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CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade

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Abstract

Chimeric antigen receptor (CAR) therapy targeting CD19 is an effective treatment for refractory B cell malignancies, especially acute lymphoblastic leukemia (ALL)¹. While a majority of patients will achieve a complete response following a single infusion of CD19 CAR T cells^{2–4}, the broad applicability of this treatment is hampered by severe cytokine release syndrome (CRS), which is characterized by fever, hypotension and respiratory insufficiency associated with elevated serum cytokines including interleukin-6 (IL-6)^{2,5}. CRS usually occurs within days of T cell infusion at the peak of CAR T cell expansion. In ALL, it is most frequent and more severe in patients with high tumor burden.^{2–4} CRS may respond to IL-6 receptor blockade, but can require further treatment with high dose corticosteroids to curb potentially lethal severity^{2–9}. Improved therapeutic and preventive treatments require a better understanding of CRS pathophysiology, which has so far remained elusive. We report here a murine model of CRS that develops within 2–3 days of CAR T cell infusion, may be lethal and is responsive to IL-6 receptor blockade. We show that its severity is mediated not by CAR T cell-derived cytokines but by IL-6, interleukin-1 (IL-1) and nitric oxide (NO) produced by recipient macrophages, which enables novel therapeutic interventions.

Author contributions

T.G. designed the study, performed experiments, analysed and interpreted the data, and wrote the manuscript. S.J.C.v.d.S. helped design and perform *in vivo* experiments, J.E. performed *in vivo* experiments, M.H. performed *in vivo* experiments, A.P. performed and interpreted histopathological studies, M.S. designed the study, analysed and interpreted the data, and wrote the manuscript.

Data availability

Data presented in Figures 2e–f and 4a–b can be accessed under accession number GSE111236.

Statistics

Statistical analyses were performed using GraphPad Prism V7 for Mac. Statistical tests included unpaired two-sample *t*-test, two-tailed; One-way ANOVA; Two-way ANOVA; log-rank Mantel-Cox test; binomial test with FDR-adjusted *p*-values. Statistical test used for each figure is described in the corresponding figure legend.

Materials Availability

Materials are available upon request to the corresponding author.

Life Sciences Reporting Summary

Further information on experimental design is available in the Life Sciences Reporting Summary.

Competing financial interests

A patent application on CRS prevention listing T.G. and M.S. as co-inventors has been filed by MSK.

To model CAR T cell-induced CRS in mice, we aimed to establish conditions whereby CD19 CAR T cells would engage a high tumor burden and initiate CRS within a few days, akin to the clinical setting^{2,3,9,10}. Whereas CRS could not be induced in mice with medullary disease, intraperitoneal tumor growth allowed for a sufficient tumor burden to accumulate and severe CRS to develop in SCID-beige within 2–3 days of CAR T cell administration (Figure 1a). Human 1928z CAR T cells reproducibly elicited an acute inflammatory response associated with reduced activity, general presentation of malaise, piloerection, weight loss (Figure 1b), and eventual mortality (Figure 1c). Remarkably, the serum cytokine profile elicited in these mice was highly similar to that reported in clinical studies^{2,11,12} (matching 18 out of 19 reported cytokines, Supplementary Table 1). Similar to the elevation of C-Reactive Protein (CRP) observed in the clinic,^{2,3,10} the murine equivalent SAA3^{13,14} significantly rose following CAR T cell administration to tumor bearing mice (Figure 1d) as were pro-inflammatory cytokines and chemokines including IL-6 (Figure 1e and Supplementary 1a). The overall levels of these cytokines, including mIL-6, mCCL2, mG-CSF, hIL-3, hIFN- γ , hGM-CSF, hIL-2 correlated strongly with CRS severity and survival (Figure 1e). Taking advantage of the xenogeneic nature of this model to discern the T cell or host cell origin of these cytokines and chemokines, we demonstrated that some cytokines such as IFN- γ and GM-CSF were products of the human CAR T cells, while others such as IL-6 were produced by endogenous murine cells (Figure 1f and Supplementary 1b). This finding establishes that the CRS cytokine signature is the result of a multicellular network and not merely a binary tumor-CAR T cell interaction. Furthermore, the lack of activity of human IFN- γ and GM-CSF on the murine cognate receptor (Supplementary Table 2) indicates that other CAR T cell-derived cytokines and/or CAR T cell activities account for CRS. Although dispensable in this model, T cell-derived IFN- γ and GM-CSF may yet contribute to CRS in other settings. Consistent with clinical CRS,¹¹ IL-15 was not differentially elevated upon CAR transfer (Supplementary 1c). In accordance with clinical experience,^{2,3,9,10} treating mice with a murine IL-6R blocking antibody prevented CRS-associated mortality (Figure 1g and Supplementary 1d).

