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Bone marrow stroma impairs CAR-T cell proliferation and function mechanistic insights

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King's College London

Faculty of Life Sciences and Medicine

School of Cardiovascular and Metabolic Medicine & Science

Thesis submitted for the degree in Doctor of Philosophy in Stem
Cell and Regenerative Medicine

“Bone marrow stroma impairs CAR-T cell proliferation and
function: mechanistic insights.”

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2023

Declaration

I hereby declare and confirm that the work presented in this study is my own, except where otherwise stated.

Roberto Savoldelli

September 2023

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Abstract

Chimeric Antigen Receptor (CAR)-T cells have proven highly successful in the treatment of some haemopoietic neoplasms. However, acute myeloid leukaemia (AML) remains an elusive target and this has been attributed to an unfavourable immunosuppressive microenvironment. We hypothesised that bone marrow mesenchymal stromal cells (BM-MSC) acquire immunosuppressive properties after exposure to the by-stander effects of activated CAR-T cells against leukemic cells. We used a CAR-T cell consisting of a CD33 binder and CD28z-OX40 costimulatory moiety. BM-MSC were exposed to the CAR following in vitro activation on CD33-coated plates for 24 hours. To model the different ways by which stromal cells can license their immunosuppressive activity, BM-MSC were exposed to the supernatant harvested from the cultures of activated CARs or in direct contact with the activated CARs themselves. In the first case, after incubation with the CAR-conditioned medium (CAR-cm), BM-MSC was investigated for their transcriptome by RNASeq. Gene ontology analysis of the transcriptome revealed a strong NF- κ B and STAT1 signature in BM-MSC exposed to the CAR-cm compared to those which were not exposed. Amongst the most upregulated molecules, we identified several chemokines (CXCL10, CCL8, CXCL8, and CXCL9), IL-6, indoleamine-2,3 dioxygenase (IDO), and most prominently PTGES and PTGS2. We then interrogated the immune functional profile by incubating by CAR-cm licensed BM-MSC with fresh CD33-activated CAR-T cells. We observed that CAR proliferation, cytotoxicity and cytokine production was significantly inhibited. CAR function was restored if Rel-A – a REL-associated protein critically involved in NF- κ B heterodimer formation, nuclear translocation and activation – was inhibited in BM-MSC by shRNA

before exposure to CAR-cm. We previously described that BM-MSC undergo apoptosis when in contact with activated cytotoxic T cells and that this also triggers their immunosuppressive capability. Therefore, we evaluated the impact of cell contact dependent mechanisms on BM-MSC licensing. CD33-activated CAR-T cells were incubated with BM-MSC and the proportion of annexin-V positive BM-MSC was assessed after 4 hours. We observed that a large fraction of BM-MSC underwent apoptosis and that this was dependent on caspase activation, but not RIP kinase 3, and the release of cytolytic granules in a contact-dependent fashion. The supernatant of apoptotic BM-MSC but not the terminally apoptotic and neither the live cells exhibited a strong inhibitory activity on CAR-T cell proliferation, cytotoxicity and effector cytokine production. Similarly to what described with the CAR-cm induced profile, BM-MSC immunosuppression required NF- κ B and was effected via the COX2 pathway. We finally tested the impact of apoMSC in vivo in a xenograft model of AML. CD33-positive KG1 cells were injected in NSG mice and 2 weeks later, CD33-specific CAR-T were administered with or without (controls) apoMSC. Peripheral blood samples were collected 7, 14, and 21 days after. We observed an inhibitory effect of apoMSC on the in vivo expansion of CAR-T cells which was accompanied by disease progression. Accordingly, the survival of the mice treated with apoMSC was markedly reduced compared to those receiving CAR-T cells only.

We conclude that BM-MSC can be induced to acquire immunosuppressive properties by activated CAR-T cells through 2 different but overlapping mechanisms, both dependent on NF- κ B and COX2 signalling pathway. Our work provides critical information to overcome resistance to CAR-T cell treatment by neutralising the negative effects of the stromal microenvironment.

Table of content:

DECLARATION	I
ACKNOWLEDGMENTS.....	II
ABSTRACT	VI
TABLE OF CONTENT:	VIII
LIST OF ABBREVIATION:.....	XII
LIST OF FIGURES:	XVII
LIST OF TABLES	XIX
CHAPTER I - INTRODUCTION	- 1 -
I.1 THE BONE MARROW MICROENVIRONMENT	- 1 -
I.2 MESENCHYMAL STROMAL CELLS DEFINITION AND BIOLOGY.....	- 2 -
I.3 MSCS PLASTICITY AND ROLE IN IMMUNITY	- 4 -
I.4 MSC DIRECT MECHANISM OF IMMUNOSUPPRESSION	- 7 -
<i>I.4.a. Soluble factors</i>	<i>- 7 -</i>
<i>I.4.b. Membrane bound factors.....</i>	<i>- 8 -</i>
<i>I.4.c. Metabolic reprogramming.....</i>	<i>- 9 -</i>
I.5 MSC INDIRECT IMMUNOMODULATION	- 11 -
<i>I.5.a. Dendritic cells</i>	<i>- 11 -</i>
<i>I.5.b. Regulatory T cells.....</i>	<i>- 12 -</i>
<i>I.5.c. Monocytes and macrophages.....</i>	<i>- 14 -</i>
I.6 UNDERSTANDING THE IN VIVO EFFECT OF MSCS.....	- 20 -
I.7 APOPTOSIS AND IMMUNE SYSTEM	- 22 -
<i>I.7.a. The apoptotic cascade.....</i>	<i>- 22 -</i>
<i>I.7.b. apoptosis a tool for the immune system</i>	<i>- 24 -</i>
<i>I.7.c. The consequences of apoptosis.....</i>	<i>- 30 -</i>
I.8 CAR-T THERAPY	- 34 -

<i>I.8.a. CAR-T cell generations and structural features</i>	<i>- 35 -</i>
<i>I.8.b. Resistance to CAR-T.....</i>	<i>- 40 -</i>
<i>I.8.b.1. Target antigen expression</i>	<i>- 40 -</i>
<i>I.8.b.2 Antigen Loss.....</i>	<i>- 41 -</i>
<i>I.8.b.3 CAR-T intrinsic factors</i>	<i>- 41 -</i>
<i>I.8.b.4 Tumors intrinsic pathway mutation</i>	<i>- 42 -</i>
<i>I.8.b.5 The tumors microenvironment.....</i>	<i>- 42 -</i>
<i>I.8.c. Armouring CAR-T</i>	<i>- 46 -</i>
I.9 AIM AND HYPOTHESIS.	- 49 -
CHAPTER II - MATERIAL & METHODS	- 50 -
II.1 CELL LINE MAINTENANCE.....	- 50 -
II.2 MSCS	- 50 -
<i>II.2.a Bone marrow derived MSC isolation</i>	<i>- 50 -</i>
II.3 GENERATION OF REL-A KNOCK DOWN (KD)MSCS	- 53 -
<i>II.3.a Plasmid DNA preparation</i>	<i>- 53 -</i>
<i>II.3.b Lentivirus production</i>	<i>- 53 -</i>
<i>II.3.c MSC transduction.....</i>	<i>- 54 -</i>
II.4 GENERATION OF APOPTOTIC MSCS AND SUPERNATANT.....	- 55 -
II.5 CAR-T.....	- 57 -
<i>II.5.a Isolation of PBMCs.....</i>	<i>- 57 -</i>
<i>II.5.b CAR-T manufacture</i>	<i>- 57 -</i>
<i>II.5.c T cell activation using recombinant CD33-Fc-6His protein</i>	<i>- 61 -</i>
II.6 MSC RE-EDUCATION	- 61 -
<i>II.6.a RNA extraction.....</i>	<i>- 62 -</i>
<i>II.6.b RNA sequencing and data analysis.....</i>	<i>- 62 -</i>
<i>II.6.c Proliferation assay with re-educated MSCs</i>	<i>- 71 -</i>
II.7 CYTOKINE LEVEL ASSESSMENT	- 75 -
<i>II.7.a ELISA for PGE2</i>	<i>- 75 -</i>

II.7.b Cytokine quantification via Luminex assay.....	- 75 -
II.8 MSC-CAR T CO-CULTURES	- 76 -
II.8.a Apoptotic assay	- 76 -
II.8.b Live cell imaging	- 76 -
II.8.c Blockade assays	- 82 -
II.9 CYTOTOXICITY ASSAYS OF AML CELLS	- 82 -
II.10 FLOW CYTOMETRY ANALYSIS.....	- 83 -
II.11 IN-VIVO EXPERIMENTS.....	- 86 -
II.12 STATISTICAL ANALYSIS	- 87 -
CHAPTER III – RESULTS: CAR-T RELEASED SOLUBLE FACTORS RE-EDUCATE MSCS TOWARDS AN IMMUNOSUPPRESSIVE PHENOTYPE.....	- 92 -
III.1 INTRODUCTION	- 92 -
III.2 SOLUBLE FACTOR RELEASED BY ACTIVATED CAR-T CELLS INDUCE MSCS RE- EDUCATION TOWARDS AN IMMUNOSUPPRESSIVE PHENOTYPE.	- 93 -
III.3 RE-EDUCATED MSCS EXPRESS INCREASING LEVEL OF COX2 AND PGE2 IN A NF-KB DEPENDENT FASHION.....	- 95 -
III.4 CAR-RE-EDUCATED MSCS ARE CAPABLE TO INHIBIT CAR-T CELL PROLIFERATION, CYTOKINE RELEASE AND CYTOTOXICITY BY A NF-KB DEPENDENT MECHANISM. ...	- 102 -
III.5 DISCUSSION	- 109 -
CHAPTER IV - RESULTS : CAR-T CELLS ARE ABLE TO INDUCE CASPASE DEPENDENT APOPTOSIS IN MSCS IN AN ANTIGEN INDEPENDENT MANNER.	- 111 -
IV.1 INTRODUCTION	- 111 -
IV.2 CAR-T CELLS INDUCE CASPASE ACTIVATION AND CONSEQUENT CELL DEATH IN MSCS IN A CONTACT DEPENDENT FASHION.	- 112 -
IV.3 CAR-T INDUCED CELL DEATH IS A “BYSTANDER EFFECT”.	- 119 -
IV.4 FUNCTIONAL IMPACT OF APOPTOTIC MSCS ON CAR-T ACTIVITY.....	- 129 -
IV.5 CAR-T INHIBITION BY APOPTOTIC MSCS MILIEU IS DEPENDENT ON THE NF- KB/COX-2 PATHWAY.	- 135 -
IV.6 DISCUSSION	- 140 -
CHAPTER V – RESULTS: <i>IN-VIVO</i> VALIDATION.....	- 143 -
V.1 INTRODUCTION	- 143 -

