities.

RESULTS

IFNγ blockade does not affect CAR-T subsets or function

To define the role of IFNγ in CAR-T function, we leveraged pharmacologic and genetic approaches to block or delete IFNγ in co-cultures of CD19-directed CAR T cells and CD19-positive leukemia and lymphoma tumor cells. Primary human T cells derived from healthy donors were transduced with a lentiviral vector encoding a CD19-specific CAR bearing a 4-1BB costimulatory domain (BBζ; **Fig. 1a-c; Supplementary Fig. S1a**). Following CAR-T expansion, IFNγ was successfully and specifically targeted with a blocking antibody (αIFNγ) in a dose-dependent manner (**Fig. 1d; Supplementary Fig. S1b**). Antibody-mediated blockade of IFNγ inhibited downstream signaling in the presence of recombinant human IFNγ, as demonstrated by reduced phospho-STAT1 (pSTAT1) and maintained stable expression of interferon gamma receptor 1 (IFNγR1) on the surface of CAR-T (**Fig. 1e**) and tumor cells, including JeKo-1 (Mantle cell lymphoma; **Fig. 1f**), Nalm6 (Acute lymphoblastic leukemia) and Raji (Burkitt's lymphoma) (**Supplementary Fig. S1c,d**). As expected, IFNγ blockade alone did not affect the expression of IFNγR1 or the viability of untransduced T cells (UTD), CAR T cells or B cell tumors (**Supplementary Fig. S1e-h**). Based on these data, we chose to move forward with two

doses of IFNγ-blocking antibody offering moderate (5μg/ml; ~60%) or high (20μg/ml; ~90%) blockade of IFNγ signaling.

To genetically knock out IFNγ in CAR T cells, four IFNγ-targeted guides and a control GFP guide were purchased from the Broad Institute and assessed for their ability to knock out IFNγ in the T cell line SMZ-1 (**Supplementary Fig. S2a-c**). The two guides that deleted IFNγ production in SMZ-1 cells compared to UTD and the GFP control were incorporated into CAR constructs, but one of these was more efficient at deleting IFNγ in primary CAR T cells while maintaining production of other cytokines; therefore, this guide was chosen for further studies (**Supplementary Fig. S2d**). To generate IFNγ knockout CAR-T, healthy donor T cells were isolated, activated, and transduced with a lentiviral vector encoding the same CAR construct with the additional modification of upstream CRISPR/Cas9 guide RNA sequences for either the T cell receptor α alone (TRAC knockout; KO) or in combination with the guide for IFNγ (IFNγKO) (**Fig. 1g-i; Supplementary Fig. S2e**). Following Cas9 mRNA electroporation, KO and IFNγKO CAR-T were isolated by removing $CD3^+$ T cells from the culture (**Supplementary Fig. 2f**), and specific deletion of IFNγ production in $CD3^-$ CAR-T was confirmed by ELISA (**Fig. 1j**) and flow cytometry (**Supplementary Fig. S2g,h**). The deletion of IFNγ did not significantly alter the subsets of CAR T cells, as the two groups had similar ratios of CD4/CD8 T cells (**Fig. 1k**), with the majority being naive ($CD62L^+CD45RO^-$) and expressing type 1 ($CXCR3^+CCR4^-CCR6^-$) or type 2 ($CCR4^+CCR6^-$) chemokine receptors (**Supplementary Fig. S2i,j**). There were no significant differences in memory subsets seen in $CD4^+$ or $CD8^+$ T cells. Like antibody blockade, IFNγKO CAR-T did not alter expression of IFNγR1 on the cell surface or compromise viability following T cell activation (**Supplementary Fig.S2k,l**).

Transcriptional analysis of knockout CAR T cells following antigen stimulation through the CAR revealed strong donor-specific grouping with a weaker clustering among the KO and IFNγKO cells by principal component analyses (**Supplementary Fig. S2m,n; Supplementary NanoString sequencing data file**). As expected, *IFNG* expression was significantly reduced in IFNγKO CAR-T compared to KO (**Supplementary Fig. 2o**). Collectively, these data demonstrate that IFNγ can be effectively blocked in CAR T cells by both pharmacologic and genetic approaches without significantly altering CAR T cell subsets, viability, or transcriptional profiles.

IFNγ is not required for effective CAR-T therapy in CD19$^+$ hematologic malignancies

We next sought to determine how the loss of IFNγ would affect CAR-T lysis of tumor cells *in vitro* and *in vivo*. ELISA analysis confirmed that while IFNγ was decreased, protein levels of cytotoxic granules, such as granzyme B, were not different in antibody-blocked CAR T cells in response to antigen stimulation with CD19$^+$ cancer cells (**Fig. 2a**). Luciferase-based assays revealed that CAR T cells lysed JeKo-1, Nalm6, and Raji tumor cells in the presence of IFNγ blockade (5 and 20μg/ml) (**Fig. 2b**). Collectively, blocking IFNγ through antibody blockade had no effect on CAR-T cytotoxicity against CD19-expressing tumor cells *in vitro*.

To determine if IFNγ was required for CAR-T anti-tumor efficacy *in vivo*, JeKo-1 lymphoma cells were intravenously (IV) injected into NSG mice seven days prior to treatment with CD19-directed BBζ CAR-T in combination with intraperitoneal injections of αIFNγ blocking antibody or control IgG antibody (**Fig. 2c**). To evaluate the efficacy of the antibody blockade *in vivo*,

serum was collected, and it was confirmed that IFNγ was reduced in mice receiving αIFNγ blockade (**Fig. 2d**). All mice receiving BBζ CAR T cells, regardless of IFNγ blockade, efficiently cleared the lymphoma (**Fig. 2e,f**). Further assessment revealed a similar CAR-T engraftment level in the blood and comparable overall survival of all treated mice (**Fig. 2g,h**). A similar protocol was followed using NSG mice bearing the more aggressive Nalm6 leukemia cells and confirmed that antibody blockade of IFNγ did not impact CAR-T persistence or efficacy *in vivo* (**Fig. 2i-n**). Interestingly, Nalm6-bearing mice that received anti-IFNγ blocking antibody displayed significantly greater survival compared to mice received BBζ + IgG or BBζ alone.

We next sought to determine how the genetic deletion of IFNγ affected CAR-T therapy of $CD19^+$ malignancies. Like the blocking antibodies, IFNγKO CAR-T had reduced production of IFNγ but maintained Granzyme B expression and effectively lysed tumor cells *in vitro* (**Fig. 3a,b**). Tumor-bearing mice treated with IFNγKO CAR-T exhibited reduced serum IFNγ levels but similar tumor clearance, engraftment, and survival as KO CAR-T-treated mice using both lymphoma (**Fig. 3c-h**) and leukemia (**Fig. 3i-n**) mouse models. Given that CD19-specific CAR T cells utilizing a CD28 costimulatory domain (28ζ) can also produce IFNγ and mediate toxicities and responses in patients, we next assessed whether IFNγ is required for tumor clearance by 28ζ CAR-T. We generated KO and IFNγKO CAR-T with a CD28 costimulatory domain (**Supplementary Fig. S3a**) and assessed their cytotoxic capacity against Nalm6 leukemia cells. Like BBζ knockout CAR-T, IFNγ KO had no impact on cytotoxicity against leukemia cells by 28ζ CAR-T *in vitro* (**Supplementary Fig. S3b**), and other than having reduced levels of IFNγ in serum (**Fig. 3o,p**) did not behave differently than IFNγ-replete CAR T *in vivo* (**Fig. 3q**). These

data confirm that IFNγ production by CAR T cells appears to be dispensable for effective BBζ and 28ζ CAR-T function in the setting of $CD19^+$ malignancies.

Given the established role of IFNγ as a marker of T cell effector function, the finding that it is dispensable for effective cytotoxicity against $CD19^+$ tumor cells was surprising. To determine if this was isolated to tumors expressing CD19, we next generated CAR T cells specific for the B-cell maturation antigen (BCMA), which is now an established target in multiple myeloma (**Supplementary Fig. S3c**). Specific antibody-mediated blockade of IFNγ production and not other cytokines was confirmed using BCMA-BBζ CAR T cells co-cultured with the BCMA-expressing multiple myeloma cell line RPMI-8226 at various effector-to-target ratios (**Supplementary Fig. S3d**). As with leukemia and lymphoma tumors, loss of IFNγ had no impact on *in vitro* cytotoxicity against two different myeloma cell lines, either by antibody blockade (**Supplementary Fig. S3e**) or IFNγ genetic knockout (**Supplementary Fig. S3f**); these data suggest that IFNγ production by CAR-T does not have an essential role in mediating cytotoxicity against hematologic malignancies.

Although the role of IFNγ in CAR-T therapy for hematologic malignancies has not been previously reported, it is thought to play a role in solid tumors. To determine if IFNγ production by CAR T cells impacts cytotoxicity against solid tumors in similar assays, we generated KO and IFNγKO CAR-T targeting mesothelin antigen with the SS1 scFv and assessed their capacity to lyse the pancreatic adenocarcinoma cell lines BxPC-3 and Capan-2 tumor cells in overnight and real-time killing assays (**Supplementary Fig. S3g-i**). We found that pancreatic tumor lines had moderate resistance to mesothelin-targeted CAR-T in the absence of IFNγ. Similarly, we found

that antibody blockade of IFNγ dampened cytotoxicity of anti-EGFR CAR T cells against the $EGFR^+$ glioblastoma cell line U87 *in vitro* (**Supplementary Fig. S3j,k**). Collectively, these data suggest that IFNγ may have differential impacts on anti-tumor efficacy of CAR T cells in hematologic versus solid malignancies.

IFNγ knockout reduces immune checkpoint proteins and increases 28ζ CAR-T proliferation

Based on previous reports of IFNγ upregulating immune checkpoint proteins, such as PD-1 (4) and CTLA-4 (5), we next sought to evaluate how the loss of IFNγ would affect the expression profile of immune checkpoint proteins in CAR T cells. Given that innate immune cells, such as macrophages, amplify the IFNγ signal and could thereby increase its upregulation of immune checkpoint proteins, we assessed how the loss of IFNγ in CAR-T affects their phenotype in the absence and presence of macrophages. To do this, we generated KO and IFNγ KO CAR-T from healthy donors, co-cultured them with Nalm6 leukemia cells at an effector-to-target (E:T) ratio of 1:10 with or without donor-matched GMCSF-activated macrophages for five days, and assessed for immune checkpoint proteins and CAR-T proliferation. Regardless of macrophage presence, IFNγKO BBζ CAR-T had similar proliferative capacity as KO CAR-T (**Supplementary Fig. 4a**) but exhibited a slightly reduced expression of the immune checkpoint proteins Lag3, PD-1 and Tim3 as shown by lower mean intensity (**Supplementary Fig. 4b**) and frequency (**Supplementary Fig. 4c**), particularly in the $CD4^+$ subset of macrophage-treated cultures.