Histopathological analyses performed 2 and 5 days following CAR T cell infusion did not reveal any evidence of graft-versus-host disease (GVHD) or tissue destruction (Supplementary 2), consistent with the initiation of this inflammatory response following tumor recognition by CAR T cells as well as the full recovery of mice surviving the CRS. Histopathological examination of the CNS and meninges 1, 2 and 5 days after CAR T cell transfer did not reveal morphological evidence of acute damage or toxicity (Supplementary 3), consistent with the absence of overt neurological symptoms (seizures, limb dyskinesia or paralysis). None of the reported pathologic findings indicative of neuropathology¹⁵ or associated with neurotoxicity (cortical laminar necrosis, hemorrhages, DIC, gliosis, vasogenic, neurotoxic or interstitial edema) in human patients¹⁶ were observed in any of the mice examined in the present study. The occurrence of subclinical functional alterations or ultrastructural morphologic changes cannot be excluded. Mice surviving CRS rapidly return to a highly active state, akin to healthy tumor free mice. No clinical neurological anomalies were noted until mice had to be sacrificed because of tumor progression.

The high serum levels of murine IL-6, a predominantly myeloid-derived cytokine, together with the presence of tumor-infiltrating myeloid cells (Figure 2a) prior to CAR T cell transfer

and more so thereafter, led us to hypothesise that tumor-associated myeloid cells are closely associated with the induction of CRS. Only after infusion of CAR T cells in the presence of tumor was toxicity observed (Supplementary 4a and Supplementary 1a), concurrent with a brisk accumulation of peritoneal neutrophils, eosinophils, dendritic cells (DCs), monocytes (Figure 2b and Supplementary 5a) and F4/80^{int-lo} Ly6C^{int-hi} macrophages (hereafter referred to as CRS-associated macrophages), which differ from the typical F4/80^{hi} Ly6C^{neg-lo} resident peritoneal phenotype (Figure 2c). The rapid elevation of myeloid cell numbers, already noticeable 18 hours after CAR T cell administration (Figure 2d), suggested that recruitment was a major contributor to this accumulation. RNAseq analyses at 18 hours showed increased expression of cell cycle promoting genes, suggesting that cell proliferation may play a secondary role in local myeloid cell accumulation.

To address whether these alterations were regional or systemic, we enumerated neutrophils, eosinophils, DCs, monocytes and macrophages in spleen, bone marrow, lungs, liver and peripheral blood (Figure 2d and Supplementary 4b). Whereas neutrophils, DCs and macrophages accumulated at the tumor site, systemic perturbations were limited to increased macrophage counts in the spleen (Figure 2d) and neutrophils in peripheral blood, coinciding with neutrophil depletion in bone marrow (Figure 2d and Supplementary 4b). The major alterations in myeloid cell distribution were thus confined to the tumor vicinity and to the spleen.

Since IL-6 is a signature cytokine of CRS, we hypothesized that the tracking down IL-6-producing cells would identify the main physiopathological sites. We therefore purified DC, macrophage and monocytic populations from peritoneum and spleen (neutrophils do not typically produce IL-6¹⁷) (Supplementary 5a and b) and performed RNAseq analysis. Remarkably, only peritoneal but not splenic DCs, monocytes and macrophages showed upregulated IL-6 transcripts (Figure 2e and f). The induction of other major pro-inflammatory cytokines was likewise confined to the CAR T cell/tumor tissue (Figure 2e and f). Combined with cell enumeration data, macrophages thus emerged to be the main overall source of IL-6. As CAR T cells were found in the peritoneum but not spleen or other organs (Supplementary 4c), these findings further suggested that IL-6 induction and myeloid activation require proximity of CAR T cells and myeloid cells, and possibly their direct interaction.

We hypothesized that arming the CAR T cells with a cell-surface ligand capable of activating myeloid cells would determine whether such a direct cellular contact can take place. To address this question, we again took advantage of our xenogeneic model to probe the potential for CD40L/CD40 interactions. Since CD40L is mainly expressed by T cells, while DCs, monocytes and macrophages express the CD40 receptor¹⁸, and since human CD40L does not functionally interact with the murine CD40 receptor¹⁹, we constitutively expressed mCD40L in human CAR T cells (Supplementary 6a and b). This modification resulted in more severe and sustained weight loss in mice (Figure 3a) and markedly increased mortality (Figure 3b). The similar overall number of recruited myeloid cells in the CAR and CAR/mCD40L treatment groups (Supplementary 6c) suggested that the increased severity of CRS was due to qualitative and not quantitative changes in the myeloid compartment. Indeed, mice receiving 1928z-mCD40L CAR T cells showed an increased