V.2 APOPTOTIC MSC ADMINISTRATION IMPAIRS CAR-T CELL PERSISTENCE AND INHIBIT THEIR ANTILEUKEMIC ACTIVITY IN-VIVO.....	- 144 -
V.3 DISCUSSION.....	- 150 -
CHAPTER VI - DISCUSSION & CONCLUSION	- 151 -
VI.1 DISCUSSION.....	- 151 -
VI.2 CONCLUSIONS	- 154 -
- BIBLIOGRAPHY -	- 157 -

List of abbreviation:

ABBREVIATION	MEANING
7AAD	7-Aminoactinomycin D
A-MEM	Alpha-Minimal Essential Medium
ACT	Adoptive Cell Therapy
AML	Acute Myeloid Leukemia
APAF	Apoptotic Protease Activating Factor 1
APC	Allophycocyanin
APC	Antigen Presenting Cells
APOMSC	Apoptotic MSC
ATP	Adenosine Triphosphate
ATP11C	Atpase Phospholipid Transporting 11C
BAI1	Brain-Specific Angiogenesis Inhibitor 1
BCL2	B-Cell Lymphoma 2
BCMA	B-Cell Maturation Antigen
CAD	Caspase-Activated Deoxyribonuclease
CAR	Chimeric Antigen Receptor
CCL	Chemokine C-C Motif Ligand
CD	Cluster Of Differentiation
CDC	Cholesterol-Dependent Cytolysins
CO	Carbon Monoxide
COX2	Cyclo-Oxygenase-2

CRS	Cytokine Release Syndrom
CSF	Colony-Stimulating Factor 1
CTLA	Cytotoxic T-Lymphocyte-Associated Protein
CTV	Cell Trace Violet
CXCL	C-X-C Motif Chemokine
CXCR	C-X-C Chemokine Receptor Type
DAMPS	Danger-Associated Molecular Patterns
DC	Dendritic Cells
DD	Death Domains
DDMSO	Dimethyl Sulfoxide
DEG	Differentially Expressed Genes
DNA	Deoxyribonucleic Acid
DR	Dependence Receptors
E:T	Effector To Target
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic
EGFR	Epidermal Grow Factor Receptor
EGTA	Ethylene Glycol-Bis-(Beta-Aminoethylether)-N,N,N',N'-Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EP	Prostaglandin E2 Receptors
FADD	FAS-Associated Death Domain Protein
FAP	Fibroblast Activating Protein
FAS-L	Fas Ligand

FBS	Fetal Bovine Serum
FDA	Food And Drug Administration
FITC	Fluorescein
FOXP3	Forkhead Box P3
GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
GVHD	Graft-Versus-Host Disease
HEK	Human Embryonic Kidney
HER	Human Epidermal Grow Factor Receptor
HLA	Human Leukocyte Antigen
HMGB1	High Mobility Group 1
HO-1	Heme Oxygenase-1
HSC	Hematopoietic Stem Cells
HSP	Heat-Shock Proteins
IBD	Inflammatory Bowel Disease
ICHTB	Imperial College Healthcare Tissue Bank
IDO	Indoleamine-Pyrrole 2,3-Dioxygenase
IFN	Interferon
IGF1	Insulin-Like Growth Factor 1
IL	Interleukin
IL1-RA	Interleukin 1 Receptor Antagonist
INOS	Inducible Nitric Oxide Synthase
IPLA2	Calcium-Independent Phospholipase A2
IRES	Internal Ribosome Entry Site

ISCT	International Society Of Cellular Theraphy
ITREGS	Induced Tregs
KD	Knock Down
LILRB2	Leukocyte Immunoglobulin-Like Receptor B2
LPC	Phospholipid Lysophosphatidylcholine
LPS	Lipopolysaccharides
MCSF	Macrophage Colony Stimulating Factor
MDSC	Myelo-Derived Suppressor
MFGE8	Like Milk Fat Globule-EGF Factor 8
MOMP	Mitochondrial Outer Membrane Permeabilization
MPC1	Mitochondrial Pyruvate Carrier 1
MSC	Mesenchymal Stromal Cells
NDUSF1	NADH Dehydrogenase Fe-S Protein-1
NK	Natural Killer
NO	Nitric Oxide
NSCLC	Non-Small-Cell Lung Cancer
NSG	NOD.Cg-Prkdcscid Il2rgtm1Wjl/Szj
NTREGS	Natural Tregs
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PC	Principal Component
PCD	Programmed Cell Death
PD1	Programmed Cell Death Protein 1

PDL1	Programmed Death-Ligand 1
PE	R-Phycoerythrin
PE-CY7	R-Phycoerythrin Cyanin 7
PEI	Polyetherimide
PGE2	Prostaglandin E2
PTGES	Prostaglandin E2 Synthetase
RIPK3	Receptor-Interacting Serine/Threonine-Protein Kinase
RNA	Ribonucleic Acid
RNASEQ	Rna Sequencing
SB	Sleeping Beauty
SCF	Stem Cell Factor
SCFV	Single-Chain Variable Fragment
SLE	Systemic Lupus Erythematosus
STAB2	Stabilin 2
STAT	Signal Transducer And Activator Of Transcription
STC1	Stanniocalcin-1
TCR	T-Cell Receptor
TGF-B	Transforming Growth Factor Beta
TH	T-Helper Cell
TLR	Toll-Like Receptor
TMEM16F	Transmembrane Protein 16F
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor

TNP	2,4,6-Trinitrophenyl
TRADD	Tumor Necrosis Factor Receptor Type 1-Associated DEATH Domain Protein
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TREGS	Regulatory T-Cell
TRUCK	T Cells Redirected for Universal Cytokine Killing
TSG-6	Tumour Necrosis Factor-Inducible Gene 6 Protein
UTP	Uridine-5'-Triphosphate
VEGF	Vascular Endothelial Growth Factor
VH	Variable Heavy
VL	Variable Light
XKR8	Xk-Related Protein 8

List of Figures:

Figure	Page
Figure SP1: graphical summarization of msc driven immunosuppression	18
Figure SP2: clinical trials involving MSC	20
Figure SP3: apoptosis and caspase activation cascade schematics	29
Figure SP4: structural components of car construct and generations	39
Figure SP5: project graphical abstract	49

FIGURE SP6: MSC phenotype	52
Figure SP7: transduction level of different batches of mscs rel-akd	54
Figure SP8: apoMSC FACS analysis	56
Figure SP9 : Representation of CAR construct	59
Figure SP10 : representative batch transduction levels and gating strategy of car-t.	60
Figure SP11: graphical representation of analysis pipeline	63
Figure SP12: rnaseq analysis step-by-step results	64
Figure SP13: cd33-fc protein coating beads and efficacy test to activate car-t	73
Figure SP14: example of car-t gating strategy for proliferation assay	74
Figure SP15: gating strategy examples of MSC-CAR co cloture after 4h to identify apoptosis in MSCs recorded by annexin-v/7aad staining and active caspase 3/7	78
Figure SP16: live cell imaging analysis pipeline	79
Figure SP17: example of gating strategy used to analyze cytotoxic assay after car and KG-1 co cloture	84
Figure SP18: example of gating strategy used to analyze intracellular level of cox2 in MSCs after car-cm re-education	85
Figure SP19: example of gating strategy used to analyze samples of murine peripheral blood.	88
Figure 1: activated car t cells re-educate MSCs to acquire an immunosuppressive phenotype.	96
Figure 2: re-educated MSCs impact on CAR-T cells later functions through an NF-κB dependent mechanism.	104

Figure 3: MSCs undergo apoptosis when in contact with activated car-t cells.	114
Figure 4: activated CAR-T cell mediated MSC apoptosis is contact-dependent and requires caspase activation.	121
Figure 5: apoptotic MSC conditioned media but not apoptotic cells for itself inhibit car-t cells functions.	131
Figure 6: MSC immunosuppression of car-t required NF-κB and was affected via the cox2 pathway.	136
Figure 7: apoptotic MSCs inhibit CAR-T antileukemic activity in-vivo.	145

List of Tables

Table	Page
Table 1: MSC donor sex and age	89
Table 2: CAR-T donor sex and age	89
Table 2: blocking and activating antibody.	90
Table 3: inhibitors and small molecules	90
Table 4: flow cytometry antibody	91
Table 5: fluorescent dies used in flow cytometry	91

Chapter I - Introduction

I.1 The bone marrow microenvironment

Bone marrow is a critical component of the hematopoietic system, playing a central role in the generation and maintenance of blood cells. It is a spongy tissue located within the cavities of certain bones, including the pelvis, skull, vertebrae, and long bones. It is composed of a complex microenvironment that includes various cell types, extracellular matrix (ECM) proteins, growth factors, and cytokines. One of the most important cell types in the bone marrow microenvironment is the mesenchymal stem/stromal cell (MSC), which plays a crucial role in the regulation of hematopoietic stem cell (HSC) activity and differentiation (Mendelson & Frenette, 2014; S. J. Morrison & Scadden, 2014).