Based on previous preclinical and clinical data, CAR-T containing the CD28 costimulatory domain have greater antigen sensitivity and cytotoxic activity but increased inhibitory proteins and reduced persistence compared to CAR-T containing the 4-1BB costimulatory domain (18,19); therefore, we next sought to determine how 28ζ CAR-T are affected by the loss of IFNγ. In contrast to BBζ CAR-T, IFNγKO 28ζ CAR-T had significantly greater expansion compared to KO CAR-T in the absence and presence of macrophages (**Supplementary Fig. 4d**). We further found that IFNγKO $CD4^+$ and $CD8^+$ CAR-T had reduced upregulation of PD-1, Tim3, and Lag3 compared to KO CAR-T (**Supplementary Fig. 4e,f**), regardless of the presence or absence of macrophages in the culture. The percentage of Lag3, PD-1, and Tim3 expressing CAR T cells was consistently lower in the IFNγ-deficient group, though it was only statistically significant for Tim3 in $CD4^+$T cells. Altogether, these data reveal that IFNγ plays a role in the upregulation of immune checkpoint proteins on CAR T cells and suggests that IFNγ signaling restricts the expansion of CAR T cells, especially those with CD28 signaling domains.

Establishing macrophage activation models to mimic patient cytokine profiles

Given the role of IFNγ in innate immune activation (1,2) and the correlation between high IFNγ serum levels and CRS/macrophage activation syndrome (MAS) in the clinic (20,21), we next sought to determine if the absence of IFNγ could mitigate CAR-mediated macrophage activation. We have treated lymphoma patients with either tisagenlecleucel or axicabtagene ciloleucel CAR-T products and have collected toxicity data and serum samples early in their course under an IRB-approved protocol. We selected serum samples from patients who later went on to develop cytokine-related toxicities, namely CRS, MAS, or ICANS, in the first two weeks after CAR-T infusion (**Fig. 4a**). Cytokine profiles were first measured from serum samples collected 2-5 days

post-infusion, prior to treatment with tocilizumab, steroids, and/or anakinra (in all but one patient who received tocilizumab early), in order to avoid skewing of cytokine profiles after cytokine-directed interventions. Serum cytokines were graphed individually (**Supplementary Fig. 5a,b**) and by mean total (**Supplementary Fig. 5c,d**). In agreement with previous studies, patients with documented CRS and/or ICANS had elevations in IFNγ, IL-6, IL-10, IP-10, MCP-1, and MIP-1β as compared to control patients who did not experience CRS or ICANS. Of note, CXCL9 and CXCL10 (IP-10) are both driven by IFNγ, upregulated in patients with HLH and CRS (12), and are reduced following clinical administration of the IFNγ blocking antibody, emapalumab (22-24)

(CXCL9 was not included in our Luminex panel).

Our goal was to mimic the patient cytokine profile *in vitro* using T cells and monocytes/macrophages from healthy donors. CAR T cells made from these donors were co-cultured with Nalm6 tumor cells, and their supernatants, which contain cytokines produced during CAR T cell activation, were collected. Monocytes from the same donors were left untreated or differentiated into various macrophage subsets (M0, M1, M2, GMCS-activated) and exposed to donor-matched supernatant from the CAR-T:Nalm6 co-cultures. Based on comparing the cytokine profiles after supernatant exposure to our patients' serum cytokine profiles, GMCSF-activated macrophages were the closest match, with increased IL-6, IP-10, and MCP-1 production compared to baseline (**Supplementary Fig. 6a-c**). We then further developed the GMCSF macrophages as *in vitro* model of human CAR T cell induced CRS. To determine how GMCSF-activated macrophages responded to circulating cytokines from patients who had developed CAR T cell induced CRS, we added serum from the axicabtagene ciloleucel (axi-cel)

or tisagenlecleucel (tisa-cel) treated patients directly to healthy donor-derived macrophages for 48 hours and then measured the cytokine profiles by Luminex (**Fig. 4b**) (of note, the sample from the patient who had received tocilizumab prior to serum collection was excluded in this experiment, due to the effect of tocilizumab on measurements of IL-6). Macrophage cultures exposed to serum from patients who went on to develop cytokine-mediated toxicities after axi-cel or tisa-cel exhibited an elevated production of IL-1α, IL-8, IP10, MCP-1, MIP-1α, and MIP-1β (**Fig. 4c**; mean values), all of which were absent or reduced in macrophage cultures treated with control patient samples (no CRS; **Supplementary Fig. 6d**; mean values).

To build fully *in vitro* models that could recapitulate the macrophage-activation cytokine profiles from patients, we refined our GMCSF macrophage activation system to test *in vitro* CAR-T/tumor interactions into contact-dependent (CAR-T + tumor cells + macrophages) and contact-independent (CAR-T + tumor cells --> supernatant to macrophages) culture systems. We first confirmed macrophage activation in the contact-dependent model by identifying increased expression of inflammatory proteins IL-1α, IL-6, and IP-10 and T cell cytokines IFNγ, IL-8, and IL-10 compared to CAR-T and tumor cell cultures alone (**Supplementary Fig. 7a**). Contact-dependent cultures with control KO BBζ and KO 28ζ CAR-T yielded broad macrophage activation as defined by elevated levels of IFNγ, IL-6, IL-8, IL-10, IL-13, MCP-1, MIP-1α, and MIP-1β in response to both Nalm6 and JeKo-1 target cells (**Fig. 4d,e**). Due to the similarity between the two cell lines, and for simplicity, we chose to move forward with just the Nalm6 leukemia model. For the contact-independent approach, supernatant from Nalm6:KO CAR-T cultures was added to donor-matched macrophages and assessed for cytokine secretion (**Fig. 4f**). Like the first model, both BBζ and 28ζ KO CAR-T elicited strong macrophage responses, as

seen by increased production of IL-1α, IL-6, IL-8, IP-10, MCP-1, MIP-1α, and MIP-1β (in E+T->M cultures) compared to CAR-T and tumor cell cultures alone (E+T; **Fig. 4g**). Both *in vitro* approaches showed similar cytokine expression patterns to the patient samples, especially following macrophage addition. Although neither approach exactly recapitulated the profile of every patient sample, all the upregulated cytokines and chemokines identified in these models have been reported in patients with CRS. Therefore, these *in vitro* systems provide a rapid, robust, and scalable model of CAR-T cell induced macrophage activation, which we could then leverage to assess the role of IFNγ in catalyzing cytokine-related toxicities, and as well as to measure the effects of other drugs and downstream cytokine pathways.

IFNγ-deficient CAR-T reduce macrophage activation in a contact-independent manner.

Prior to determining how IFNγ augments macrophage activation, we first confirmed the generation of mature macrophages based on the upregulation of CD86 and inducible nitric oxide synthase (iNOS) (**Fig. 5a**). Next, we sought to verify that IFNγ could trigger measurable IFNγ receptor signaling in GMCSF-activated macrophages by showing increased phosphorylation of JAK1 and STAT1 in response to recombinant human IFNγ (**Fig. 5b**). To determine how the loss of IFNγ affects the contact-dependent cell culture model introduced in **Fig. 4b**, donor-matched KO or IFNγKO CAR-T were co-cultured with $CD19^+$ Nalm6 cells plus or minus donor-matched GMCSF-activated macrophages and supernatant was collected at 6, 24, 48, and 72 hours (**Supplementary Fig. 7b-e**). Cytokine analysis of BBζ and 28ζ cultures revealed that while KO and IFNγKO CAR-T had similar inflammatory profiles in the absence of macrophages (**Supplementary Fig. 7c**), macrophage-containing IFNγKO cultures exhibited decreased

activation/function as shown by lower levels of IL-1β, IL-6, IP-10, and MCP-1 (**Supplementary Fig. 7e**).

To determine if this reduction in macrophage activation was contact-dependent, supernatant from CAR-T:Nalm6 co-cultures was added to donor-matched GMCSF-activated macrophages for 48 hours, and then resulting supernatants were collected and analyzed for cytokines (**Fig. 5c**). Like our initial co-culture system, we found that macrophage activation was diminished when using supernatant from either BBζ or 28ζ IFNγKO CAR-T cultures, suggesting that this effect is not contact-dependent (**Fig. 5d,e**). To confirm that the reduced levels of cytokines/chemokines in these cultures was specifically and mechanistically due to the loss of IFNγ, we added IFNγ blocking antibody to KO CAR-T or added recombinant IFNγ to IFNγKO CAR T cultures to see if the macrophage phenotypes could be reversed simply by the deletion or addition of IFNγ. We found that manipulation of IFNγ alone was able to reverse the functional profile of macrophages in both BBζ (**Fig. 5f,g**) and 28ζ (**Fig. 5h,i**) cultures as shown by mean total expression (**Fig. 5f,h**) and fold-change (**Fig. 5g,i**), thereby confirming the dominant role of IFNγ in macrophage response to CAR-T activation.

In addition to measuring cytokine production as a functional assay of macrophage activation, we confirmed reduced activation of macrophages in response to BBζ and 28ζ IFNγKO CAR-T by measuring surface expression of the macrophage activation markers CD86/CD69 and downstream IFNγ receptor signaling pathway (pJAK1, pJAK2, pSTAT1) (**Fig. 5j,k; Supplementary Fig. 8a,b**). Furthermore, macrophages treated with IFNγKO CAR-T showed

reduced expression of the checkpoint inhibitory molecule PD-L1. Overall, these data further confirm that CAR T cells induce macrophage activation in an IFNγ-dependent manner.

Although two murine *in vivo* CRS models have been reported, these approaches are quite challenging, because of the need to start with large numbers of T cells to account for CRISPR editing and isolation of successful knockout CAR T cells (25), and because of the lack of cross-reactivity between human and mouse IFNγ (26). Therefore, we sought to establish a hybrid *in vivo/in vitro* model in which macrophage activation could be assessed. To this end, serum from Nalm6-bearing, KO or IFNγKO CAR-T-treated mice was added to donor-matched macrophages *in vitro* and then profiled by Luminex (**Fig. 6a**). At baseline, mouse serum (prior to its addition to macrophages), had slightly higher but not significant (aside from IFNγ) cytokine levels after treatment with KO CAR T cells compared to those treated with untransduced T cells (UTD), IFNγKO CAR-T, or mice with tumor alone (**Fig. 6b,c**). However, the differences in cytokine profiles among the groups were further amplified when mouse serum was added to donor-matched GMCSF-activated macrophages. In particular, the macrophage-activation associated cytokines identified in our lymphoma patients (IL-6, IP10, MCP-1, MIP-1α) were elevated when adding sera from KO CAR-T treated mice compared to IFNγKO CAR-T in both BBζ (**Fig. 6d-f**) and 28ζ (**Fig. 6g-i**) cultures. Fluorescent imaging confirmed reduced macrophage activation, IFNγ receptor signaling and PD-L1 expression in macrophages exposed to BBζ or 28ζ IFNγKO CAR-T (**Fig. 6j,k; Supplementary Fig. 8c,d**). We also noted that the absolute levels of human cytokines recovered from mouse sera were much lower than in the supernatants recovered from *in vitro* co-cultures of CAR T cells with tumor cells (as indicated by the scales in the heatmaps of **Fig. 4** compared to **Fig. 6**). However, the levels of human cytokines recovered from mouse sera

were closer in scale to the concentrations recovered from human patients. Thus, using hybrid mouse/human mixed *in vivo/in vitro* models enabled us to evaluate macrophage activation in response to CAR-T therapies and demonstrate the principal role of IFNγ in this interplay.

IFNγ blockade reduces macrophage activation to a greater extent than current clinical biologic approaches.