proportion of CRS-associated macrophages (Figure 3c). Notably, while cell-surface expression of CD40 was exclusive to macrophages and DCs in peritoneal myeloid cells (Supplementary 6e), only macrophages down-regulated CD40 expression (Figure 3d and Supplementary 6d and f), an expected consequence of its ligation by CD40L.^{20–22} In line with the increased severity of CRS, levels of murine inflammatory cytokines were significantly increased, including IL-6, which is known to be directly induced by CD40L signaling²³ (Figure 3e). These findings establish that CAR T cell-macrophage interactions can take place at tumor sites and furthermore that such interactions, albeit not obligatory, have the potential to aggravate CRS severity. Interestingly, expression of endogenous CD40L positively correlates with CAR expression in human CD4 T cells (Supplementary 6e), which suggests that such interactions potentially take place in the clinical setting. The genetic manipulation of receptor-ligand pairs in our model further serves as a proof of concept that species barriers can be exploited to investigate immunological interactions between adaptive and innate immune responses in xenogeneic tumor models. Altogether, our enumeration and expression profiling of tumor and splenic myeloid cells, compounded by the exacerbation of CRS via engineered CD40/CD40L interaction, designate CRS-associated macrophages as a major mediator of CRS severity.

To further define the contribution of macrophages to CRS, we investigated the role of inducible Nitric Oxide Synthase (iNOS), an enzyme predominantly expressed by macrophages upon their activation.²⁴ In line with a requirement for proximity to CAR T cells, only peritoneal but not splenic or bone marrow myeloid populations significantly increased iNOS production in CRS (Figure 3f). Macrophages showed the highest induction (Figure 3f) and were numerically the most abundant iNOS-expressing population (Supplementary 7a). Aberrant NO production is known to cause vasodilation and hypotension^{25,26}, which are common features of clinical CRS that require vasopressor administration⁹. We treated mice with either one of two iNOS inhibitors, L-NIL²⁷ and 1400W²⁸. Both alleviated clinical toxicity (Figure 3g and Supplementary 7b), including mortality under conditions of severe CRS (Figure 3h). These findings further support the direct role of macrophage-derived products in CRS and may provide a novel means to mitigate CRS severity.

The direct involvement of iNOS in CRS pathophysiology prompted us to further examine the role of IL-6 and IL-1, both of which are inducers of iNOS.^{29,30} Our RNAseq data in myeloid cell types harvested at the onset of CRS showed that the type 1 IL-1 receptor (IL1R1), which is required for functional IL-1 signaling, was exclusively upregulated in tumor-associated myeloid cells but not splenic cells (Figure 4a and b). Conversely, splenic myeloid cells upregulated only the type 2 IL-1 receptor (IL1R2), which does not functionally signal and serves as a decoy receptor. We also observed upregulation of IL-1 receptor antagonist (IL1RN/IL-1Ra) in splenic myeloid cells (Figure 4b), which suggested an adaptive response to inhibit IL-1 signaling outside the tumor bed.³¹ We hypothesized that endogenous IL-1 suppression is insufficient to inhibit the pro-inflammatory effects of IL-1 but that a pharmacological intervention could mitigate CRS symptoms. IL-1 blockade by Anakinra, an IL-1 receptor antagonist, did indeed abrogate CRS-related mortality (Figure 4c). In order to obtain more insight in the protective mechanism of IL-1 blocking and assess how it compares to IL-6 blockade, we assessed the impact of Anakinra on macrophage

iNOS expression levels. Both blockades resulted in similarly reduced iNOS⁺ macrophage fractions (Figure 4d and Supplementary 7c), establishing downregulation of iNOS as one unifying mechanism by which IL-6 and IL-1 blockades can abate CRS. Combined IL-1/IL-6 blockade however did not further decrease the fraction of iNOS⁺ macrophages (Figure 4d), suggesting that the inhibition afforded by these two blockades operates through a common pathway.

To prevent CRS mortality without exogenous intervention, we generated CAR T cells that constitutively produce IL-1 receptor antagonist (IL1RN/IL-1Ra) (Figure 4e and Supplementary 8a). First, we demonstrated that this novel construct protects from CRS-associated mortality (Figure 4f and Supplementary 8b). Significantly, CAR T cell-derived serum cytokine levels were unaffected (Figure 4g), consistent with the blockade of endogenous CRS pathways independent of CAR T cell activation. Next, we assessed whether anti-tumor activity might be affected by evaluating 1928z-mIL1Ra T cells in the CAR “stress test” at limiting CAR T cell doses in NSG mice³². At two different dose levels, 1928z-mIL1Ra matched the therapeutic efficacy of control 1928z-LNGFR CAR T cells (Figure 4h and Supplementary 8c and d). Therefore, we not only identified a novel actionable target for CRS but also designed a CAR construct that autonomously prevents CRS-associated mortality in mice.