MSCs are multipotent cells that can differentiate into a variety of mesodermal cell types, such as osteoblasts, chondrocytes, and adipocytes. However, their role in the bone marrow extends beyond their differentiation potential. MSCs secrete various cytokines and growth factors, such as interleukin (IL)-6, IL-8, and transforming growth factor beta (TGF- β), which are crucial for the maintenance of the HSC niche (Méndez-Ferrer et al., 2010; Sánchez-Aguilera & Méndez-Ferrer, 2017)

The HSC niche is a specialized microenvironment that supports the self-renewal and differentiation of HSCs. It is composed of various cell types, including MSCs, osteoblasts, endothelial cells, and immune cells, as well as ECM proteins and growth factors. MSCs play a crucial role in the regulation of

HSC activity and differentiation within this niche. For example, MSCs have been shown to promote HSC quiescence and self-renewal through the secretion of C-X-C motif chemokine 12 (CXCL12), a chemokine that binds to the C-X-C chemokine receptor type 4 (CXCR4) receptor on HSCs (Sugiyama et al., 2006). MSCs also regulate HSC differentiation by producing various growth factors, such as TGF- β and insulin-like growth factor 1 (IGF-1), which can promote myeloid or lymphoid lineage commitment (Frenette et al., 2013).

Other cell types within the bone marrow microenvironment also play important roles in the regulation of haematopoiesis. Osteoblasts, for example, are important regulators of HSC activity and differentiation, and have been shown to promote HSC quiescence through the secretion of various growth factors, such as angiopoietin-1 (Ehninger & Trumpp, 2011). Endothelial cells, on the other hand, play a crucial role in the maintenance of the HSC niche by providing structural support and producing various growth factors, such as vascular endothelial growth factor (VEGF) and stem cell factor (SCF) (Butler et al., 2010).

1.2 Mesenchymal stromal cells definition and biology

In 1970, Friedenstein and colleagues discovered fibroblast-like cells from guinea-pig bone marrow (Friedenstein et al., 1970) that were highly replicative in vitro, could sustain self-maintenance, form colony units, and differentiate into various mesenchymal cell lineages. These cells were initially considered "stem cells" due to their high clonogenic potential and multi-potency features

and were systematically referred to as mesenchymal stem cells from the early 1990s (Caplan, 1991).

While cells with analogous characteristics have been successfully isolated and expanded from almost all other tissues (da Silva Meirelles et al., 2006), including placenta (Igura et al., 2004), umbilical cord (Erices et al., 2000), adipose tissue (Zannettino et al., 2008), and dental pulp (Gronthos et al., 2000), successful MSC isolation depends on several poorly defined factors, and MSC heterogeneity has been described within species (Hass et al., 2011), tissue preparations (Bieback et al., 2008; in 't Anker et al., 2003), and even within the same donor isolations (Mets & Verdonk, 1981; Yang et al., 2018).

Due to its ubiquitous presence, MSCs are believed to be part of the supportive network of most organs, but there is still a lack of understanding of whether there are common mesenchymal progenitors throughout the body or if the progenitors are specialized and site-specific. Their ontogeny is also point of debate, to date there are no evidence that can attribute their origin to a specific germ line, however studies have described mesenchymal like cells originated from both mesoderm and ectoderm layers (Crisan et al., 2008; da Silva Meirelles et al., 2008). The International Society of Cellular Therapy (ISCT) proposed minimal and largely incomplete criteria to define MSC in 2006, which included (1) plastic adherence, (2) a phenotype characterized by Cluster of Differentiation (CD) CD73+, CD90+, CD105+, CD45-, CD34-, CD14-, CD19-, MHC-II- and the ability to differentiate into mesenchymal cell types like chondrocytes (Aggrecan+, COL2A1+, SOX9+), osteoblast (Runx2+, ALP+, Osteoclastin+) and adipocytes (PPARy+, FABP4+, Adipsin+) (Dominici et al., 2006). However, this definition has been criticized for its substantial overlap

with the traditional characterization of other stromal cells, such as fibroblasts and pericytes (Crisan et al., 2008; Haniffa et al., 2007; Sudo et al., 2007). Therefore, it is plausible to consider that these fibroblast-like cells are equivalent or related cell types to MSCs (Haniffa et al., 2009; Sudo et al., 2007).

I.3 MSCs plasticity and role in immunity

Stromal cells, including MSCs, have a wide distribution across tissues and are thought to have crucial roles in maintaining tissue function and responding to diseases. Their interactions with other cell types in the tissue are vital for ensuring proper organ function, with a strong regulatory relationship between stromal and immune cells during inflammatory responses. The immunobiology of stromal cells is marked by features such as plasticity, immunogenicity, and immunosuppression, which contribute to their importance in physiological and pathological processes.

MSCs are known for their functional plasticity, which is dependent on their microenvironment. Exposure to soluble inflammatory mediators and cell-cell contact mechanisms play a crucial role in modulating the functional status of MSC. Pro-inflammatory cytokines like Interferon Gamma (IFN- γ), Tumour Necrosis Factor Alpha (TNF- α), and IL-1b "license" MSC to acquire immunosuppressive abilities by inducing the expression of immunosuppressive molecules such as Indoleamine-pyrrole 2,3-dioxygenase (IDO) (Krampera et al., 2006). In some cases, cytokine-primed MSC can also upregulate molecules promoting antigen-presenting cell-like functions (Chan et al., 2006). Additionally, specific Toll-Like Receptor (TLR)-activating ligands can induce MSC to acquire pro-inflammatory functions (Romieu-Mourez et al., 2009; Waterman et al., 2010). Human and murine MSC express different TLRs (Liotta

et al., 2008; Pevsner-Fischer et al., 2007), and their activation can modulate immune responses. TLR-4-activated MSC produce pro-inflammatory mediators such as IL-1b, IL-6, IL-8, and TGF-b, recruit innate immune cells, and fail to suppress T cell activation. TLR-3-primed MSC produce immunosuppressive molecules like IDO and Prostaglandin E2 (PGE2), which inhibit T cell proliferation. This suggests that MSC can be polarized towards a more pro-inflammatory phenotype (MSC1) or an anti-inflammatory one (MSC2), similar to the polarization observed in macrophages (Waterman et al., 2010). However, it remains to be determined if these two phenotypes exist in nature.

In addition to soluble factors, cell-cell contact mechanisms also contribute to the functional plasticity of MSC. Recent studies have shown that activated cytotoxic cells can license MSC by inducing caspase-dependent apoptosis, and the consequent efferocytosis of apoptotic MSC by professional phagocytes results in potent immunosuppression both in vitro and in vivo (Galleu et al., 2017). However, it remains unclear whether the apoptotic MSC themselves are able to directly exert immunosuppressive effects.

MSCs were initially believed to be immune privileged, as they effectively suppressed T cell proliferation in mixed lymphocyte reactions even though they were of allogeneic origin (Le Blanc et al., 2003). However, subsequent in vitro experiments showed that, although MSC exhibited potent anti-proliferative abilities towards lymphocytes, they induced alloantigen priming and failed to elicit T cell proliferation due to compensatory immunosuppressive mechanisms (Klyushnenkova et al., 2005). Intravenous MSC injections were found to trigger systemic inflammatory responses in mice, as evidenced by increased numbers of macrophages in the lungs, along with elevated levels of CXCL1, Monocyte chemoattractant protein 1 (MCP1), and IL-6 in the serum (Hoogduijn et al., 2013). Interestingly, these animals developed an attenuated

immune reaction to LPS a few days after infusion. Similarly, the administration of MSC in a heart transplant model resulted in T cell infiltration in the heart and subsequent graft rejection if immunosuppressants were not co-administered (Eggenhofer et al., 2012). Similar findings were observed in a mouse model of graft-versus-host disease (Nauta et al., 2006).

Studies tracking the fate of MSCs in animal models have revealed that allogeneic MSCs persist for a shorter duration after infusion compared to syngeneic cells and are capable of inducing immune memory (Zangi et al., 2009). In clinical studies, evidence of MSC immunogenicity has been reported, with the presence of anti- Human Leukocyte Antigen (HLA) donor-specific antibodies detected in patients who received MSC infusions. However, the occurrence of anti-HLA donor-specific antibodies was not consistently associated with a particular clinical outcome (García-Sancho et al., 2017; Mayer et al., 2013).

MSCs possess the ability to interact with a wide range of cells of the immune system and influence their phenotype and function efficiently. The mechanisms employed by MSCs enable them to be potent immunomodulatory entities that can affect immune responses in different conditions. MSCs can directly exert their effect towards adaptive and innate effector cells, resulting in immunosuppression. Alternatively, MSCs can communicate with myeloid and regulatory cells, which can indirectly modulate immune responses to achieve a more potent and long-lasting immunosuppressive effect. Both of these mechanisms can occur simultaneously in vivo and are not exclusive. It is important to note that immunosuppression occurs with both autologous and allogeneic MSC sources, indicating that it is not antigen-specific and is not restricted by major histocompatibility complex (Cheung & Dazzi, 2018).