We next wanted to compare how targeting IFNγ in CAR T cells would compare to current clinical practices for treating CRS and macrophage activation, which are typically based on blocking IL-6R (27) (tocilizumab) or IL-1 (28) (with anakinra, an IL-1RA competitor). To this end, we leveraged our contact-independent co-culture system and used blocking antibodies against IFNγ, IL-1Rα, and IL-6R in cultures of macrophages with conditioned media from IFNγ-replete BBζ or 28ζ CAR-T co-cultured with $CD19^+$ targets (**Supplementary Fig. 9a**). Cultures treated with αIFNγ blockade showed significant reduction in IFNγ, IL-1α, IL-8, and IP-10 as well as slightly reduced levels of IL-6 and MCP-1 compared to no treatment or traditional anti-IL1Rα and IL-6R blocking strategies (**Supplementary Fig. 9b**). Although we primarily chose to focus on GMCSF-activated macrophages due to their cytokine profile, we verified that IFNγ-deplete CAR-T cultures yielded reduced macrophage activation (as measured by IL-6 production) in M0, M1, M2 and mixed macrophage populations similar to that of the GMCSF-activated subset (**Supplementary Fig. 9c**). While macrophages play a role in the development of CRS (26), it has also been reported that monocyte-derived IL-1 and IL-6 contribute to CRS and neurotoxicity (25,29). To determine how IFNγ inhibition affects monocyte function, we repeated these experiments using donor-matched monocytes and saw a reduction of IFNγ, IL-6, IP-10, and MCP-1 using both BBζ and 28ζ CAR constructs (**Supplementary Fig. 9d,e**). Overall, these

data suggest that blocking IFNγ reduces macrophage and monocyte activation at least as well as, if not more than, current clinical approaches.

Finally, we sought to determine how various blocking antibodies would affect the macrophage response to serum collected from B-cell lymphoma patients treated with tisagenlecleucel or axicabtagene ciloleucel CAR-T products (introduced in **Fig. 4**). We used these serum samples and added it to healthy donor GMCSF-activated macrophages in the absence or presence of blocking antibodies. Macrophage responses after 48 hours were assessed by measuring cytokine production and targeted RNA sequencing (**Fig. 7a**). We found that blocking IFNγ in sera from tisagenlecleucel patients led to similar macrophage responses compared to blockade of IL-1Rα (αIL1Rα) and IL-6R (αIL6R), with additional reductions in IFNγ, IL-1α, IL-6, and IP10 (**Fig. 7b; Supplementary Fig. 10a**). Serum collected from the patient who had already received three doses of tocilizumab prior to collection (orange dot) revealed increased expression of IP-10 that was only reduced by IFNγ blockade. Furthermore, all patients had increased IL-6 levels following αIL6R blockade *in vitro*. Given the likely role of IL-6 in ICANS (25,30), which can be especially elevated in patients treated with tocilizumab (31) and is simulated herein, these data highlight the potential for IFNγ blockade to manage or prevent both CRS and ICANS.

Transcriptional analysis revealed a distinct profile of differentiated genes for macrophages treated with serum from tisagenlecleucel-treated patients in the presence of IL-6R, IL-1Rα, or IFNγ blocking antibodies compared to control macrophages devoid of antibody blockade (**Supplementary Fig. 10b; Supplementary NanoString sequencing data file**). Pathway scoring in tisagenlecleucel patients showed strong clustering between no treatment/αIL6R as

well as between αIFNγ/αIL1Rα (**Fig. 7c; Supplementary Fig. 10c**), with the former group having heightened angiogenesis and ECM remodeling pathways compared to αIFNγ and αIL1Rα. While macrophages treated with patient serum plus αIL1Rα or αIFNγ blockade clustered together, these subsets maintained distinct transcriptional profiles (**Supplementary Fig. 10d**). Building on our previous findings showing that IFNγ affects immune checkpoint protein expression, αIFNγ-treated macrophages exhibited upregulation of costimulatory genes, including *DPP4* and *ICOSLG* (**Fig. 7d; Supplementary Fig. 10e,f**), which have been implicated in enhanced CAR-T therapy(32,33), and a reduction of coinhibitory genes such as *HAVCR2* and *PDCD1LG2* (**Fig. 7e; Supplementary Fig. 10g**). Similar findings occurred in cultures treated with sera from patients who received axicabtagene ciloleucel with an additional reduction of MIP-1β (**Fig. 7f-i; Supplementary Fig. 10h-n**). Collectively, these data reveal that IFNγ blockade could reduce macrophage activation and potentially mitigate major cytokine-related toxicities (CRS, MAS, and ICANS) in the clinic. Furthermore, blocking IFNγ increased costimulatory genes and reduced immune checkpoint genes to a similar or greater extent than current clinical approaches, which could enhance CAR-T function and persistence in patients, thus improving the both the efficacy and toxicity profiles of this powerful therapy.

DISCUSSION

Although IFNγ production is frequently used as a measure of antigen-specific T cell and CAR-T effector function and potency, we hypothesized and demonstrated that IFNγ production was not essential for anti-tumor efficacy in hematologic malignancies. Our findings are supported by recent data from Singh et. al., who reported that in an unbiased genome-wide CRISPR screen of Nalm6 leukemia cells, defects in programmed cell death pathways mediated resistance to CAR-T

cell mediated cytotoxicity (34), but there was no evidence for a role of IFNγR or its signaling pathway proteins in conferring resistance to CD19-directed CAR T cells. We focused on hematologic malignancies for testing our hypothesis on the role of IFNγ because these are the currently approved and successful indications for CAR T cells and where the incidence and severity of cytokine-related toxicities are clinically significant. We recognize that IFNγ may play a different role in solid tumors, based on data presented here as well as in in other emerging work from our laboratory that identified IFNγ receptor signaling as a resistance pathway in solid tumors(10).

Our data also reveal that despite being dispensable for direct cytotoxicity of CAR T cells, IFNγ plays two major roles in this context: 1) IFNγ dampens T cell proliferation, especially in CAR-T containing a CD28 costimulatory domain, and mediates upregulation of inhibitory checkpoint proteins and 2) IFNγ drives macrophage activation and subsequent production of pro-inflammatory cytokines/chemokines. Checkpoint blockade therapy has been combined with CAR-T therapy to increase CAR-T persistence with modest effects (35,36), and combinations of IL-1R and IL-6R blockades have been used to mitigate clinical toxicities driven by macrophage activation (37,38). Data herein suggests that both of these goals, of reducing immune checkpoint proteins and macrophage activation, could be simultaneously accomplished by blocking or deleting IFNγ. In this study, we found that immune checkpoint protein expression was reduced, but not absent, on both BBζ and 28ζ IFNγKO CAR-T; however, this only translated to a significantly greater *in vitro* proliferation in 28ζ CAR-T. These findings suggest that while IFNγ depletion could enhance CAR-T survival and persistence via reduced immune checkpoints, there are most likely additional factors driving the rapid growth of 28ζ CAR-T. We recognize that

clinically, reduction of immune checkpoint proteins also has potential to elicit or amplify toxicities not driven by IFNγ and its downstream effects (39). In addition, *in vivo* antibody blockade of IFNγ would be expected to affect all T cells, not just CAR-T cells, and may result in increased susceptibility to infections.

The strength of our work lies in the use of dual, non-overlapping approaches (antibody blockade and genetic deletion) to specifically examine the role of a single cytokine; the use of CAR T cells bearing both costimulatory domains that are in widespread use (4-1BB and CD28); the use of multiple specificities, target antigens (CD19, BCMA, EGFR, mesothelin), and tumor histologies (leukemia and lymphoma, multiple myeloma, glioblastoma, and pancreatic adenocarcinoma, respectively); and the development of novel, scalable, and robust models of *in vitro* and hybrid *in vivo/in vitro* macrophage activation that were optimized to mimic cytokine profiles from patient-derived samples. Although we did not test the impact of IFNγ blockade or deletion in immune competent models, recent work from the Fry lab showed that blocking IFNγ in an immune-competent mouse model of leukemia did not diminish tumor clearance in mice treated with wildtype CAR-T, supporting our findings (40). Our work focused on the interaction of CAR-produced IFNγ with macrophages and monocytes; however, IFNγ is known to interact with other cells, including stromal and endothelial cells (41), which were not tested herein. While immunocompetent mouse models would allow us to more fully test the impact of IFNγ on other hematopoietic cells and non-hematopoietic tissues, the differences in human and murine biology, in addition to the those in tumor-associated antigens and CAR constructs for these species, may not provide a true picture of the biology most relevant to human patients. For example, syngeneic models do not develop the clinical syndrome of CRS the way patients do (for

example, mice do not develop fever or measurable hypotension or cognitive dysfunction), and the lack of cross-reactivity with IFNγ/IFNγR also makes it especially difficult to study this specific axis in mice (26).

A sophisticated humanized model of CRS has been published (25), but the complexities inherent to this model have made it challenging for widespread implementation; one finding from this model was that IFNγ was one of the most upregulated cytokines that led to enhanced production of IL-1 and IL-6. Here, we focused evaluating more scalable *in vitro* and hybrid models that could best recapitulate the cytokine and chemokine profiles observed in patients as they develop CRS (i.e. IFNγ, IL-6, IP-10, MCP-1, MIP-1β) and that have been previously correlated clinically with cytokine-mediated syndromes, including CRS, MAS, and HLH (12). Based on various *in vitro* models testing co-cultures and supernatants from CAR T cells with tumors and macrophages that had been derived from monocytes through various differentiation protocols (including monocytes, M0, M1, M2, and GM-CSF-stimulated macrophages), we found that the *in vitro* culture system using supernatants from CAR T cells cultured with tumor cells, added to GM-CSF-macrophages, best recapitulated the cytokine profiles observed in patients; our hybrid model, using sera from tumor-bearing mice treated with CAR T cells, also replicated the cytokine profiles from patients when added to GM-CSF macrophages, but generally produced lower levels of IL-6 than the *in vitro* models, likely due to lower overall concentrations of human IFNγ recovered from mouse sera. Although no model perfectly recapitulates the human clinical toxicity, we are optimistic that these *in vitro* and hybrid models will prove to be useful, scalable and of interest to the field.

In vitro comparisons of IFNγ blockade or knockout in the CAR T cell product with currently used clinical strategies suggested that targeting IFNγ could mitigate major cytokine-related toxicities to a greater extent than existing approaches. Although early intervention with tocilizumab did not interfere with CAR-T efficacy in clinical trials, it also did not prevent or ameliorate neurotoxicity, and inflammatory cytokines such as IFNγ and MIP-1β remained elevated (37). A clinical trial using prophylactic administration of tocilizumab was found to reduce severe CRS but increased neurotoxicity, likely due to elevated IL-6, which tocilizumab does not reduce (38). While clinical trials are still ongoing, an early report on the use of anakinra prophylaxis in patients with multiple myeloma treated with CAR-T reported fewer grade 2 events in patients and reduced need for corticosteroids, but the overall frequency of cytokine release syndrome was similar to the non-randomized control group, though there were no grade 3 or higher events reported (42). Collectively, these studies reveal that although early/prophylactic intervention with antibodies targeting the IL-1 and IL-6 pathways do not diminish CAR-T efficacy, they may not entirely mitigate CRS or neurotoxicity. A recent study has suggested that like IFNγ, upstream activators of macrophage activation, such as GM-CSF, can be targeted to reduce CAR-mediated toxicities (43). While a direct comparison between IFNγ and GM-CSF blockade was initially planned herein, only 3 of the 12 patients in this study had detectable GM-CSF in their sera, suggesting that this cytokine may not be a key mediator of CRS in our sample of lymphoma patients.