We demonstrate here that recipient myeloid cells play a critical role in the pathogenesis of CRS. We establish that activated CAR T cells recruit and activate myeloid cells, and that co-localized myeloid cells are the main source of IL-6. Macrophages contribute to CRS pathophysiology through their production of IL-6 and iNOS. The aggravation of CRS by CAR T cells expressing mCD40L demonstrates that CAR T cells and macrophages can functionally interact within the tumor microenvironment. Whether CAR T cell – macrophage contact is required for CRS remains to be determined, since CRS could develop in our model in the absence of a functional CD40/CD40L axis. CAR T cells may activate myeloid cells through a variety of other pathways including cytokines, other cell contact-dependent pathways and Toll-like receptor stimulation. Our findings on the importance of CAR T cell-macrophage proximity suggest that the incidence and severity of CRS in CAR therapies will at least in part depend on the extent of myeloid infiltration in the targeted tumor, which may be variable among hematological and solid tumors³³.

Selectively modulating macrophage activity with CD40L, iNOS inhibitors or Anakinra revealed the central role played by macrophages and provides insights for novel therapeutic interventions. We identified IL-1 as a novel actionable target, suitable to treat acute CRS and diminish its severity. Although circulating IL-1 levels were below detection, as observed in CD19 CAR T cell recipients undergoing CRS,¹¹ it is noteworthy that other IL-1 driven inflammatory conditions associated with low or undetectable circulating IL-1 have been found to resolve with the administration of Anakinra³⁴.

Our findings that both IL-1 signaling and iNOS inductions are critical determinants of severe CRS potentially explain why SCID-beige develop more severe CRS. SCID-beige mice and NSG mice differ in their genetic background (C.B17-Scid, BALB/c and NOD/LtSz-Scid, NOD/LT, respectively) and the impaired IL-1 response to IFN- γ priming and LPS

stimulation found in the NSG background.³⁵ It is plausible that additional developmental and maturation defects of monocytes and macrophages in NOD mice³⁶ contribute to reduced macrophage reactivity and diminished CRS in NSG mice. Moreover, NSG mice lack the common γ_c chain receptor, which is essential for signaling through IL-2, IL-15 and other cytokines. Peritoneal macrophages lacking γ_c are defective in NO production primed by IL-15 signaling³⁷, a cytokine that is present in our mouse model.

Our findings informed the design of an IL-1Ra-secreting CAR construct that can demonstrably prevent CRS-related mortality while maintaining intact anti-tumor efficacy. The benefits of an IL-1 blockade through IL-1Ra are especially intriguing given the latter's ability to cross the blood brain barrier³⁸, in contrast to tocilizumab⁹. As human microglia activated by IL-1 may produce iNOS and proinflammatory cytokines,^{39,40} blocking IL-1 could potentially not only protect from severe CRS but also reduce the severity of CAR T cell-related neurotoxicity.

In addition to the toxicity in itself, CRS carries a high cost because of the stringent patient monitoring and supportive therapy it may require. CRS toxicity and its management costs presently hamper the broad use of CAR therapy. This study demonstrates that CAR T cells may be engineered to mitigate these burdens without requiring exogenous intervention.

Materials and methods

Cell culture

Burkitt Lymphoma Raji cells and NALM-6 pre-B-ALL cells were obtained from ATCC. Raji GFP-FLuc (Fluc: Firefly Luciferase) and NALM-6-GFP-FLuc cells were cultured in RPMI (Invitrogen) supplemented with 10% FBS (HyClone), 10mM HEPES (Invitrogen), L-Glutamine 2mM (Invitrogen), NEAA 1x (Invitrogen), 0.55mM β -mercaptoethanol, 1mM Sodium Pyruvate (Invitrogen), Penicillin-Streptomycin 50U/ml (Invitrogen). Raji and NALM-6 cells were routinely tested for mycoplasma and found negative.

T cells, source and handling

Buffy coats from anonymous healthy donors were purchased from the New York Blood Center (IRB exempted) and were handled following all required ethical and safety procedures. The researchers were blind to any covariate characteristics.

T cells, isolation and culture

Primary human T cells were purified from buffy coats of healthy donors by negative magnetic selection (Pan T Cell Isolation Kit, Miltenyi). Purified T cells were cultured in XVIVO 15 (Lonza) supplemented with 5% Human Serum AB (Gemini), 10mM HEPES, 2mM GlutaMax (Invitrogen), 1x MEM Vitamin Solution (Invitrogen), 1mM Sodium Pyruvate (Invitrogen), Penicillin-Streptomycin 50U/ml (Invitrogen), 60U/ml recombinant IL-2.