I.4 MSC direct mechanism of immunosuppression

I.4.a. Soluble factors

MSCs possess immunomodulatory properties through various mechanisms. MSCs secrete immunomodulatory cytokines such as TGF- β , IL-6, and human HLA-G that directly act on effector immune cells. TGF- β is a potent factor released by MSCs that inhibits T cell proliferation by inducing cyclin-dependent kinase inhibitors and eliminating proliferative mediators. In vitro, MSCs express TGF- β after contact with activated T cells, and its neutralization with specific monoclonal antibodies is sufficient to inhibit MSC anti-proliferative response (Nasef et al., 2007; Nicola et al., 2002). Similarly, IL-6 mediates PGE2 release from MSCs and orchestrates subsequent immunosuppression in a model of murine arthritis (Deng et al., 2016). Additionally, HLA-G is involved in the immunomodulatory action of MSCs as they secrete the soluble form of HLA-G5 after contact with stimulated T cells in an IL-10 dependent manner (Selmani et al., 2008). Furthermore, Tumour necrosis factor-Inducible Gene 6 Protein (TSG-6) is another soluble molecule that is employed by MSCs to modulate inflammation. Pro-inflammatory cytokines like TNF- α and IL-1 β can stimulate the secretion of TSG-6, which in turn modulates inflammation. The MSC-related anti-inflammatory roles of TSG-6 have been documented in vivo in a murine model of myocardial infarction, pancreatitis, and colitis (R. H. Lee et al., 2009; Sala et al., 2015).

I.4.b. Membrane bound factors.

In addition to the soluble factor-mediated immunosuppression, membrane-bound molecules on MSCs play an essential role in their immunomodulatory effects. One such membrane-bound molecule is HLA-G, a non-classical major histocompatibility complex molecule. HLA-G can inhibit the activation of various immune cells, such as T cells, natural killer (NK) cells, B cells and dendritic cells, leading to a potent immunosuppressive effect. HLA-G binds to its receptors, such as the leukocyte immunoglobulin-like receptor B2 (LILRB2) and induces immune tolerance by inhibiting T cell proliferation by disrupting cell cycle, lowering the IFN- γ levels, increasing IL-10, and inducing T cell apoptosis (Giuliani et al., 2011). Moreover, HLA-G can modulate the activity of DCs by reducing the expression of co-stimulatory molecules and down-regulating the production of pro-inflammatory cytokines (Götherström et al., 2004). Another membrane-bound molecule involved in MSC-mediated immunomodulation is programmed death-ligand 1 (PD-L1), which interacts with its receptor programmed cell death protein 1 (PD-1) on T cells to inhibit their activation and proliferation (Sheng et al., 2008). The expression of PD-L1 on MSCs can be upregulated by pro-inflammatory cytokines, such as IFN- γ , further enhancing their immunomodulatory effects (Augello et al., 2005). In addition, the engagement of the Notch receptor on T cells with its ligand Jagged-1 on MSCs can also contribute to the immunosuppressive effects of MSCs by inducing T cell anergy and promoting regulatory T cell (Tregs) differentiation (Liotta et al., 2008). Moreover, TLR3 activation in MSCs can upregulate Jagged-1 expression and contribute to MSC-mediated immunosuppression through Notch-dependent PGE2 production (Qiu et al., 2018). Thus, membrane-bound molecules on MSCs play a crucial role in their immunomodulatory effects, and understanding their mechanisms of action

could help develop more effective MSC-based therapies for various immune-mediated disorders. Schematics of MSC immunosuppression is depicted in figure SP1.

1.4.c. Metabolic reprogramming

One of MSC most recognized and extensively studied mechanisms involves the reprogramming of immune cells via metabolic enzymes. The enzyme IDO is the best-characterized enzyme in this process. IDO promotes the conversion of tryptophan to kynurenine, which leads to the depletion of the essential amino acid tryptophan and the accumulation of its toxic by-products. This results in the suppression of immune cell activation and proliferation, affecting a wide range of targets including T cells, NK cells, and dendritic cells (François et al., 2012; Krampera et al., 2006; Ryan et al., 2007; Tipnis et al., 2010). Importantly, IDO expression is not constitutive but is induced by IFN- γ , usually released by activated T cells, NK cells, and monocytes. Exogenous addition of IFN- γ in the cultures is sufficient to elicit MSC-mediated immunosuppressive effects also in B lymphocytes (Krampera et al., 2006).

Although the majority of studies have documented the fundamental function of IDO in MSC-mediated immunosuppression, some circumstances have shown that human MSCs lacking both IFN- γ receptor 1 and IDO still exert immunosuppression. This suggests that other mechanisms are likely to take part in MSC-mediated immunosuppression (Gieseke et al., 2007). Another metabolic enzyme that plays an important role in MSC-mediated immunosuppression is cyclo-oxygenase-2 (COX-2), which regulates the synthesis of PGE2 from arachidonic acid. PGE2 acts as a potent

immunosuppressive factor and enhances immunosuppression in different ways, including the inhibition of T cell proliferation and suppression of IL-2 and IFN- γ (Aggarwal & Pittenger, 2005; Spaggiari et al., 2009).

Murine MSCs, on the other hand, employ the metabolic action of the enzyme inducible nitric oxide synthase (iNOS) to exert immunosuppression. iNOS catalyses the production of nitric oxide (NO) from L-arginine, which affects macrophage and T-cell functions. iNOS is induced in MSCs after exposure to several pro-inflammatory cytokines and efficiently inhibits splenocyte proliferation when in close proximity to the target cells. Inhibition of Signal transducer and activator of transcription (STAT) 5 phosphorylation was described as a possible mechanism mediating T cell suppression (Ren, Zhang, et al., 2008; Sato et al., 2007).

In rats, the metabolic pathway elicited by stromal cells relies on the enzyme heme oxygenase-1 (HO-1), which is induced in MSCs by pro-inflammatory cytokines and stress-related stimuli. HO-1 catalyses the conversion of heme into biliverdin, releasing free iron and carbon monoxide (CO), which exert strong anti-inflammatory and anti-oxidative properties. Chabannes and colleagues demonstrated that inhibition of HO-1 together with iNOS partially restored T cell proliferation in vitro and was sufficient to abolish the protective activity of MSCs in heart allograft rejection in vivo (Chabannes et al., 2007)

I.5 MSC indirect immunomodulation

In addition to the direct effect of MSC membrane bound and secreted factors on the functions and proliferation of lymphocytes and NK cells, the interaction of MSC with other immune regulatory cells plays a crucial role in promoting long-term immunosuppressive effects. While most studies have shown that myeloid cells such as monocytes, macrophages, and dendritic cells play an important role in mediating MSC-induced immunosuppression, there is also strong evidence supporting the importance of Tregs cells in suppressing inflammation and promoting immunomodulation.

I.5.a. Dendritic cells

Dendritic cells (DCs) are specialized Antigen Presenting Cells (APC) that present antigens on both MHC class I and II molecules, stimulating CD4 and CD8 T cells (Mellman & Steinman, 2001). The maturation state of DCs influences their behaviour, with immature DCs being better at phagocytosis but less capable of priming T cells due to lower expression levels of costimulatory molecules such as CD80 and CD86 and cytokines like IL-12 (Mellman & Steinman, 2001; Ramasamy et al., 2007). It has been shown that MSCs can impair the differentiation and antigen-presenting function of DCs through various mechanisms. Soluble factors secreted by MSCs such as IL-6 (Djouad et al., 2007), TSG-6 (Y. Liu et al., 2014), and PGE2 (B. Zhang et al., 2009) prevent DC differentiation and maturation (Jiang et al., 2005). Additionally, MSCs can polarize mature DCs into a regulatory-like phenotype that produces less IL-12 but more IL-10 and TGF- β , inhibiting T cell priming (Ramasamy et al., 2007). Furthermore, MSCs can induce

a regulatory-like DC phenotype through cell-cell contact and Jagged-1 expression, leading to the expansion of CD4⁺CD25⁺ forkhead box P3 (FOXP3) ⁺ Tregs (Cahill et al., 2015). This regulatory DC phenotype expresses lower levels of HLA-DR and CD86.

Recent studies have also shown that MSCs can inhibit DC differentiation and function through the downregulation of TLRs on DCs(Jiang et al., 2005). TLR signalling is essential for DC maturation, and MSCs can downregulate TLR expression on DCs by secreting factors such as TGF- β and IDO. MSCs can also induce a tolerogenic DC phenotype by promoting the secretion of immunomodulatory cytokines such as IL-10, IL-27, and IL-35. This phenotype leads to the expansion of Tregs and the suppression of effector T cells(Li et al., 2008). Moreover, MSCs can inhibit DC migration to lymphoid tissues by downregulating the expression of chemokine receptors on DCs. This inhibition of DC migration limits their ability to prime T cells, contributing to the immunosuppressive effects of MSCs(Rasmusson et al., 2007).