There has been understandable hesitation to administer IFNγ blocking antibodies to patients with severe cytokine-related toxicities, especially since these are frequently reversible with existing clinical measures, and because the risk of abrogating an anti-tumor response in patients with

relapsed or refractory disease has been considered high. Furthermore, blocking antibodies to IFNγ received their first and only FDA approval in November 2018, for primary HLH, and they are still expensive and difficult to obtain for off-label uses. Our data suggests that IFNγ may not be critical for CAR-T efficacy in hematologic malignancies but could mitigate cytokine-related toxicities by reducing macrophage/monocyte activation and function, specifically IL-1α, IL-6, IP-10, and MCP-1. In agreement with our hypothesis, recent clinical work by McNerney et. al revealed that IFNγ blockade can mitigate CRS in CAR-T patients without compromising anti-tumor efficacy(17). Our data suggest that IFNγ deletion from CAR T cell products, is a specific and viable strategy that could improve CAR T cell efficacy and also prevent or mitigate cytokine-related toxicities in hematologic malignancies.

METHODS

CAR design

Four CAR constructs (two anti-CD19, one anti-BCMA, and one anti-EGFR) were synthesized and cloned into a second-generation lentiviral backbone under the regulation of a human EF-1α promoter. For CD19, one construct contained a CD8 transmembrane domain in tandem with an intracellular 4-1BB costimulatory domain and a CD3ζ signaling domain (BBζ), while the second was identical, but with a CD28 costimulatory domain replacing 4-1BB (28ζ). For BCMA and EGFR constructs, the CD8 transmembrane domain was in tandem with intracellular 4-1BB and a CD3ζ signaling domain. Eight additional constructs were created as described above but containing guide RNA sequences for TRAC (AGAGTCTCTCAGCTGGTACA) +/- IFNγ (CCAGAGCATCCAAAAGAGTG). Pilot studies were performed using multiple guide RNA sequences from the Brunello library (44) to each target, but only the most effective guides were

used for the final constructs, including: TRAC (AGAGTCTCTCAGCTGGTACA) and IFNγ (CCAGAGCATCCAAAAGAGTG). Using these guide sequences, KO and IFNγKO CAR constructs were made to target CD19 (two with 4-1BB, two with CD28), BCMA (two with 4-1BB) and mesothelin (two with 4-1BB). All constructs contained a transgene coding the fluorescent reporter, mCherry, to determine transduction.

CRISPR/Cas9 Guide Selection

The IFNγ-producing T cell line, SMZ-1, was transduced to constitutively express CBG-GFP and stable Cas9 with puromycin resistance for selection. Four IFNγ-targeted guides (G1=CCAGAGCATCCAAAAGAGTG, G2=TGAAGTAAAAGGAGACAATT, G3=TGCAGGTCAGATGTAG, G4=TTCTCTTGGCTGTTACTGC) were purchased from the Brunello library (44) (Broad Institute) and assessed for their ability to knockout IFNγ in SMZ-1 cells. A GFP guide was used as a control to verify Cas9 activity and rule out non-specific IFNγ targeting by knocking out GFP in SMZ-1 cells without affecting IFNγ production. Two of the four guides (G1 and G2) deleted IFNγ production in SMZ-1 cells compared to UTD and GFP control. These guides were incorporated into CAR constructs, and although both specifically reduced IFNγ production in the SMZ-1 cell line, only guide 1 effectively reduced IFNγ in primary CAR T cells; therefore, this guide was chosen for further studies.

CAR-T production

Human T cells were isolated from the peripheral blood of anonymous healthy donor leukapheresis product (Stem Cell Technologies) purchased from the MGH blood bank under an Institutional Review Board-approved protocol. T cells were re-suspended in R10 media (RPMI

1640 + 10% FBS + Pen/Strep) supplemented with 20IU/ml IL-2 and activated using anti-CD3/CD28 Dynabeads (Life Technologies) at a 1T:3B ratio. Twenty-four hours post-activation, T cells were transduced with one of the lentiviral vectors encoding anti-CD19, BCMA, mesothelin, or EGFR CAR constructs described above. Cells were de-beaded five days post-activation and expanded in the presence of 20IU/ml IL-2. For knockout CAR-T, T cells were re-suspended at $5e^6$/100µl in Opti-Mem (Thermo Fisher) after de-beading on day 5 and electroporated with 10µg Cas9 mRNA (TriLink) at 360V x 001ms. Three to five days later, $CD3^-$ T cells were isolated by column purification (EasySep Human APC Positive Selection Kit II; STEMCELL Technologies) or flow-based cell sorting using the BD FACSAria. Deletion of CD3 (TRAC) and IFNγ was confirmed by flow cytometry and ELISA. Transduction efficiency was determined using mCherry expression by flow cytometry. For all functional assays, CAR-T and KO CAR-T were normalized for transduction efficiency.

Mice and cell lines

NSG mice were purchased from Jackson Laboratory and bred under pathogen-free conditions at the MGH Center for Comparative Medicine. All experiments were performed according to protocols approved by the MGH Institutional Animal Care and Use Committee. The human SMZ-1, JeKo-1, Nalm6, Raji, MM.1S, RPMI-8226, BxPC-3, Capan-2, and U87 cell lines were purchased from American Type Culture Collection (ATCC). All cell lines were authenticated and regularly tested for mycoplasma. Cell lines were engineered to constitutively express click beetle green luciferase (CBG) and enhanced GFP (GFP) prior to sorting on FACSAria (BD) to obtain a ≥99% CBG-GFP^+ population. SMZ-1, JeKo-1, Nalm6, Raji, MM.1S, RPMI-8226, and BxPC-3 cell lines were cultured in R10 media (RPMI1640 + 10%FBS +

Penicillin/Streptomycin). Capan-2 and U87 cell lines were culture in D10 media (DMEM + 10%FBS + Penicillin/Streptomycin).

Blocking antibodies

IFNγ was pharmacologically blocked with 0.1-20μg/ml Purified NA/LE Mouse Anti-Human IFN-γ Clone NIB42 or control anti-IgG1 Mouse Isotype Control Clone 107.3 (BD Biosciences). Blocking and control antibodies were refreshed every 24 hours as needed. To block IL-1Rα and IL-6R, Il1rα Monoclonal Antibody (10309) (Thermo Scientific) and Tocilizumab (Selleck Chemicals) were added to cultures at 10μg/ml and 5μg/ml, respectively, and refreshed every 24 hours as needed.

ELISA

CAR T cells were activated for 6 hours using Cell Activation Cocktail without Brefeldin A (BioLegend) or at varying E:T ratios with target (Nalm6, JeKo-1, MM.1S, RPMI-8226) cells for 18 hours and supernatant was collected. Cytokine levels of IFNγ, IL-2, GM-CSF, TNF-α and Granzyme B was measured according to manufacturer's protocol using Human DuoSet ELISA kits (R&D).

Flow cytometry

The following antibodies were purchased for flow cytometry from BioLegend: CCR6-PE/Cy7 (Clone G034E3), CD3-APC (Clone OKT3), CD45RO-APC, CD62L-FITC (Clone DREG-56), CTLA-4-PE/Cy7 and CXCR3-BV421; BD Biosciences: CD4-v450 (Clone SK3), CD8-v500 (Clone SK1), Lag3-AF647 (Clone T47-530), PD-1-BV786 (Clone EH12.1) and Tim3-BV711

(Clone 7D3); R&D Systems: CCR4-AF488; Abcam: IFNγR1 (Clone EPR7866) and Thermo Scientific: DAPI. For extracellular staining, cells were stained in the dark for 20 minutes at room temperature in BD Horizon Brilliant Stain Buffer (BD Biosciences) and washed twice with FACS Buffer (PBS + 2% FBS) prior to acquisition. To measure viability, cells were left untreated or activated non-specifically through Cell Activation Cocktail (BioLegend) for 6 hours or in an antigen-specific manner using $CD19^+$ Nalm6 target cells at a 1:1 ratio for 18 hours. DAPI was added to cultures at 1:500 and analyzed immediately. Cells to be saved for analysis at later time points were fixed using Fixation Buffer (BioLegend) according to protocol. For phophoflow, cells were resuspended at $1e^6$/ml and left untreated, treated with αIFNγ (1-20μg/ml) and/or given 10ng/ml recombinant human IFNγ (BioLegend) for 20 minutes at 37°C. Cells were fixed at 1:1 ratio with BD Cytofix (BD Biosciences) for 15 minutes at room temperature. Following centrifugation, cells were resuspended in Perm Buffer III (BD Biosciences) at $2e^6$/ml, vortexed vigorously and put on ice for 30 minutes. Cells were stained with PhosphoStat1 Tyr701 (Clone 58D6; Cell Signaling) in 1% PBSA followed by anti-Rabbit IgG (H+L), F(ab')2 Fragment AF647 (Cell Signaling). All cells were run on a Fortessa X-20 (BD) and analyzed using FlowJo software.

NanoString

CAR T cells were combined with Nalm6 cells at a 1E:10T ratio for 5 days. Remaining cells were collected and CAR-T ($mCherry^+GFP^-$) were isolated using flow-based sorting on the BD FACSAria. For patient samples, serum was added to GMCSF-activated macrophages in culture in the presence or absence of blocking antibodies to IFNγ, IL-1Rα, or IL-6R for 48 hours. CAR-T from Nalm6 cultures and macrophages from patient cultures were lysed using RLT buffer to

obtain RNA and code set probes were hybridized with RNA by PCR for 18 hours at 65°C according to NanoString protocol. nCounter gene expression assays (NanoString Technologies) were performed using NanoString XT CAR-T Panel Standard + Customized PLUS panel (CAR-T) or nCounter Myeloid Innate Immunity Panel (macrophages). Hybridized RNA was loaded into nCounter MAX cartridges, run on the nCounter MAX and quantified using nSolver or nSolver Advanced software. For advanced analysis, donor/patients as covariates were considered. Normalized data was used for Log_2 scores and gene counts shown herein.

***In vitro* killing assays**

For single time-point cytotoxicity assays, CAR T cells were incubated with luciferase-expressing target cells at indicated E:T ratios for 18 hours. Remaining luciferase activity was measured using the Luciferase Assay System (Promega) with a Synergy Neo2 luminescence microplate reader (Biotek). Percent specific lysis was calculated using the following equation: % Specific lysis = [(total relative luminescence units (RLU) / target cells only RLU) x100]. For real-time cytotoxicity assays using plate-bound (via CD9) Nalm6 cells, cell index was recorded as a measure of cell impedance using the xCELLigence RTCA SP instrument (ACEA Biosciences). Graphs for % Cytolysis were calculated on the ACEA software to show percent target killing by CAR-T groups compared to tumor only.

NSG xenograft models

6–8-week-old male and female NSG mice were intravenously (IV) injected with $1e^6$ JeKo-1 or Nalm6 CBG-GFP^+ cells. Seven days later, mice were randomized and left untreated (tumor only; TO) or IV injected with $1e^6$ CAR T cells and tumor burden was measured by bioluminescence

using an Ami spectral imaging apparatus and analyzed with IDL software following intraperitoneal (IP) injection of D-Luciferin substate solution (30mg/ml; no blinding). Mice receiving IFNγ blocking antibody (InVivoMab anti-human IFNγ; Bio X Cell) or control IgG (InVivoMab mouse IgG1 isotype control; Bio X Cell) were IP injected with 12mg/kg of the appropriate solutions 1 hour prior to CAR-T injection. Antibodies were re-administered IP every 24 hours for the first 5 days and then maintained with 1 injection/week through day 21. For most experiments, mice were bled on days 3 and 14 post-CAR-T injection to monitor IFNγ expression/cytokine profiles and CAR-T persistence, respectively. In all experiments, bioluminescence was measured weekly.