Mice

Mice were treated under a protocol approved by the MSKCC Institutional Animal Care and Use Committee, according to all relevant animal use guidelines and ethical regulations. CRS Model: 6–8 week old female C.B.Igh-1b/GbmsTac-Prkdc^{scid}Lyst^{bgN7} (SCID-beige) mice (Taconic) were intraperitoneally injected with 3 million Raji-GFP-Fluc cells and tumors were left to grow for 20 days. Tumor burden was evaluated by in vivo bioluminescent imaging two days prior to CAR T cell transfer. Outlier mice with extreme burdens (either too high or too low compared to most) were excluded from the experiment before CAR T cell infusion. No mice were excluded afterwards at any point. Mice were injected intraperitoneally with 30 million CAR+ T cells in PBS supplemented with 2% Human Serum. Control mice received PBS supplemented with 2% Human Serum. Stress test model: 6–8 week old male NOD.Cg-Prkdc^{scid}Il2rg^{tmWjl}/SzJ (NSG) mice (Jackson Laboratory) were inoculated with 0.5e6 NALM-6-GFP-Fluc cells by tail vein injection followed by 0.2e6 or 0.5e6 CAR T cells four days later. Bioluminescence imaging utilized the Xenogen IVIS Imaging System (Xenogen) with Living Image software (Xenogen) for acquisition of imaging datasets. Tumor Burden was assessed as previously described⁴¹.

Mouse treatment

Anakinra was administered intraperitoneally at 30mg/kg once per day for 5 days, starting 5 hours prior to CAR T cell transfer, or PBS (vehicle) for control mice. Anti murine IL-6 (clone MP5–20F3, BioXcell) or isotype (Rat IgG1, clone HRPN, BioXcell), anti murine IL-6R (clone 15A7, BioXcell) or isotype (Rat IgG2b, clone LTF-2, BioXcell) and anti murine IL-1b (clone B122, BioXcell) or isotype (Armenian Hamster IgG, clone Armenian Hamster IgG, BioXcell) were administered intraperitoneally once per day at 25mg/kg for the first dose and 12.5mg/kg for subsequent doses for 5 days starting 5 hours prior to CAR T cell transfer. L-NIL (Enzo Life Sciences) or 1400W (Cayman Chemical) were administered intraperitoneally at 5mg/kg once per day for 5 days starting 5 hours prior to CAR T cell transfer, or PBS (vehicle) for control mice.

Flow Cytometry

Antibodies were titrated for optimal staining. The following fluorophore conjugated antibodies were used (“h” prefix denotes anti-human, “m” prefix denotes anti-mouse): hCD4 BUV395 (clone RPA-T4, BD), hCD8 PE-Cy7 (clone SK1, eBioscience), hCD3 PerCP-eFluor710 (clone OKT3, eBioscience), hCD19 BUV737 (clone SJ25C1, BD), hLNGFR BB515 (clone C40–1457, BD), hCD40L BV421 (clone TRAP1, BD), mF4/80 BV421 and BV711 (clone T45–2342, BD), mLy6C Alexa Fluor 647 and BV786 (clone ER-MP20, AbDSerotec and clone HK1.4, BioLegend respectively), mGR-1 (clone RB6–8C5, BD), mMHCI BB515 (clone 2G9, BD), mCD11c BV650 (clone N418, BioLegend), mLy6G APC-Fire750 (clone 1A8, BioLegend), mSIGLEC-F PE-CF594 (clone E50–2440, BD), mCD40 BV786 (clone 3/23, BD), mCD40L PE (clone MR1, BD), mCD11b BUV395 (clone M1/70, BD), mNOS2 PE-Cy7 (clone CXNFT, eBioscience). For flow cytometry with live cells 7-AAD (BD) was used as a viability dye. For flow cytometry with fixed cells eFluor506 fixable viability dye (eBioscience) was used. Fc receptors were blocked using Fc Receptor Binding Inhibitor Antibody Human (eBioscience) and Fc Block Mouse (Miltenyi).

Cells were fixed using the Intracellular Fixation and Permeabilization Buffer Set (eBioscience) according to the manufacturer's instructions. For CAR staining a Alexa Fluor 647 conjugated goat anti-mouse antibody was used (Jackson ImmunoResearch). For cell counting, Countbright beads were used (Invitrogen) according to the manufacturer's instructions.

Retroviral Vector Constructs and Retroviral Production

The 1928z-LNGFR construct has been previously described⁴². 1928z-mCD40L and 1928z-mIL1RN were prepared using standard molecular biology techniques. To obtain the 1928z-mCD40L construct, the cDNA for murine CD40L was inserted in the place of LNGFR. To obtain the 1928z-mIL-1Ra construct, the cDNA for murine IL-1Ra was inserted in the place of LNGFR. Plasmids encoding the SFG γ -retroviral (RV) vector⁴³ were prepared as previously described⁴². gpg29 (H29) cells were transfected to produce VSV-G pseudotyped retroviral supernatants. These supernatants were used to transduce stable retroviral-producing 293T cell lines as previously described⁴⁴. Retroviral supernatants carrying the various CAR genetic constructs harvested from 293T cell lines were used to transduce human T cells. T cells were activated with CD3/CD28 T cell Activator Dynabeads (Invitrogen) immediately after purification at a 1:1 bead-to-cell ratio. After 48 hours of bead activation, T cells were transduced with retroviral supernatants by centrifugation on Retrofectin (Takara)-coated plates in order to obtain 1928z-LNGFR, 1928z-mCD40L or 1928z-mIL-1Ra CAR T cells. Transduction efficiency was verified three days later by flow cytometry. CAR T cells were injected in mice 7 days after the first T cell activation.