I.5.b. Regulatory T cells

Tregs are categorized into two types: natural Tregs (nTregs) and induced Tregs (iTregs)(Sakaguchi et al., 2008). nTregs are matured and derived from the thymus and constitutively express CD4, CD25, and FOXP3, while iTregs are generated from peripheral naive T cells in the presence of specific cytokines(Sakaguchi et al., 2008). Both nTregs and iTregs exhibit similar in vitro immunosuppressive functions such as anti-proliferation of stimulated T cells

and the production of immunosuppressive cytokines like IL-10 and TGF- β (English et al., 2009). nTregs have additional regulatory mechanisms, including the killing of effector T cells, metabolic disruption, and inhibition of DC maturation and function (Vignali et al., 2008). MSCs have been demonstrated to induce the generation of Tregs both in vitro and in vivo. For example, MSCs can induce the generation of Tregs in T-Helper (Th)1 (Maccario et al., 2005), Th2 (Kavanagh & Mahon, 2011), and Th17 (Luz-Crawford et al., 2013) environments to dampen their corresponding inflammations. This Treg induction is mediated by cell-cell contact, production of soluble factors, and interaction with the myeloid compartment (Cahill et al., 2015). MSCs induce functional Tregs through the secretion of HLA-G5 (Selmani et al., 2008), IDO (Su et al., 2014), COX2/PGE2 (English et al., 2009; Ghannam et al., 2010), TGF- β (Nemeth et al., 2010), and HO-1 (Mougiakakos et al., 2011). MSCs can also cooperate with immature dendritic cells and M2-like macrophages to induce Tregs. In one study, MSCs skew mature DCs to the immature form, and co-culture of these immature DCs with CD4+CD25-FOXP3- T cells generated more CD4+CD25+FOXP3+ Tregs (Cahill et al., 2015). In another example, MSCs promote monocytes' differentiation into M2-like macrophages expressing CD206 and IL-10 and secrete Chemokine C-C motif ligand 18 (CCL18) to promote Treg induction (Melief et al., 2013). The interplay between MSCs, the MPS, and Tregs has been proposed as fundamental to tease out the dynamics of stromal cell immunomodulation, particularly about the impact on the long-term effects in vivo (Cheung & Dazzi, 2018).

Recent studies have demonstrated that MSCs can induce the generation of Tregs through epigenetic modifications. For instance, MSCs can increase the expression of FOXP3, a transcription factor critical for Treg function, through histone modifications and DNA methylation (Duffy et al., 2011; Oh et al., 2019).

Additionally, MSCs can induce Treg generation by transferring mitochondria to T cells. Mitochondrial transfer from MSCs to T cells enhances T cell bioenergetics and promotes Treg differentiation (W. Zhang et al., 2004; Z. Zhou & You, 2016).

I.5.c. Monocytes and macrophages

Monocytes are highly versatile immune cells capable of migrating from blood to tissues and differentiating into macrophages or dendritic cells, depending on the inflammatory milieu (Serbina et al., 2008; Swirski et al., 2009). MSCs have been shown to recruit monocytes through the secretion of CCL2 during infection and tissue repair (L. Chen et al., 2008; C. Shi & Pamer, 2011). In a coxsackievirus B3-induced heart injury animal model, infusion of MSCs led to a reduction in pro-inflammatory monocytes (Ly6C high and Ly6C int) and an increase in anti-inflammatory monocytes (Ly6C low), promoting tissue repair (Miteva et al., 2017). These Ly6C low monocytes are thought to differentiate into alternatively activated (M2) macrophages during tissue repair (Geissmann et al., 2010). In a mouse model of allergic airway inflammation, CCL2-deficient MSCs were unable to recruit monocytes and suppress airway inflammation, highlighting the importance of CCL2 in monocyte recruitment by MSCs (Takeda et al., 2018). Interestingly, IFN- γ treatment can upregulate CCL2 production by MSCs, underscoring the significance of MSC activation status (Takeda et al., 2018). While the exact mechanisms of MSC-mediated monocyte recruitment remain unclear in some studies, the influx of monocytes or monocyte-like myeloid cells following MSC infusion has been reported to be important for tissue repair (Ko et al., 2016).

Macrophages are a type of immune cell with remarkable plasticity that responds to environmental cues (Mosser & Edwards, 2008). They typically reside in different tissues in the body and are specialized in different functions depending on their anatomical locations, maintaining homeostasis (C. Shi & Pamer, 2011). Upon encountering immune stimuli, macrophages are divided into two main categories: the classically activated (M1) macrophages that produce a variety of pro-inflammatory cytokines and promote Th-1 or Th-17 development, and the M2 macrophages that produce anti-inflammatory cytokines and promote Th-2 and Tregs development (Martinez & Gordon, 2014). During the resting state, most macrophages in different tissues are derived from embryonic yolk sac progenitors rather than haematopoiesis, but they can also be replaced by infiltrating monocytes during inflammation (Gomez Perdiguero et al., 2015; Taylor & Gordon, 2003).

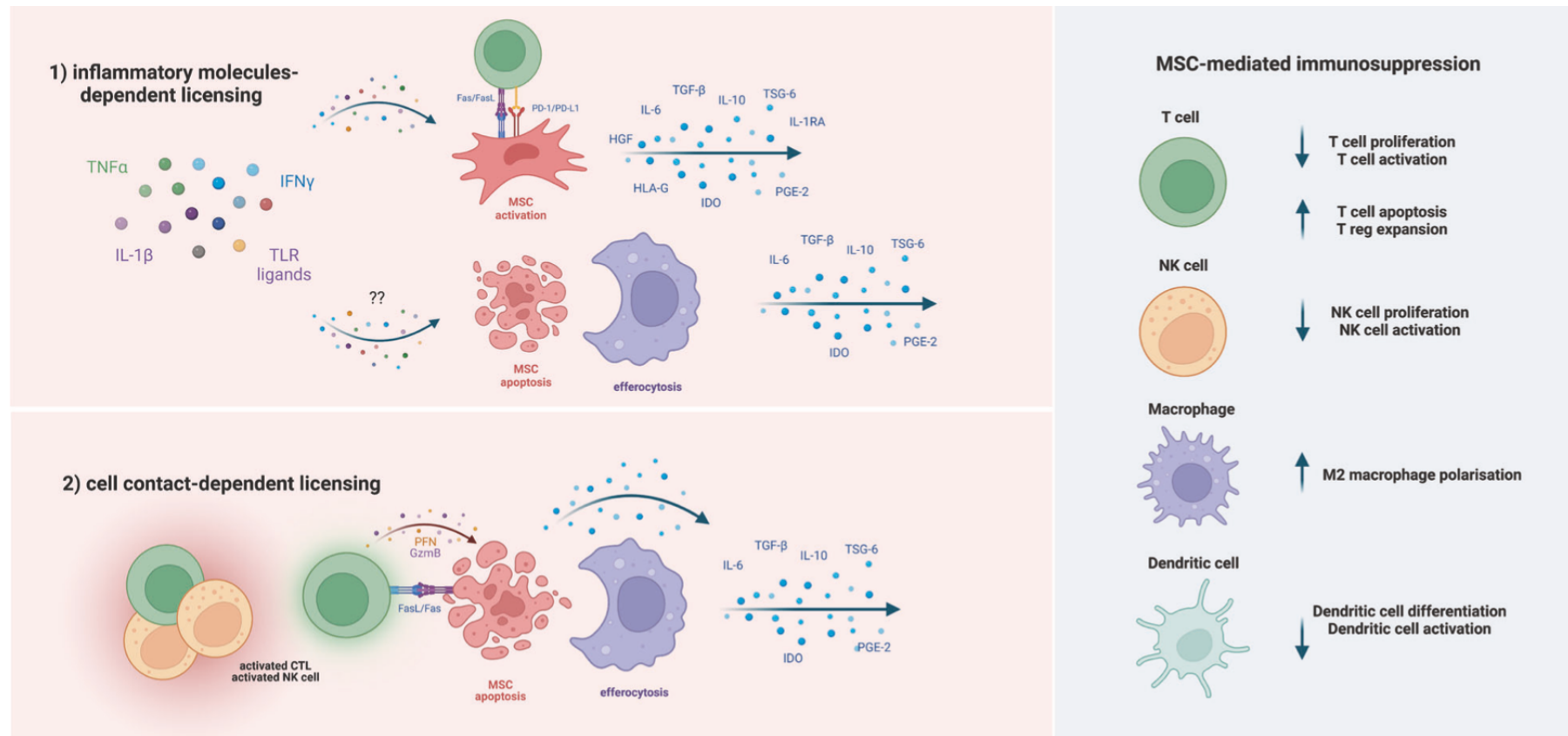
Studies have shown that MSCs can promote monocyte differentiation into M2 macrophages. When treated with colony-stimulating factor 1 (CSF-1/M-CSF), MSCs up-regulate the M2 surface marker CD206 and increase the production of IL-10 and TGF- β in monocytes. The phagocytic efficiency of these macrophages against *Escherichia coli* is also improved, thus confirming M2 differentiation (J. Kim & Hematti, 2009). It has been suggested that IDO and COX2/PGE2 are responsible for MSC-mediated M2 differentiation (Ge et al., 2010). The secretion of IL-10 is dependent on the expression of IDO in MSCs, as inhibition of IDO reverses this effect (François et al., 2012). On the other hand, MSCs can differentiate monocytes into M2-like macrophages through their COX2/PGE2 activity under the induction of Macrophage colony stimulating factor (M-CSF) (Maggini et al., 2010). These M2-like macrophages suppress T cell proliferation and NK cell activation through IL-10 and TGF- β secretion (Chiossone et al., 2016).

Recently, it has been shown that MSCs can also reprogram monocyte differentiation through metabolic activities such as lactate production (Selleri et al., 2016). When monocytes are co-cultured with MSCs in the presence of IL-4 and Granulocyte-Macrophage colony stimulating factor (GM-CSF), they fail to differentiate into DC and instead acquire an M2 profile (Selleri et al., 2016). These M2-like macrophages promote Th-2 or Tregs differentiation from naïve CD4 T cells. Furthermore, an in vitro study demonstrated that MSCs can induce M2 polarization by reducing the secretion of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-12p70 while enhancing the secretion of IL-10 in Lipopolysaccharides (LPS)-stimulated macrophages through COX2/PGE2 activity (Selleri et al., 2016).