Incucyte

Healthy donor-derived KO and IFNγKO CAR-T targeting CD19 were combined with Nalm6 leukemia cells at 10:1, 1:1, or 1:10 effector-to-target ratios with or without donor-matched GMCSF-activated macrophages and proliferation of $mCherry^+$ CAR T cells was assessed for five days on the Incucyte. Anti-CD19 28ζ KO and IFNγKO CAR-T were cultured at a 1:1 ratio with Nalm6 cells and tumor burden (GFP) was monitored for five days. Anti-mesothelin BBζ KO and IFNγKO CAR-T were cultured at a 1:1 ratio with BxPC-3 or Capan-2 cells and tumor burden (GFP) was monitored for five days.

Innate cell isolation and differentiation

PBMCs were isolated from healthy donors using Ficoll-Paque PLUS (GE Healthcare, C987R36) and monocytes were purified with a kit from StemCell (Catalog #19359). Recombinant human carrier-free cytokines were purchased from BioLegend: GM-CSF and IFNγ; Shenandoah

Biotechnology: IL-4, IL-13 and M-CSF and Sigma-Aldrich: Lipopolysaccharide (LPS). Monocytes were plated and differentiated into various macrophage subsets as previously described (45): M0 (25ng/ml M-CSF), GMCSF-activated (5ng/ml GM-CSF), M1 (5ng/ml GM-CSF, 20ng/ml IFNγ, 100ng/ml LPS) and M2 (25ng/ml M-CSF, 20ng/ml IL-4, 20ng/ml IL-13). Following macrophage differentiation, co-cultures with donor-matched CAR-T and/or target cells were constructed as described below and in figure legends. For all cultures, macrophages were left on original plates and washed briefly with PBS prior to addition of CAR-T and/or tumor cells. The ratio of 50 CAR-T : 1 macrophage or monocyte was consistent throughout experiments and was based on previous work (29).

CAR-T, macrophage, and tumor co-cultures

For contact-dependent studies, donor-matched macrophages were generated, CAR T cells/target cancer cells were added at varying tumor quantities: low (10E:1T:0.02M), moderate (1E:1T:0.02M), or high (1E:10T:0.02M), and supernatant was collected for analysis at 6, 24, 48 or 72 hours. CAR-T plus macrophage cultures were included as a control and exhibited a similar functional profile as CAR-T alone. For contact-independent studies, CAR-T and Nalm6 cells were cultured at a 1:1 ratio overnight. After 24 hours, supernatant was collected and added to donor-matched GMCSF-activated macrophages for 48 hours prior to supernatant collection and analysis.

Luminex

Supernatant was collected from co-cultures as denoted in figure legends and cytokine production was measured using the Human Inflammatory Panel 20-plex ProcartaPlex Panel (Thermo Fisher) and run on the FLEXMAP 3D System (Luminex).

Macrophage activation from mouse serum

6–8-week-old male and female NSG mice were intravenously injected with $1e^6$ JeKo-1 or Nalm6 CBG-GFP$^+$ cells. Seven days later, mice were randomized and left untreated (tumor only; TO) or IV injected with $1e^6$ KO/IFNγKO CAR T cells and tumor burden was measured by bioluminescence as described above (no blinding). Serum was collected from mice 3 days post-CAR-T injection and either saved for Luminex or added directly to donor-matched GMCSF-activated macrophages in culture. 48 hours later, supernatant was collected and assessed for function using the Human Inflammatory Panel 20-plex Luminex kit and macrophages were imaged using fluorescent microscopy.

Fluorescent microscopy

Monocytes from healthy donors were plated on iBidi glass-bottom 8 well slides and kept in 5ng/ml GMCSF for 7 days prior to use. Recombinant human IFNγ, supernatant from CAR-T/tumor culture, or serum from mice was collected and added directly to washed macrophages for 48 hours. Cells were fixed and permeabilized using the Molecular Probes Image iT kit according to protocol (Thermo Fisher). Cells were stained with unconjugated primary antibodies purchased from Thermo Fisher: CD69, CD86, PD-L1 or Cell Signaling: pJAK1 (Clone D7N4Z), pJAK2 (Clone C80C3), pSTAT1 (Clone 58D6), iNOS (CloneD6B6S) overnight at a concentration of 1:100 - 1:200. Secondary anti-rabbit antibodies conjugated to AF647 or AF549

(Cell Signaling) were used at 1:500 for detection. Molecular Probes Actin Green 488 (Thermo Fisher) was used for actin staining and slides were mounted using Prolong Gold Antifade Reagent with DAPI (Cell Signaling). Macrophages were imaged on the Zeiss Observer Microscope at 63x with similar exposures between all samples in each experiment.

Patient samples

Annotated serum samples from lymphoma patients treated with CAR T cell products (tisagenlecleucel or axicabtagene ciloleucel) at our institution were obtained after written informed consent under a protocol (16-206) approved by the Dana-Farber/Harvard Cancer Center IRB. All patient samples used herein were collected 2-5 days after CAR-T infusion and in all but one patient, collection was performed prior to treatment with tocilizumab or anakinra. Patient samples were assessed for cytokines/chemokines by Luminex or added to healthy donor macrophages (+/- blocking antibodies) *in vitro* for 48 hours prior to assessment.

Data Availability

The data generated in this study are available within the article and its supplementary data files.

Statistical Analysis

All analyses were performed with GraphPad Prism v8 software. Data were presented as means ± s.e.m. with statistically significant differences determined by tests as indicated in figure legends. For experiments with multiple groups, correction for multiple comparisons was applied. All *n* values given are biologic replicates unless otherwise stated.

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Figure Legends

Fig. 1: IFNγ can be pharmacologically and genetically blocked in CAR T cells.
a-c, CAR T cells were generated from healthy donors and IFNγ signaling was disrupted using αIFNγ blocking antibodies. **d,** BBζ CAR-T were activated with PMA/Ionomycin in the presence of αIFNγ antibody or IgG control and assessed by ELISA, *n=5*. **e,f,** IFNγR1 and pSTAT1 expression in CAR-T (**e**) and JeKo-1 lymphoma cells (**f**) treated with (+) or without (-) 10ng/ml IFNγ +/- αIFNγ blocking antibody (μg/ml) as shown by MFI (left) and percent positive cells (right), *n=3*. **g-i,** BBζ CAR T cells genetically lacking TRAC (KO) or TRAC and IFNγ (IFNγKO) were generated from healthy donors. **j**, KO and IFNγKO CAR-T were activated with PMA/Ionomycin and assessed by ELISA, *n=5*. **k**, CD4 and CD8 populations were determined by flow cytometry, representative of *n=5*. Data are shown as mean ± s.e.m. with *P* values by unpaired t-tests (**d,j**) or one-way ANOVA (**e,f**). *P:* **<0.01, ***<0.001, ****<0.0001, ns=not significant.

Fig. 2: IFNγ blockade does not diminish CAR-T efficacy *in vitro* or *in vivo*.
a, BBζ CAR-T were combined with Nalm6 tumor cells overnight at various E:T ratios in αIFNγ blocking antibody (0, 5, 20μg/ml) and IFNγ and Granzyme B production was determined by ELISA, *n=5*. **b,** Luciferase-based specific lysis of JeKo-1, Nalm6 and Raji tumor cells by BBζ CAR-T with αIFNγ blocking antibodies, *n=5*. **c-h**, NSG mice were IV injected with JeKo-1 tumor cells and treated with BBζ CAR-T +/- αIFNγ or IgG control antibodies as shown in **c**. IFNγ expression in serum collected from mice three days post-treatment with BBζ CAR +/- antibodies (**d**). Tumor growth was tracked by bioluminescent imaging (**e,f**), CAR-T persistence in the blood determined on day 14 post-CAR injection (**g**) and overall survival was monitored throughout (**h**). **i-n**, Experiments described in **c-h** were repeated using the Nalm6 tumor model. For all experiments, *n=3-5 mice/group*; repeated with 3 healthy donors. Data are shown as mean ± s.e.m. with *P* values by one-way ANOVA or Log-rank (Mantel-Cox test) for Kaplan Meier curves. *P:* *<0.05, **<0.01, ***<0.001, ****<0.0001, ns=not significant.

Fig. 3: IFNγKO CAR-T clear lymphoma and leukemia tumors *in vitro* and *in vivo*.
a, BBζ KO/IFNγKO CAR-T were combined with Nalm6 tumor cells overnight at various E:T ratios) and IFNγ and Granzyme B production was determined by ELISA, *n=5*. **b,** Luciferase-based specific lysis of JeKo-1, Nalm6 and Raji tumor cells by BBζ KO/IFNγKO CAR-T, *n=5*. **c-h**, NSG mice were IV injected with JeKo-1 tumor cells and treated with BBζ KO or IFNγKO CAR-T as shown in **c**. IFNγ expression in serum collected from mice three days post-treatment with BBζ CAR-T (**d**). Tumor growth was tracked by bioluminescent imaging (**e,f**), CAR-T persistence in the blood determined on day 14 post-CAR injection (**g**) and overall survival was monitored throughout (**h**). **i-n**, Experiments described in **c-h** were repeated using the Nalm6 tumor model. **o-q**, NSG mice were IV injected with Nalm6 tumor cells and treated with 28ζ KO or IFNγKO CAR-T (**o**). Mice were assessed for IFNγ in the serum (**p**) and measured weekly using bioluminescence (**q**). For BBζ experiments, *n=3-5 mice/group*; repeated with 3 healthy donors. For 28ζ experiment, *n=5 mice/group*; repeated with 2 healthy donors. Data are shown as mean ± s.e.m. with *P* values by one-way ANOVA or Log-rank (Mantel-Cox test) for Kaplan Meier curves. *P:* *<0.05, **<0.01, ***<0.001, ****<0.0001, ns=not significant.

Fig. 4: Establishing macrophage activation models to simulate lymphoma patient cytokine profiles.

a, Swimmer plots for lymphoma patients in this study, including dates of serum collection (black arrow), anakinra treatment (red inverted triangle), tocilizumab treatment (red square), CRS grading (red gradient) and peak ICANS days (blue gradient). **b,c,** Serum from patients receiving tisagenlecleucel or axicabtagene ciloleucel CAR-T products was collected 2-5 days post-CAR treatment, added to healthy donor-derived GMCSF-activated macrophages and cytokines were assessed 48 hours later. Luminex data was graphed by heatmap to highlight upregulated proteins in serum alone (top) and following addition to macrophages (bottom) in tisagenlecleucel and axicabtagene ciloleucel patients, *n=5 patients/CAR-T product*. **d,e**, Healthy donor BBζ and 28ζ KO CAR-T were generated and combined at a 1E:1T:0.02M ratio with donor-matched macrophages and JeKo-Nalm6 or JeKo-1 cells for 48 hours and serum was analyzed by Luminex, *n=5*. **f,g**, Healthy donor KO CAR-T were generated and combined with Nalm6 at a 1:1 ratio overnight before serum was collected and added to donor-matched macrophages for 48 hours. Serum from the E:T cultures (top) and E:T:M (bottom) was collected, analyzed by Luminex, and graphed by BBζ (left) or 28ζ (right), *n=5.* Data are shown as heatmaps depicting mean values with *P* values by unpaired t-tests. *P:* *<0.05, **<0.01, ***<0.001, ****<0.0001.