Serum collection

Blood was collected from mice by tail-clip or by retro-orbital bleeding. After collection blood was left to clot for 30 minutes at room temperature. Following clotting blood was centrifuged at 6000g for 10minutes at 4 degrees. Serum was aliquoted in tubes in order to prevent multiple freeze-thaw cycles, and was immediately stored at -80°C until analysis.

Cytokine measurements

Serum/plasma cytokines were measured using Cytometric Bead Arrays (BD) or ELISA kits for mouse IL-1Ra (Thermo-Fisher), mouse SAA3 (Millipore) and mouse IL-15/IL-15R complex (Thermo-Fisher), as per the manufacturer's instructions.

Animal pathology

Mice were transferred to the pathology core facility of Memorial Sloan Kettering where they were sacrificed by cardiac puncture. Tissues obtained were fixed in 10% buffered formalin and were further processed for H&E staining and immunohistochemistry.

RNA extraction and Transcriptome Sequencing

Cells were sorted directly into 750ul of Trizol LS (Invitrogen). The volume was adjusted to 1ml with PBS and extraction was performed according to instructions provided by the manufacturer. After ribogreen quantification and quality control of Agilent BioAnalyzer, total RNA underwent amplification using the SMART-seq V4 (Clontech) ultra low input

RNA kit for sequencing. For 2–10 ng of total RNA, 12 cycles of amplification were performed. For lesser amount (0.13 to 2 ng), 13 cycles of amplification were performed. Subsequently, 10 ng of amplified cDNA was used to prepare Illumina HiSeq libraries with the Kapa DNA library preparation chemistry (Kapa Biosystems) using 8 cycles of PCR. Samples were barcoded and run on HiSeq 2500 1T, in a 50bp/50bp Paired end run, using the TruSeq SBS Kit v3 (Illumina). An average of 38.5 million paired reads were generated per sample and the percent of mRNA bases was over 77% on average.

RNAseq Analysis

The output FASTQ data files were mapped (2 pass method) to the target genome (MM10 assembly) using the STAR RNA aligner, resolving reads across splice junctions (ENSEMBL assembly). The first mapping pass uses a list of known annotated junctions from ENSEMBL. Novel junctions found in the first pass are then added to the known junctions, after which a second mapping pass is performed using the RemoveNoncanonical flag. After mapping, the output SAM files were post-processed using PICARD tools to add read groups, AddOrReplaceReadGroups, sort the files and convert to BAM format. The expression count matrix for the mapped reads was then computed using HTSeq. Finally, DESeq was used to normalise the full dataset and analyze differential expression between sample groups.

Program	Version
HTSEQ	htseq/HTSeq-0.5.3
PICARD	picard/picard-tools-1.124
R	R/R-3.2.0
STAR	star/STAR-STAR_2.5.0a
SAMTOOLS	samtools/samtools-0.1.19

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: A mouse model of CRS recapitulates clinical CAR T cell-induced CRS.

a. Schematic of mouse model. Raji tumour cells are intraperitoneally injected in mice and allowed to grow for three weeks, where they eventually grow into vascularized solid tumour masses. Thirty million CAR T cells are transferred and mice are monitored over the following days. Mice are sacrificed and cells are obtained for analysis through peritoneal lavage and tissue harvesting for further analysis

b. Weight change of tumour bearing mice after 1928z CAR T cell transfer. Weight per mouse is normalised to starting weight pre-CAR transfer (Tumour only n=12 mice, Tumour + CAR n=18 mice). Experiments monitoring weight under CRS conditions were performed in at least 20 independent experiments. (Two-way ANOVA).

c. Percent survival of mice after 1928z CAR T cell transfer (Tumour only n=12 mice, Tumour + CAR n=18 mice). Experiments monitoring survival under CRS conditions were performed in at least 20 independent experiments. (Log-rank Mantel-Cox test).

d. Serum levels of murine SAA3 at 42 hours post 1928z CAR T cell transfer as measured by ELISA (No Tumor no CAR n=5 mice, tumour only n=5 mice, CAR only n=5 mice, tumour + CAR n=7 mice). SAA3 levels under CRS conditions were measured at least in two independent experiments. (Unpaired two sample *t*-test, two-tailed).

e. Cytokine levels 4.5 hours before (pre-car) and 24 hours post 1928z CAR T cell transfer (CRS or Severe CRS). Mice that eventually died from CRS were grouped under Severe CRS while mice that survived but suffered greater than 10% weight loss were grouped under CRS. “m” prefix denotes murine origin while “h” prefix denotes human origin. Cytokine levels were measured by Cytokine Bead Array (CBA). (Severe CRS n=5 mice, CRS n=10 mice, pre-CAR n=16 mice). Stratification of cytokines based on survival were performed at least in three independent experiments. (Unpaired two sample *t*-test, two-tailed).