These primed macrophages also exhibit a low intensity of costimulatory molecules CD86 and HLA-DR, which limits their ability to stimulate CD4 T cell proliferation (Németh et al., 2009). This effect has also been observed in a murine sepsis model where administration of murine bone marrow-derived MSCs improved organ function and life span compared to the control group, and the beneficial effects were abolished when using neutralizing antibodies against IL-10 and its receptor or clodronate-liposomes (van Rooijen & Hendrikx, 2010) to deplete myeloid phagocytes (Nemeth et al., 2009).

In addition to COX2/PGE2, MSCs secrete other soluble factors such as TSG-6 and interleukin 1 receptor antagonist (IL-1RA) that can polarize macrophages towards the M2 phenotype (Arena et al., 1998; H. Choi et al., 2011; K. C. Lee et al., 2015; Luz-Crawford et al., 2016). Moreover, MSCs can also induce M2 macrophage polarization through mitochondrial transfer, which modulates oxidative phosphorylation in conditioned macrophages resulting in a decrease of TNF- α secretion, improvement of phagocytosis and increase of CD206 expression (T. J. Morrison et al., 2017). Figure SP1 summarize the interaction between MSCs and immune cells.

Figure SP1: graphical summarization of MSC driven immunosuppression



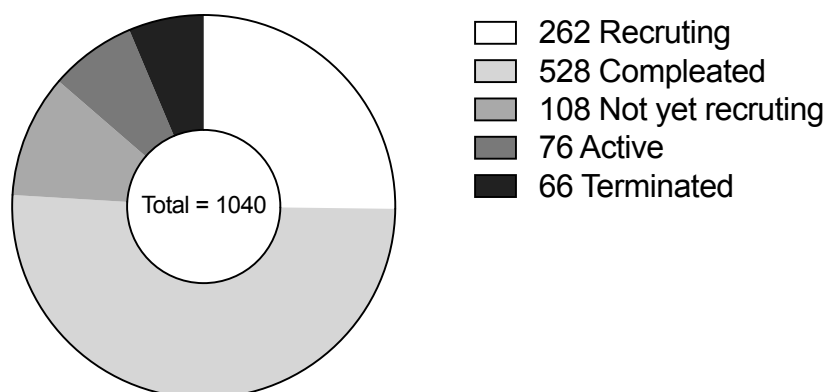
Graphical summarization of MSC driven immunosuppression.

This can be achieved by being stimulated with certain cytokines like IFN- γ , TNF- α , IL-1 β , or TLR ligands found in inflamed environments, or through direct interaction with activated cytotoxic T and NK cells. (1) MSCs Licensed by Soluble Factors: MSCs activated by these soluble agents turn into powerful immunomodulating cells. They release a set of immune-dampening molecules such as IDO, PGE2, TGF- β , TSG-6, IL-10, IL-6, HGF, soluble HLA-G, and IL-1RA. Additionally, they activate the PD-1/PD-L1 and Fas/FasL pathways. The mix of these soluble molecules in their surroundings can also prompt MSCs to start self-destruction processes, after which they are absorbed by monocytes/macrophages. (2) MSCs Licensed by Cytotoxic Cells: These MSCs experience a fast caspase-driven self-destruction and are then consumed by monocytes/macrophages. As a result, these monocytes/macrophages turn into anti-inflammatory cells, emitting similar immune-inhibiting molecules. As they undergo apoptosis, the MSCs also release caspase-controlled immune-modulating agents. The culmination of these licensing procedures effectively modulates the immune response by restraining T cells, NK cells, and DCs. It also promotes M2 macrophage differentiation and the growth of Treg cells. Adapted from (Giacomini et al., 2023)

I.6 Understanding the in vivo effect of MSCs

A large bulk of studies have been shown that MSC produce in vivo immunomodulation, making them an attractive option for treating various inflammatory-related disorders such as graft-versus-host disease (GvHD), Crohn's disease, inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), and myocardial inflammation (Y. Shi et al., 2018). As of today, many clinical trials are actively recruiting or were completed as shown in figure SP2. One of the reasons such an extensive activity can be attributed to their easy accessibility from human tissues, ease of expansion during culturing, and total safety (N. Kim & Cho, 2015). However, most of the clinical trials are still at the early stages, thus highlighting the poor understanding of their mechanisms of action and these mechanisms have not been considered when designing clinical trials.

Figure SP2: clinical trials involving MSC (based on clinical trial.gov)



Moreover, there is a paradox of a long-term immunomodulatory effect produced by the infusion of a large number of MSCs that do not engraft (Fitzsimmons et al., 2018). Therefore, it is not surprising to see that patients exhibit an extensive variability in clinical responses to MSC, despite the release criteria being relatively uniform (Trento et al, Biol Blood Marrow Transplant. 2018) (Galipeau & Sensébé, 2018).

However, a recent study by Galleu et al. has shed light on this paradoxical situation by providing new insights that can reconcile the mechanism of action with the short-term engraftment of the cells. The study used a murine GvHD model and demonstrated that intravenously infused human MSCs first accumulate in the lungs and rapidly undergo caspase-dependent apoptosis initiated by recipient activated NK and cytotoxic T cells. The process of MSCs undergoing apoptosis is essential to trigger immunosuppression, as the number of GVHD effector T cells is significantly reduced only when caspase-3 is detected in MSC. The study showed that apoptotic MSCs (ApoMSCs) are rapidly engulfed by CD11b⁺ and CD11c⁺ murine myeloid phagocytes and induce IDO expression after efferocytosis. By depleting the myeloid phagocytes and inhibiting the IDO activity in the murine model, immunosuppression is totally prevented. This study not only solves the paradox about why short-lived MSCs can induce immunosuppression but also provides new insights into how apoptosis in MSCs orchestrates myeloid cells to deliver immunosuppression in vivo (Galleu et al., 2017).

I.7 Apoptosis and immune system

I.7.a. The apoptotic cascade

Apoptosis, a type of regulated cell death, is a fundamental process that eliminates unwanted cells, and plays important roles in tissue homeostasis and immune defence system (Elmore, 2007; Fuchs & Steller, 2011; Galluzzi et al., 2018a; Kägi et al., 1994). The morphological stages of apoptosis include condensation of nucleic and cytoplasmic compartments, membrane blebbing, and fragmentation into apoptotic bodies (Elmore, 2007; Galluzzi et al., 2018b). Apoptosis also engages a protease cascade to execute cell death, primarily through caspases that cleave their targets' peptide bonds at specific aspartic acid residues (McIlwain et al., 2013). Active caspases cleave other pro-caspases, forming an amplification of the cascade, and can also cleave a variety of proteins, including DNA-degrading enzymes, leading to DNA fragmentation (Slee et al., 2001; Walsh et al., 2008). Among the caspases, pro-caspase/caspase 3 is the most dominant executioner to exert apoptosis, being the prime target of both caspase-8 and caspase-9, and outweighing caspase-6 and -7 regarding the downstream target of proteolysis they can mediate (Slee et al., 2001; Walsh et al., 2008).

Apoptotic cascades can be classified into two main pathways depending on the stimuli: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is initiated by extracellular ligands that bind to death receptors, which are members of the TNF receptor family (TNFR) including TNFR-1, cd95 (Fas), TNF-related apoptosis-inducing ligand (TRAIL) TRAILR-1/DR4 and TRAILR-2/DR5

(Locksley et al., 2001). Upon binding, the intracellular domain of these receptors recruits adapter proteins FAS-associated death domain protein (FADD) and Tumour necrosis factor receptor type 1-associated DEATH domain protein (TRADD) through their death domains (DDs) (Chinnaiyan et al., 1995; Hsu et al., 1995; Kischkel et al., 2000). The molecular mechanism of procaspase-8 activation involves the first dimerization of procaspase-8 and the following proteolytic cleavage to acquire the full catalytic activity and range of substrate binding (Hsu et al., 1995). Activated caspase-8 leads to the downstream cleavage of executioner pro-caspases like pro-caspase-3 to complete apoptosis (Hsu et al., 1996; Kischkel et al., 1995). Post-translational regulations, such as phosphorylation of the tyrosine-380 of caspase-8, can also engage as an additional insurance to regulate the enzymatic activity of caspase-8. Compared to caspase-8, less studies have focused on caspase-10 activation and its function (Dickens et al., 2012; Hughes et al., 2009). Another category of surface receptor governing apoptosis is the dependence receptors (DRs), which constantly transmit apoptotic signals to the cells and only stop when corresponding ligands bind to the receptors (Kischkel et al., 2000). The intrinsic pathway, on the other hand, is initiated by the release of mitochondria protein cytochrome c as a result of mitochondrial outer membrane permeabilization (MOMP) when the cells are overloaded with stress or injury signals. The decision making of MOMP is regulated by the pro-apoptotic and anti-apoptotic members B-cell lymphoma 2 (BCL2) protein family (Kluck et al., 1997). Cytoplasmic cytochrome c binds to an adaptor protein called Apoptotic protease activating factor 1 (APAF1), which triggers the oligomerization to form a heptamer named apoptosome. During the formation of the apoptosome, initiator procaspase-9 is recruited and activated after the assembly (Zou et al., 1997) Active caspase-9 then cleaves down-stream executioner procaspases to

induce apoptosis. In some cells, there is a crosstalk between the extrinsic and intrinsic apoptotic pathway (Acehan et al., 2002). Type I cells such as thymocytes and mature lymphocytes do not involve mitochondria pathway in the amplification of apoptotic cascade after Fas-stimulation, whereas in type II cells, caspase 8 can cleave a protein called BID, which serves as a pro-apoptotic protein under the apoptosis regulator (BCL2) family governing the mitochondria membrane permeabilization (Barnhart et al., 2003; Hao & Mak, 2010). Truncated BID translocates into the membrane of mitochondria, and this results in pore formation as well as the release of cytochrome c, initiating the intrinsic pathway in apoptosis (Yin et al., 1999). However, the mechanism of how DRs engage with procaspase system to initiate apoptosis remains to be explored. Simplified schematics of the extrinsic apoptotic pathway is depicted in figure SP3.