Fig. 5: IFNγKO CAR-T reduce macrophage activation in a contact-independent manner.

a, Monocytes were isolated from healthy donors and expanded to generate GMCSF-activated macrophages prior to immunofluorescent staining, representative of *n=2* (magnification 63x). Scale bars = 10μm. **b**, GMCSF-activated macrophages were generated in healthy donors and left untreated (NT; top) or given 10ng/ml recombinant human IFNγ (bottom) for four hours prior to staining for pJAK1 and pSTAT1 by fluorescent microscopy, representative of *n=2* (magnification 63x). Scale bars = 10μm. **c-e**, KO/IFNγKO CAR-T were generated from healthy donors and combined with Nalm6 cells for 24 hours prior to supernatant collection and addition to donor-matched GMCSF-activated macrophages (**c**). 48 hours later, supernatant was collected from macrophages, and function assessed by Luminex for BBζ (**d**) and 28ζ (**e**), *n=3*. **f,g**, Using the protocol from **c**, supernatant from BBζ cultures was added to macrophages and left untreated or IFNγKO CAR-T was given 10ng/ml rh IFNγ and KO CAR-T supernatant was supplemented with 20μg/ml αIFNγ blocking antibody. Cytokines were assessed by Luminex and graphed as a heatmap of mean expression (**f**) and fold-change (**g**), *n=5*. **h,i**, Using the protocol from **c**, supernatant from 28ζ cultures was added to macrophages and left untreated or IFNγKO CAR-T was given 10ng/ml rh IFNγ and KO CAR-T supernatant was supplemented with 20μg/ml αIFNγ blocking antibody. Cytokines were assessed by Luminex and graphed as a heatmap of mean expression (**h**) and fold-change (**i**), *n=5*. Macrophages from cultures **d** and **e** were fixed and stained for CD69, pJAK1 and PDL1 for both BBζ (**j**) and 28ζ (**k**), representative of *n=2* (magnification 63x). Scale bars = 10μm. Data in are shown as mean ± s.e.m. with *P* values by unpaired t-tests. *P:* *<0.05, **<0.01.

Fig. 6: Serum from tumor-bearing mice treated with IFNγKO CAR-T yield reduced macrophage responses *in vitro*.

NSG mice were intravenously injected with Nalm6 tumor cells and left untreated (tumor only) or given untransduced T cells (UTD) or KO/IFNγKO CAR-T. Three days post-CAR injection, serum was collected and used directly for Luminex assessment or added to donor-matched macrophages. 48 hours later, supernatant was collected and assayed for cytokine expression. **a**, Schematic of experimental layout. **b,c,** Serum from BBζ and 28ζ CAR-treated mice collected directly from mice was assayed by Luminex and graphed by mean values (**b**) and fold-change expression (**c**). **d-i**, Serum from mice was added to macrophages for 24 hours prior to collection and Luminex assessment for BBζ (**d-f**) and 28ζ (**g-i**) groups. Data are shown as mean value (**d** and **g**), fold-change expression (**e** and **h**), and cytokine level (**f** and **i**). **j,k**, Following supernatant collection, macrophages were stained for CD69, pJAK1, and PDL-1 expression for both BBζ (**j**) and 28ζ (**k**) subsets (magnification 63x). Scale bars = 10μm. Experiments were performed at 3-5 mice/group and repeated with 4 healthy donors. Data in **c**, **e**, and **h** are shown as mean ± s.e.m. with *P* values by unpaired t-tests. Data in **f** and **i** are shown as mean ± s.e.m. with *P* values by one-way ANOVA. *P:* *<0.05, **<0.01, ***<0.001, ****<0.0001, ns=not significant.

Fig. 7: Blocking IFNγ reduces macrophage activation in lymphoma patients to a greater extent than current biological approaches.

a, Serum from patients receiving tisagenlecleucel or axicabtagene ciloleucel CAR-T products was collected 2-5 days post-CAR treatment, added to healthy donor-derived GMCSF-activated macrophages +/- blocking antibodies to IFNγ, IL-1Rα and IL-6R and were assessed 48 hours later. **b-f**, Cultures receiving serum from tisagenlecleucel patients were assessed by Luminex (**b**) or NanoString (**c-e**). Experiments above were repeated using serum from axicabtagene ciloleucel (**f-i**). NanoString analysis for both groups was graphed as pathway score heatmap (**c,g**) and normalized gene counts (**d,e,h,i**). Data are shown as mean ± s.e.m. with *P* values by one-way ANOVA. *P:* *<0.05, **<0.01, ***<0.001, ns=not significant.

Fig. 1

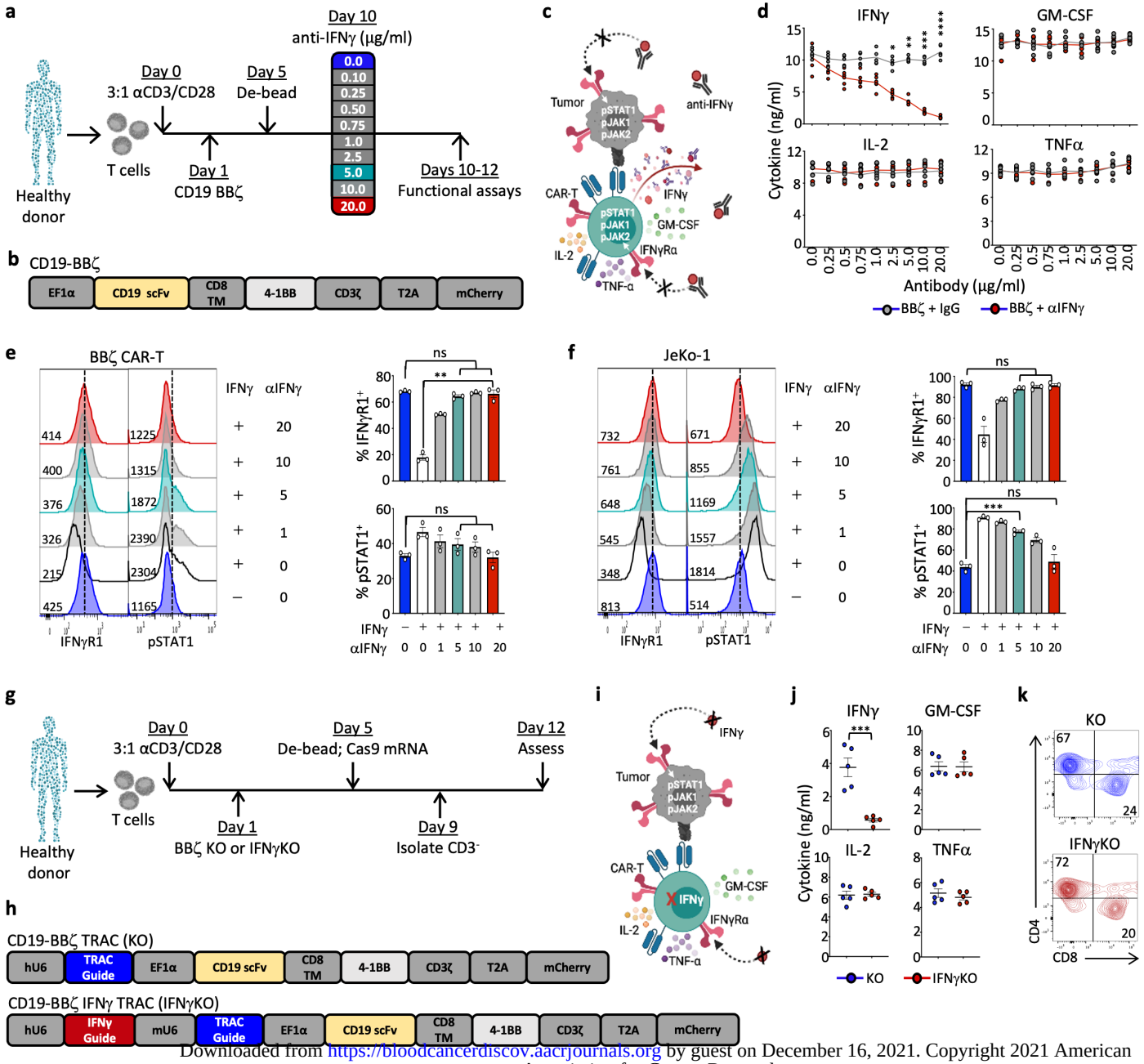
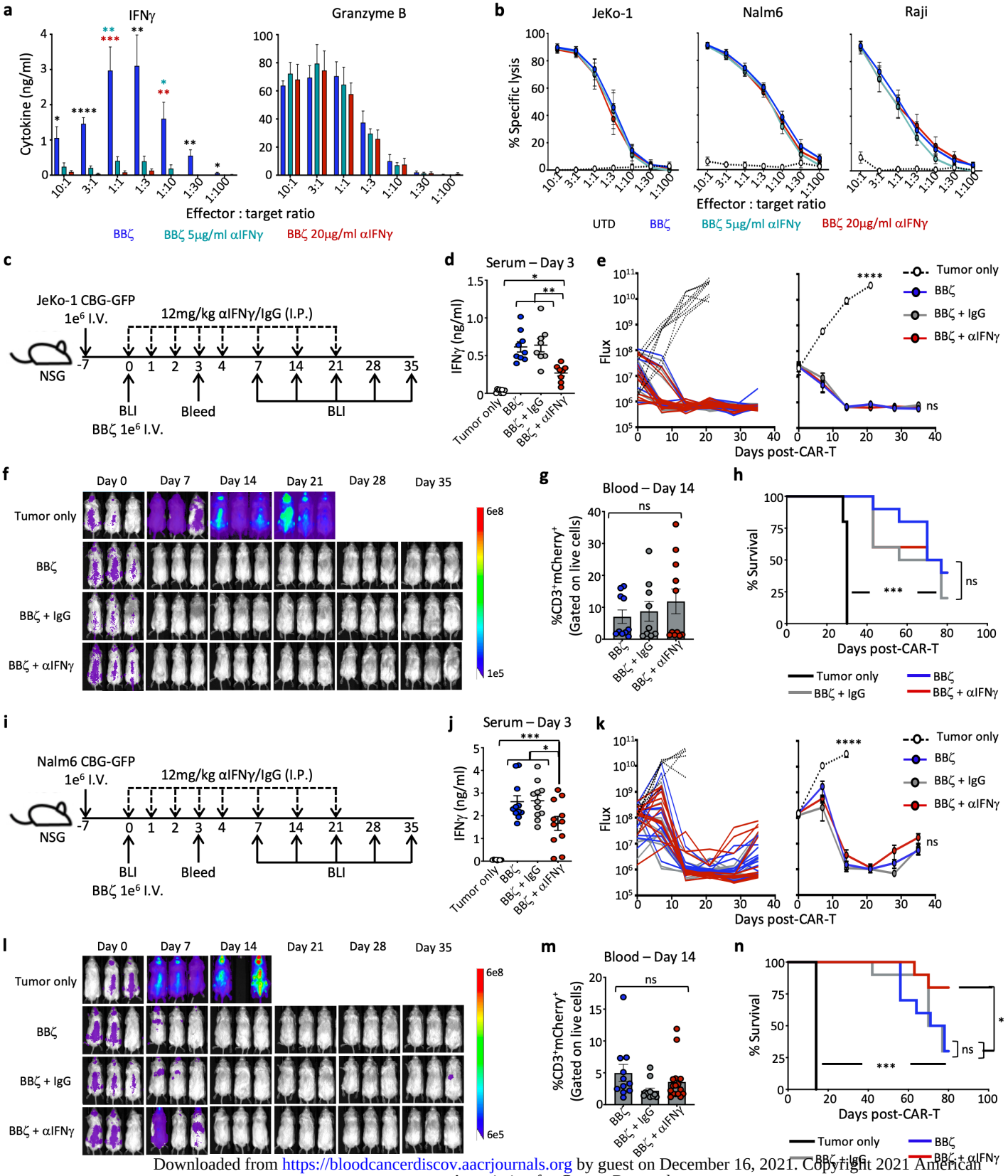