f. Species of origin of pro-inflammatory cytokines. (Human cytokines from n=15 mice, Mouse cytokines from n=15 mice).

g. Percent survival of tumour bearing mice treated with 1928z CAR T cells that received anti-murine IL-6R blocking antibody or isotype (vehicle). (Vehicle n=6 mice, anti-mIL-6R n=5 mice). Experiments measuring survival after blocking murine IL-6R were performed once. (Log-rank Mantel-Cox test).

All data are means $\pm$ s.e.m.

Figure 2: Tumour – CAR T cell interactions trigger myeloid cell recruitment and activation.

a. Immunohistochemical staining for Mac2 of sections from 3-week Raji tumour explants.

Mice had not received CAR T cells. Immunohistochemical staining of tumor explants for Mac 2 was performed once.

b. Absolute counts of myeloid cell populations obtained by peritoneal lavage 68 hours after 1928z CAR T cell transfer. Phenotypes were analyzed by flow cytometry and absolute quantification was performed by the addition of counting beads. (No tumor no CAR n=5 mice, CAR only n=5 mice, Tumour only n=6 mice, Tumour + CAR n=7 mice). Enumeration of myeloid cells under these conditions was performed at least 10 times. (Unpaired two sample *t*-test, two-tailed).

c. Representative flow cytometric plot showing Total Peritoneal Macrophages within the gated population (Resident Peritoneal Macrophages F4/80^{hi} Ly6C^{neg-lo} and CRS-Associated

Macrophages F4/80^{int-lo} Ly6C^{int-hi}). Cells were obtained from peritoneal lavage. Analysis of peritoneal macrophages for levels of F4/80 and Ly6C was performed at least 10 times.

d. Absolute counts of myeloid cell populations obtained from multiple organs 18 hours after 1928z CAR T cell transfer (Tumour only n=4 mice, Tumour + CAR n=4 mice).

Enumeration of cells retrieved from peritoneum, spleen and bone marrow under CRS conditions was performed at least 3 times. (Unpaired two sample *t*-test, two-tailed).

e and f. Fold change of pro-inflammatory cytokine gene expression in myeloid populations as determined by RNAseq analysis. Fold change was determined by comparing each population under tumour only and tumour + CAR conditions. Significant downregulation (green bars), significant upregulation (red bars), no significant change (gray bars). Gene expression levels were determined from cells obtained from Tumour only n=9 mice and Tumour + CAR n=9 mice. Cells from three mice of the same group were randomly pooled to create one biological replicate group to the end of obtaining three biological replicate groups of Tumour only mice and three biological replicate groups of Tumor + CAR mice. (Binomial test, FDR-adjusted p-values).

All data are means $\pm$ s.e.m. except in (d) which are means $\pm$ s.d.

Figure 3: Modulation of macrophage function determines CRS severity.

a. Weight change of tumour bearing mice after 1928z CAR T cell transfer. Weight per mouse is normalised to starting weight pre-CAR transfer (Tumour only n=8 mice, 1928z-LNGFR n=7 mice, 1928z-mCD40L n=5 mice). Results from one experiment. (Two-way ANOVA).

b. Percent survival of tumour bearing mice treated with 1928z-LNGFR or 1928z-mCD40L CAR T cells. (1928z-LNGFR n=16 mice, 1928z-mCD40L n=13 mice). Results pooled from two independent experiments. (Log-rank Mantel-Cox test).

c. Representative flow cytometric plot showing Total Peritoneal Macrophages within the gated population (Resident Peritoneal Macrophages and CRS-Associated Macrophages). Cells were obtained by peritoneal lavage. Analysis of peritoneal macrophages for levels of F4/80 and Ly6C in the presence of the 1928z-mCD40L CAR are representative of one experiment of cells obtained from Tumour only n=8 mice, 1928z-LNGFR n=7 mice, 1928z-mCD40L n=5 mice. Experiment was performed once.