I.7.b. apoptosis a tool for the immune system

Both extrinsic and intrinsic apoptotic pathways are critical components of the immune system that eliminate foreign, transformed, or pathogen-infected cells (Barry & Bleackley, 2002; Kägi et al., 1994). Cytotoxic CD8 T cells and NK cells express death receptor ligands such as Fas-ligand (Fas-L) (Rouvier et al., 1993) or TNF superfamily member 10 (TRAIL) (Brincks et al., 2008; Zamai et al., 1998), which engage with target cells and induce extrinsic apoptotic pathways by activating caspase-8 and DISC (Hsu et al., 1995). In addition, cytotoxic lymphocytes use a novel pathway that requires cell-cell contact and involves the secretion of granules containing perforin and a family of proteases called granzymes (Barry & Bleackley, 2002; Kägi et al., 1994).

The mechanism by which perforins deliver granzymes into target cells has undergone some debate. Currently, the most accepted theory is that perforins form pores using a mechanism similar to bacterial cholesterol-dependent cytolysins (CDC) whereby they bind and oligomerise on the membrane surface. Embedded perforins assemble into a pre-pore and then small clusters of α -helices in each monomer unwind and insert into the membrane of the target cells to form a pore, allowing granzymes to cross the membrane (Podack et al., 1985; Tweten, 2005). Multiple microscopic and kinetic studies have confirmed this phenomenon (Lopez, Jenkins, et al., 2013; Lopez, Susanto, et al., 2013). An alternative pathway has suggested that granzymes and perforins are first internalised and then merged with the endosomes in target cells, where perforin is needed to exit the granzymes into the cytosol of target cells (Browne et al., 1999; Thiery et al., 2011). There is also a study suggesting granzymes can be delivered without perforin but via binding on the mannose-6-phosphate receptor followed by endocytosis (Motyka et al., 2000). However, this concept has been recently challenged by different groups, arguing that the presence of perforin is still needed (Lopez, Jenkins, et al., 2013; Motyka et al., 2000).

Perforin is crucial for granzyme lytic functions, as evidenced by in vivo studies showing that perforin-deficiency impairs lymphocyte-mediated cytotoxicity and immunosurveillance in murine tumour models (Smyth et al., 2000). Among the granzyme family, granzyme B is the only type of granzyme reported to cleave pro-caspases to initiate apoptosis. For example, initiator pro-caspase 8, -9, and -10 as well as even executioner pro-caspase -3, -6, and -7 can be cleaved directly by granzyme B (Cullen & Martin, 2008). Additionally, granzyme B

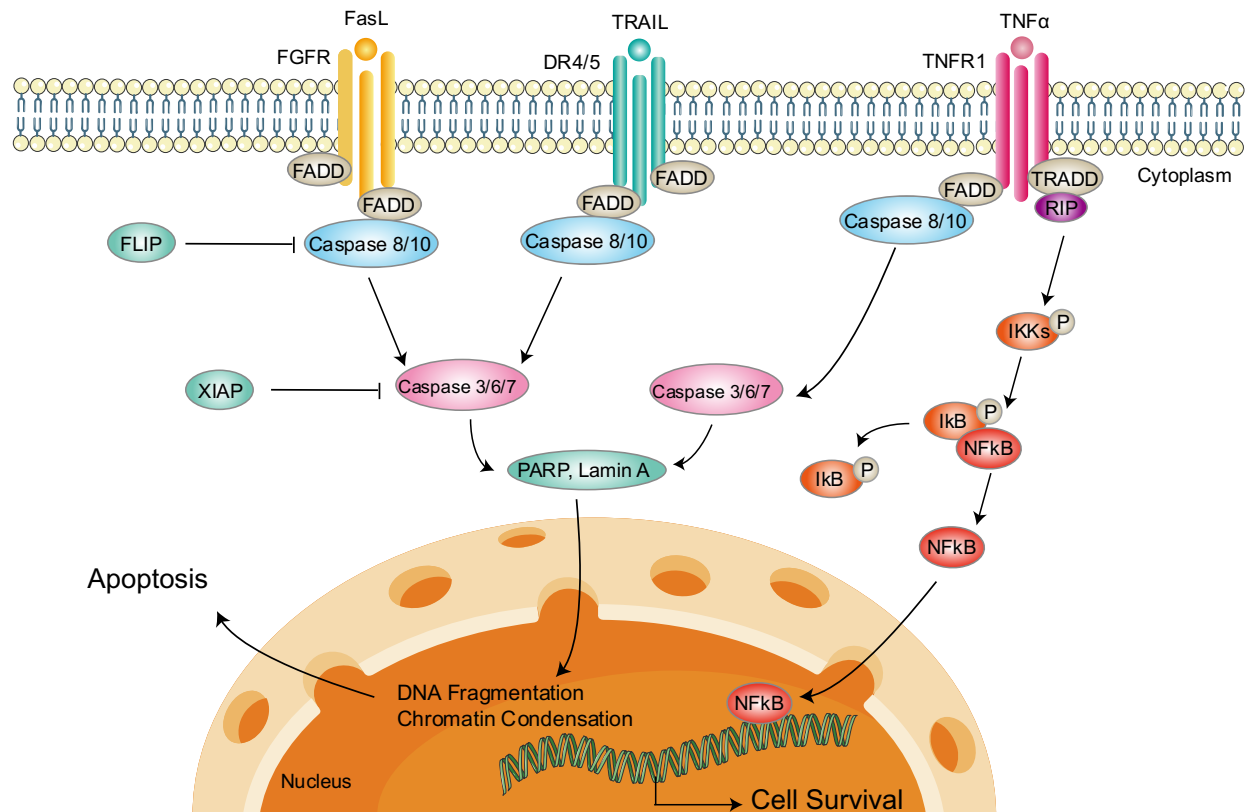
truncates BID to initiate the permeabilization of mitochondria membrane and release of cytochrome c (Heibein et al., 2000). In contrast, granzyme A, another abundant granzyme in the granules, induces caspase-independent cell death, which cannot be prevented by the addition of caspase inhibitor or overexpression of anti-apoptotic protein BCL2 (Martinvalet et al., 2005). Yet, granzyme A might serve as a complementary role to granzyme B to execute cell death.

The clearance of apoptotic cells is crucial for tissue homeostasis and involves the process of efferocytosis, which is distinct from microbial phagocytosis (Elliott & Ravichandran, 2016; Nagata & Tanaka, 2017). Unlike necrotic cell death, apoptotic cells do not induce inflammation in the surrounding tissue (Kerr et al., 1972), and this has become a hallmark of this Programmed cell death (PCD). Apoptotic cells release molecules known as "find-me" signals, which recruit phagocytes in the microenvironment (Chekeni et al., 2010; Elliott et al., 2009; Kischkel et al., 1995; Truman et al., 2008; Weigert et al., 2006, 2010). The activation of caspase-3 or -7 leads to the release of cytosolic nucleotides like Adenosine triphosphate (ATP) and Uridine-5'-triphosphate (UTP), which recruit monocytes by binding to their purinergic receptor P2Y21 (Elliott et al., 2009). Phospholipid lysophosphatidylcholine (LPC) is another "find-me" signal that recruits monocytes and primary macrophages (Peter et al., 2008). Other "find-me" signals include S1P and CX3CL1, but the evidence linking the production of these molecules to caspase activation remains ambiguous (Truman et al., 2008; Weigert et al., 2006, 2010). Chemokines such as CCL2/MCP-1 and IL-8 are also released during apoptosis to recruit monocytes, but their production appears to be overall caspase-independent (Cullen & Martin, 2008).

In addition to recruiting phagocytes, apoptotic cells also actively induce efferocytosis through their secretome (Elliott et al., 2009). The most well-characterized "eat-me" signal is phosphatidylserine (PtdSer), which is located at the inner plasma membrane in viable cells but translocate to the outer membrane during apoptosis (Elliott & Ravichandran, 2016). This is due to the cleavage of ATPase phospholipid transporting 11C (ATP11C) by caspase-3 or -7, which leads to the malfunction of ATP production and the loss of ability to maintain PtdSer at the inner membrane. Scramblases such as Xk-Related Protein 8 (XKR8) actively expose PtdSer to the outer membrane during apoptosis due to the cleavage of inhibitory protein sequence by caspase-3/-7 (Segawa et al., 2014), while transmembrane protein 16F (TMEM16F) externalizes PtdSer via a calcium-dependent mechanism (Suzuki et al., 2013). Phagocytes recognize exposed PtdSer directly through surface receptors like brain-specific angiogenesis inhibitor 1 (BAI1) and stabilin 2 (STAB2) to initiate engulfment (Elliott & Ravichandran, 2016). Alternatively, PtdSer can be recognized by bridging proteins like milk fat globule-EGF factor 8 (MFGE8), which then bind to $\alpha V\beta 3$ integrin (Elliott et al., 2009).