Fig. 2



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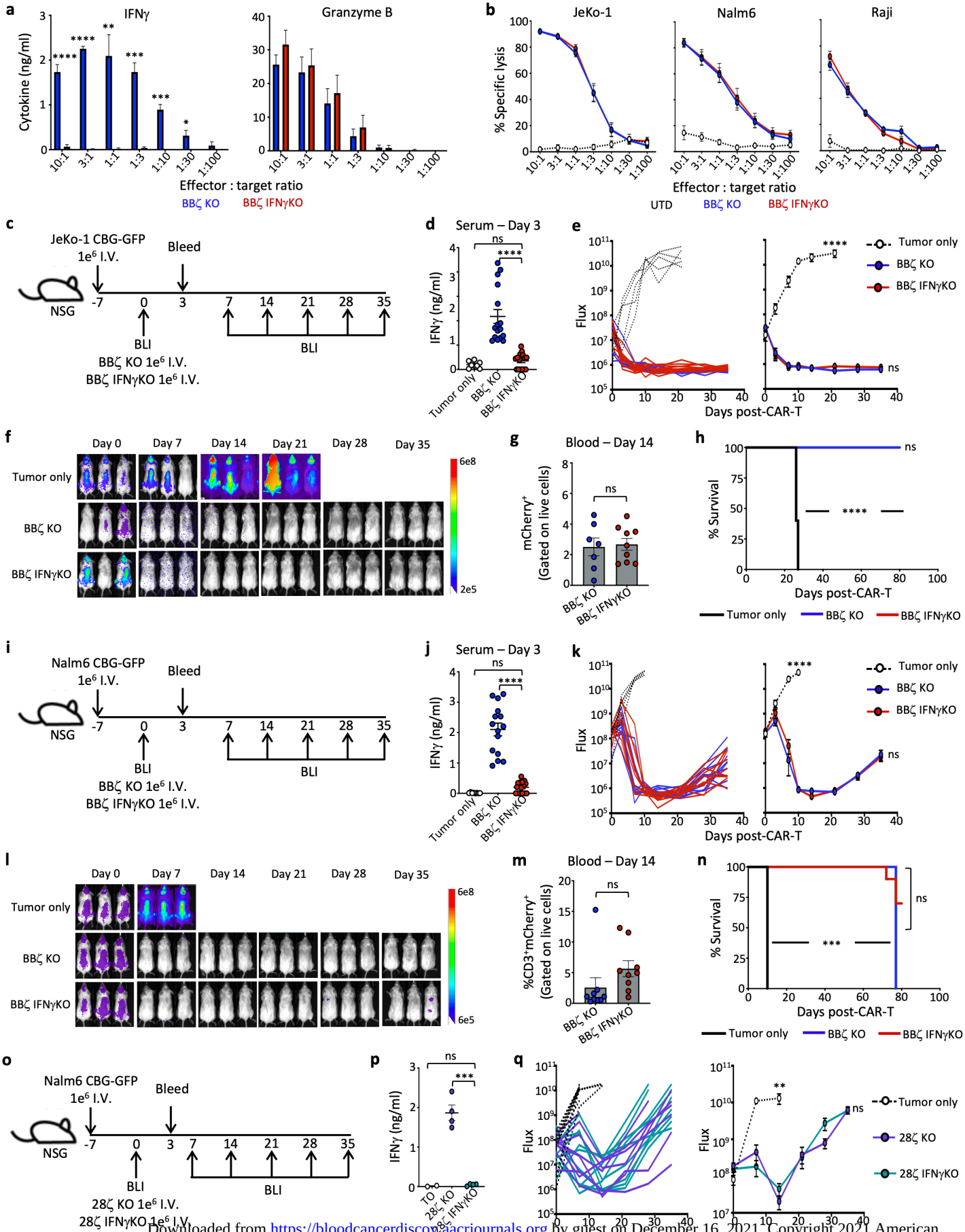


Fig. 4

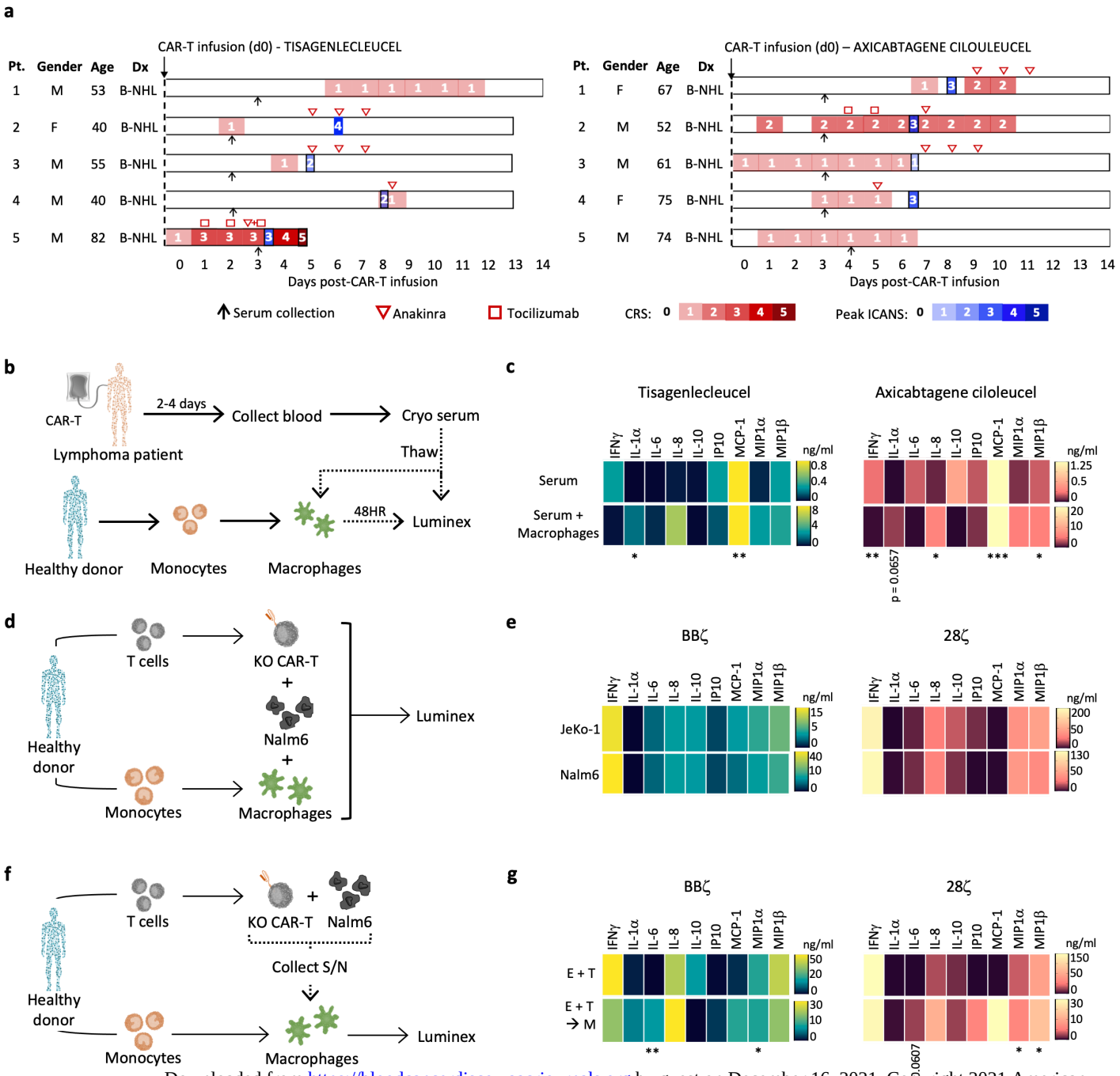
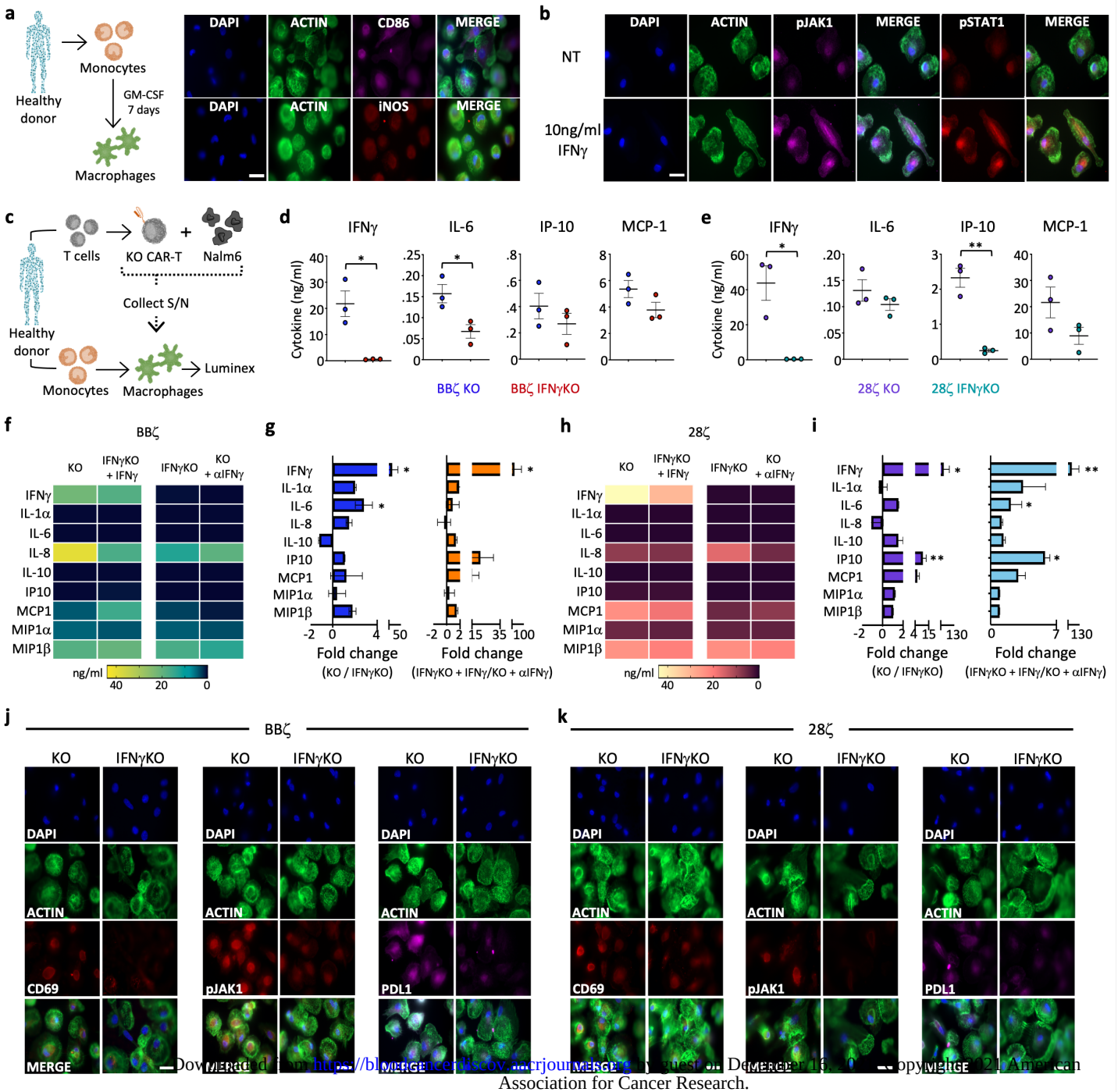