- d. Percent of CD40+ total peritoneal macrophages, obtained by peritoneal lavage at 61 hours post 1928z-LNGFR or 1928z-mCD40L CAR T cell transfer, analyzed by flow cytometry (Tumour only n=8 mice, 1928z-LNGFR n=7 mice, 1928z-mCD40L n=5 mice). Analysis of peritoneal macrophages for surface expression of CD40 in the presence of the 1928z-mCD40L CAR was performed once. (Unpaired two sample *t*-test, two-tailed).
- e. Serum levels of murine cytokines at 18 hours post CAR T cell transfer. Cytokine levels were measured by Cytokine Bead Array (CBA). (Tumour only n=8 mice, 1928z-LNGFR n=7 mice, 1928z-mCD40L n=7 mice). Serum cytokine measurements when using the 1928z-mCD40L CAR were performed once. (Unpaired two sample *t*-test, two-tailed).
- f. Percent of myeloid populations from peritoneum, spleen and bone marrow expressing iNOS protein in tumour only mice and tumour + CAR mice. iNOS expression was determined by intracellular flow cytometry. (For peritoneum n=14 mice per group, for spleen and bone marrow n=10 mice per group). For peritoneum results are shown from three pooled independent experiments. For spleen and bone marrow results are shown from two pooled independent experiments. (Unpaired two sample *t*-test, two-tailed).
- g. Weight change of tumour bearing mice after 1928z CAR T cell transfer. Weight per mouse is normalised to starting weight pre-CAR transfer. Mice were treated with L-NIL or vehicle (PBS). (Tumour only n=7 mice, CAR + L-NIL n=7 mice, CAR + Vehicle n=8 mice). Weight measurement under conditions of CRS was performed once with L-NIL and once with 1400W (Supplementary 7b). (Two-way ANOVA).
- h. Percent survival of tumour bearing mice after 1928z CAR T cell transfer receiving 1400W or Vehicle (PBS). (Vehicle n=20 mice, 1400W n=19 mice). Results pooled from three independent experiments. (Log-rank Mantel-Cox test).
- All data are means $\pm$ s.e.m.

Figure 4: IL-1Ra protects from severe CRS without compromising antitumour efficacy.

a and b. Fold change of IL-1 signaling component gene expression in myeloid populations as determined by RNAseq analysis. Fold change was determined by comparing each population under tumour only and tumour + CAR conditions. Significant downregulation (green bars), significant upregulation (red bars), no significant change (grey bars). Gene expression levels were determined from cells obtained from Tumour only n=9 SCID-beige mice and Tumour + CAR n=9 SCID-beige mice. Cells from three mice of the same group were randomly pooled to create one biological replicate group to the end of obtaining three biological replicate groups of Tumour only mice and three biological replicate groups of Tumour + CAR mice. (Binomial test, FDR-adjusted p-values).

c. Percent survival of tumour bearing SCID-beige mice after 1928z CAR T cell transfer receiving Anakinra or Vehicle (PBS). (Anakinra n=11 mice, Vehicle n=10 mice). Results pooled from two independent experiments. (Log-rank Mantel-Cox test).

d. Percent of peritoneal macrophages expressing iNOS at 18 hours post CAR T cell transfer. SCID-beige mice were treated with isotype, murine IL-6 blocking antibody, Anakinra or

murine IL-6 blocking antibody + Anakinra. (Tumour only n=4 mice, Isotype n=3 mice, Anti-mIL-6 n=3 mice, Anakinra n=3 mice, Anti-mIL-6 + Anakinra n=4 mice). Measurement of %iNOS+ macrophages with isotype or anti-mIL-6 blockade was performed in two independent experiments. Measurement of %iNOS+ macrophages with isotype, Anakinra or Anakinra + anti-mIL-6 blocking antibody was performed once. (One-way ANOVA).

e. Levels of murine IL-1Ra in supernatants of 1928z-LNGFR and 1928z-mIL1Ra transduced CAR T cells after 48 hours in culture as determined by ELISA. One sample analyzed in duplicate for each CAR construct. Measurement of IL-1Ra in supernatants of 1928z-mIL1Ra CAR T cells was performed once.

f. Percent survival of tumour bearing SCID-beige mice after 1928z-LNGFR or 1928z-mIL1Ra CAR T cell transfer. (1928z-LNGFR n=22 mice, 1928z-mIL1Ra n=18 mice). Results from three pooled independent experiments. (Log-rank Mantel-Cox test).

g. Serum levels of murine cytokines from SCID-beige mice surviving CRS at 18 hours post CAR T cell transfer. Tumour bearing mice received 1928z-LNGFR or 1928z-mIL1Ra CAR T cells. Cytokine levels were measured by Cytokine Bead Array (CBA) (1928z-LNGFR n=3 mice, 1928z-mIL1Ra n=3 mice). Results representative of three independent experiments. (Unpaired two sample *t*-test, two-tailed).

h. Percent tumour free survival of NSG mice receiving 0.2e6 or 0.5e6 1928z-LNGFR or 1928z-mIL1Ra CAR T cells. Tumours were injected intravenously on Day -4 and CAR T cells on Day 0. (Tumour only n=4 mice, 0.2e6 1928z-LNGFR n=7 mice, 0.2e6 1928z-mIL-1Ra n=7 mice, 0.5e6 1928z-LNGFR n=11 mice, 0.5e6 1928z-mIL-1Ra n=11 mice). Experiment was performed once. (Log-rank Mantel-Cox test).

All data are means $\pm$ s.e.m.