Fig. 5



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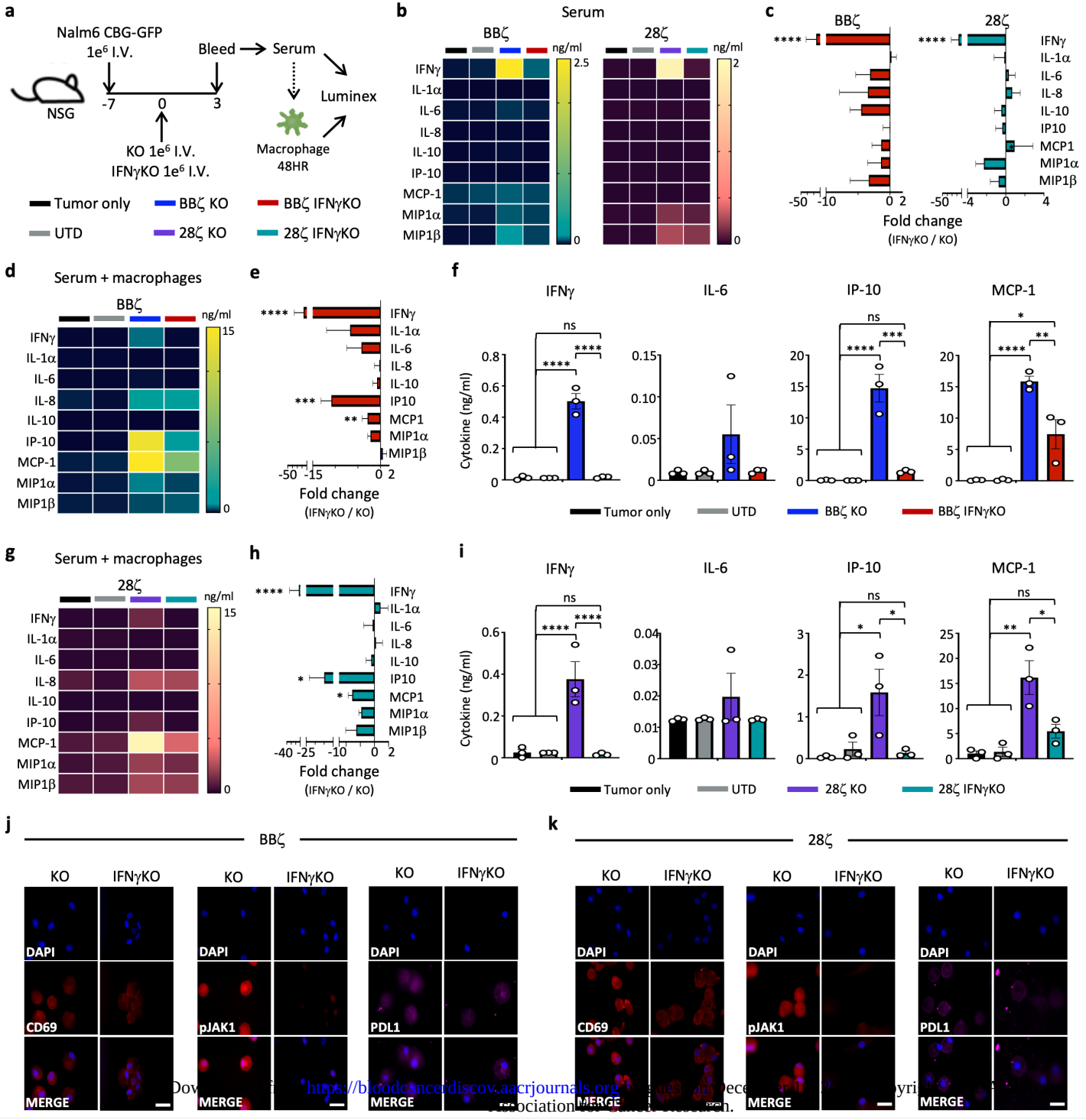
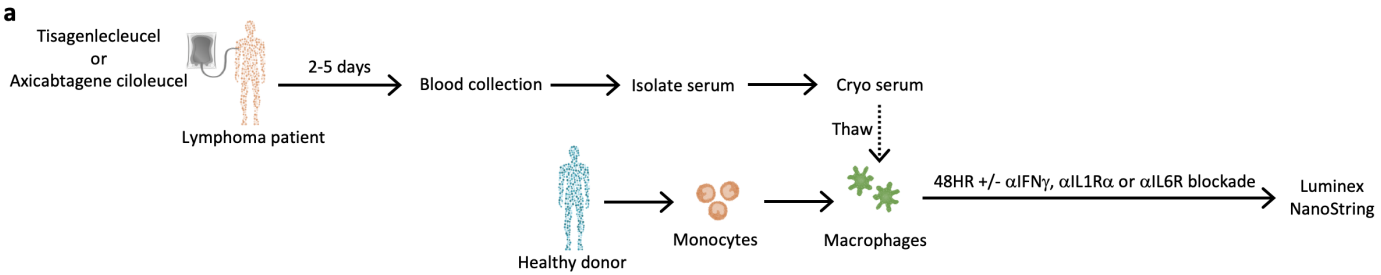
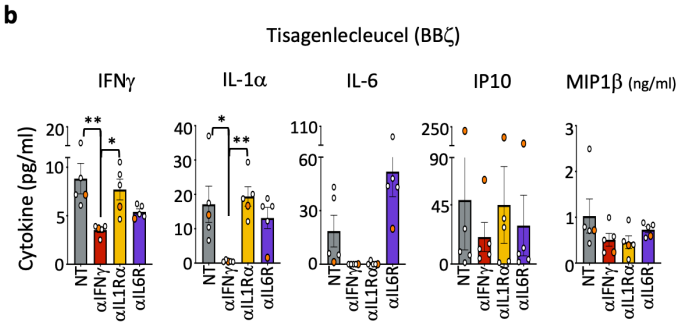
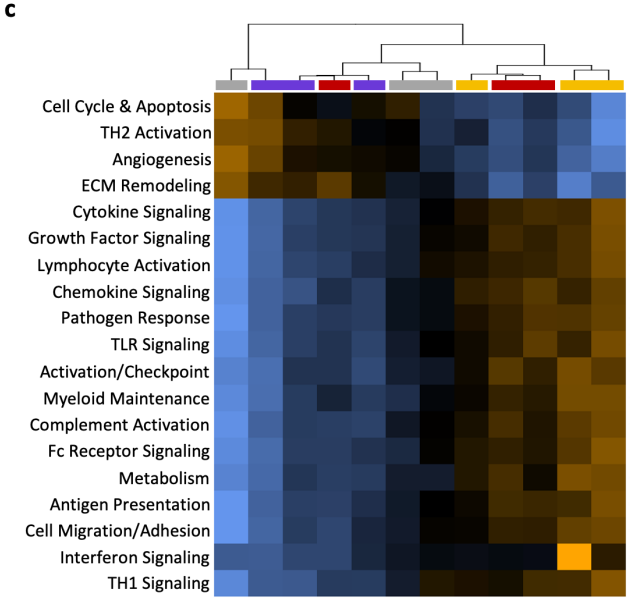
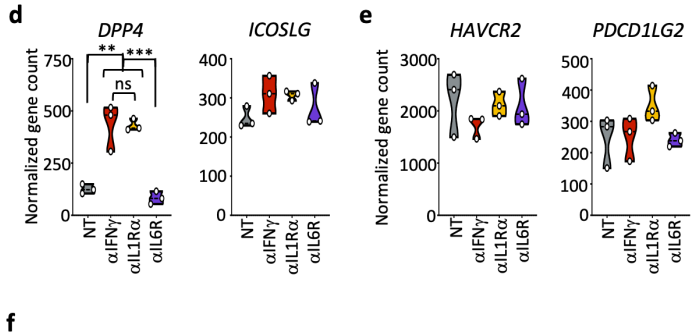


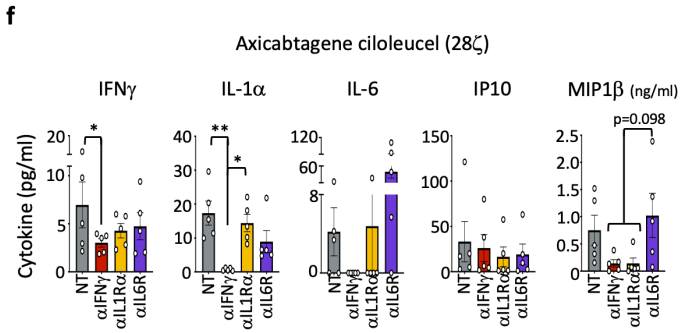
Fig. 7

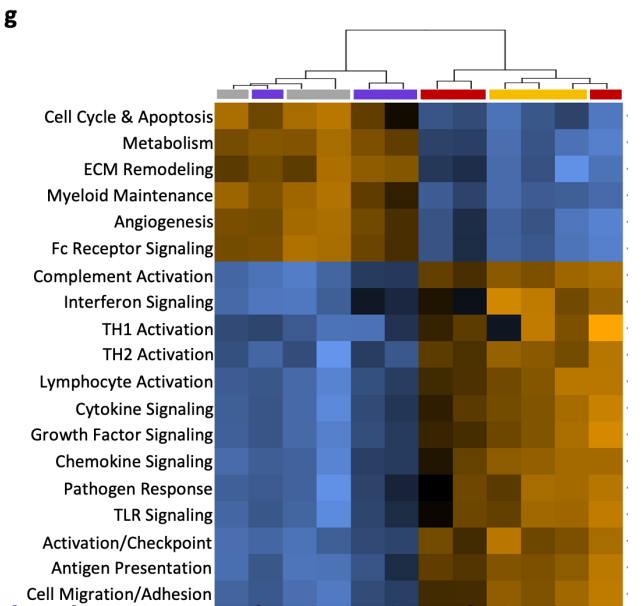


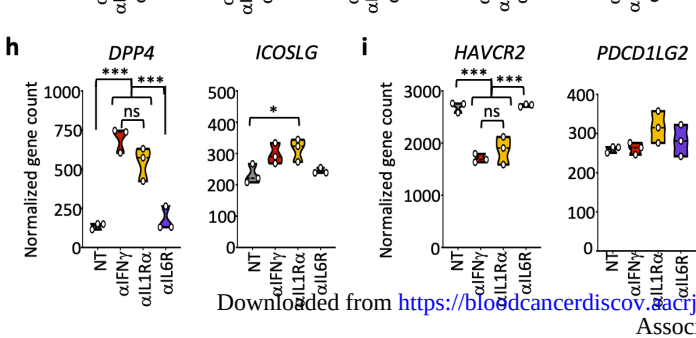












Blockade or deletion of IFNg reduces macrophage activation without compromising CAR-T function in hematologic malignancies

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