Crucial aspect is also the expression of “don’t eat me signals” which, on the other hand, grant immune evasion from phagocytic cells. Among those signals CD47 have been reported to be one of the key players by interacting with SIRP α and delivering an anti-phagocytosis signal to monocyte and macrophages as well as dendritic cells (Willingham et al., 2012). CD47 expression in MSCs have been described as one of the drivers of their immune-evasive properties (Horrigan et al., 2017) . In the context of apoptosis several groups demonstrated how the expression level of CD47 are downregulated in caspase dependent apoptosis in different cell types via activated caspase 3 and 7 cleavage (Lawrence et al., 2009; Lv et al., 2015; H. S. Wang et al., 2016) Those findings supports the assumption that despite the expression of “don’t eat me signals” such as CD47 by live MSCs, once apoptotic cascade is terminated, those cells lose the ability to deliver those signals, resulting in being efferocytosed by phagocytic cells.

Figure SP3: Apoptosis and caspase activation cascade schematics



Apoptosis and caspase activation cascade schematics

The schematics represents the different triggers of extrinsic apoptosis pathways and the cascade of caspase family protein activation to execute cell death process. Adapted from (Ashkenazi, 2008).

I.7.c. The consequences of apoptosis

Efferocytosis has been linked to the absence of inflammation and immune activation (Kerr et al., 1972; Nagata & Tanaka, 2017). Conversely, impaired clearance of apoptotic cells has been associated with chronic autoimmune diseases (Baumann et al., 2002; Herrmann et al., 1998), implicating the physiological role of efferocytosis in silencing inflammation. Monocytes and macrophages, which are professional phagocytes responsible for apoptotic cell clearance during tissue repair, secrete anti-inflammatory cytokines like TGF- β , IL-10, and PGE2 and suppress the release of pro-inflammatory molecules like TNF- α , IL-1 β , and IL-12 upon efferocytosis or in the presence of apoptotic cells (Byrne & Reen, 2002; Fadok et al., 1998; Tassiulas et al., 2007; Voll et al., 1997). In addition, macrophages release growth factors like VEGF and HGF to aid in tissue repair (Amano et al., 2004; Golpon et al., 2004).

Similarly, DCs polarize into an immunosuppressive or tolerogenic phenotype upon efferocytosis. Tolerogenic DCs, referred to as immature DCs, express lower levels of MHC-II and co-stimulatory markers CD80 and CD86, which are less efficient at stimulating or priming T cells (Mellman & Steinman, 2001). Upon efferocytosis, the maturation of DCs is halted, and the expression of MHC-II, CD40, CD80, CD83, and CD86 is down-regulated, while the secretion of pro-inflammatory cytokines like IFN- γ , TNF- α , IL-12, and IL-23 is reduced, and anti-inflammatory molecules like TGF- β and IL-10 are elevated (Ren, Su, et al., 2008). Metabolic enzymes like IDO and iNOS are up-regulated upon efferocytosis in dendritic cells, with IDO catalysing the depletion of tryptophan and iNOS facilitating the production of NO to suppress T cell proliferation

(GuangXian Zhang, 2014; Ren, Zhang, et al., 2008; Wu et al., 2013; Zhong et al., 2012). An in vivo study also showed that dendritic cells, upon efferocytosis, induce Tregs via the expression of PD-L1. Collectively, the evidence suggests that efferocytosis may play a role in preventing T cell activation and proliferation to establish immune tolerance (Mazzoni et al., 2002).

Apoptotic cells use various mechanisms to prevent the activation of immune responses beyond efferocytosis. Caspase activation inhibits the release and activity of intracellular pro-inflammatory molecules called damage-associated molecular patterns (DAMPs) (Martin et al., 2012). DAMPs can be classified into two categories: (1) molecules that become pro-inflammatory when they are released or exposed during cell lysis, such as single-stranded RNA, genomic DNA, heat-shock proteins (HSPs), High mobility group 1 (HMGB1), and ATP (Rock & Kono, 2008), and (2) molecules that constitutively exhibit immunomodulatory properties, like IL-1 α and IL-33, that are stored inside cells but released during cell lysis (Marichal et al., 2011). Caspase activation inactivates DAMPs, and one example is genomic DNA, which can initiate DC maturation, macrophage activation, and subsequent immune activity. One of the suggested mechanisms of aluminium in vaccines is to trigger the release of genomic DNA as DAMPs to activate immune responses (Oppenheim & Yang, 2005). Caspase activation causes the activation of DNases, like caspase-activated deoxyribonuclease (CAD), to initiate DNA fragmentation, providing an explanation why apoptotic cells do not trigger immune activation (Enari et al., 1998). Additionally, caspase activation impedes the stimulating pathways induced by the sensing of genomic DNA or RNA, such as the RIG-I/IRF-3 pathway, involved in antiviral immunity (Rajput et al., 2011). Caspase-8 cleaves RIPK1 to halt the expression of IFNs, and apoptotic caspases prevent the

production of type I IFNs in response to mitochondrial DNA through the cGAS/STING pathway (Rongvaux et al., 2014; White et al., 2014).

HMGB1 is a chromatin-binding factor that can be released during necrotic cell death but proteolysis of STK4/MST1 by active caspases during apoptosis results in chromatin condensation and prevents the release of HMGB1 (Scaffidi et al., 2002). NADH dehydrogenase Fe-S protein-1 (NDUSF1), another caspase substrate, can trigger a series of oxidation reactions to eliminate the stimulating activity of HMGB1 (Kazama et al., 2008). Activated NDUSF1 also interferes with the production of ATP during apoptosis (Ricci et al., 2004). Lastly, IL-33 is also a caspase substrate that can be cleaved or inactivated by active caspase-3 and -7, thereby losing their ability to activate immune responses (Lüthi et al., 2009). In summary, these studies have shown that caspase activation actively prevents the release of DAMPs and reduces their inflammatory properties, thereby sustaining the anti-inflammatory state of apoptotic cells even in the late-apoptotic stage (Birge & Ucker, 2008; Cocco & Ucker, 2001).

Studies have shown that apoptotic cells can release molecules that have immunosuppressive effects, in addition to undergoing efferocytosis and inactivating DAMPs. In one study, murine apoptotic T cells were found to release both latent and active forms of TGF- β , and it was speculated that this source of TGF- β was from existing TGF- β rather than newly synthesized TGF- β during apoptosis (W. J. Chen et al., 2001). Another study showed that the production of apoptotic murine T cells occurs via the Fas-L/Fas activation, which results in the production of the immunosuppressive cytokine IL-10. This cytokine promotes Th2 differentiation via APCs, whereas apoptotic T cells

isolated from IL-10 knockout mice or living T cells promote Th1 differentiation (Gao et al., 1998). Annexin I is secreted by human apoptotic cells to promote efferocytosis and dampen the IL-6 signalling in LPS-activated monocytes (Pupjalis et al., 2011). While it is clear that apoptotic cells release soluble factors, the upstream pathways regulating these soluble factors remain elusive. However, one study reported that activation of caspase-3, as a result of radiotherapy, leads to the production of immunosuppressive PGE2 from apoptotic tumour cells via calcium-independent phospholipase A2 (iPLA2) activation. Caspase-3 activation is also correlated with an increased rate of tumour relapse and mortality, suggesting caspase-3-mediated PGE2 production as a novel mechanism for tumour cells to escape immunosurveillance and promote tumour relapse (Huang et al., 2011).

I.8 CAR-T therapy

Chimeric Antigen Receptor (CAR) T cells have emerged as a ground-breaking approach in cancer immunotherapy, with significant clinical impact in the treatment of haematological malignancies and potential applications for solid tumour (June et al., 2018). The strategy of CAR T-cell therapy, a type of Adoptive Cell Therapy (ACT), involves genetically modifying T-cells to express a chimeric receptor that enables the recognition and binding of desired antigens, leading to selective T-cell activation (Gross et al., 1989; Z et al., 1993). The first report of CAR T-cell therapy was by a group in Israel in 1989, where they fused the Variable regions of an anti-2,4,6-trinitrophenyl (TNP) antibody (SP6) with the Constant regions of the α - or β - chain of the T-cell receptor (TCR), resulting in the chimeric chain dimerizing with the endogenous TCR chains and leading to signal transduction (Gross et al., 1989). Importantly, the cells recognized the target in an MHC-independent manner and were named "T-bodies"(Z et al., 1993) .

Over the following decades, the field witnessed rapid advancements, culminating in the approval of the first CAR-T cell therapy, tisagenlecleucel (Kymriah), by the US Food and Drug Administration (FDA) in 2017 for the treatment of relapsed/refractory B-cell acute lymphoblastic leukaemia. Shortly after, axicabtagene ciloleucel (Yescarta) was approved for the treatment of certain types of large B-cell lymphomas.

I.8.a. CAR-T cell generations and structural features

CARs are intricate molecules, constructed from domains sourced from a variety of proteins. At their core, CAR structures can be broken down into four essential components: (1) an outward-facing antigen-binding domain, (2) a pivotal hinge region, (3) a transmembrane segment, and (4) internal signalling domains.

Most CARs feature an antigen-binding segment that includes a single-chain variable fragment (scFv), which is a by-product of antibodies. This scFv integrates the variable light (VL) and variable heavy (VH) sections of a monoclonal antibody, bridged by an adaptable protein connector. In the context of a CAR, the scFv is displayed on the T cell's exterior, playing a crucial role in recognizing a tumour-associated antigen on the surface of tumour cells (Sternier & Sternier, 2021). Differently, the CAR antigen-binding sections might also be formed from peptides that engage with specific receptors on intended cells (Papa et al., 2015) or lectins adept at discerning particular carbohydrate fragments on the exteriors of target cells (Meril et al., 2020).

In a departure from TCRs, which latch onto peptides via MHC, CARs initiate T cell responses by forming a direct connection to a tumour-associated antigen on the target cell's facade. This suggests that CAR T cells predominantly align with tumour-associated antigens present on a tumour cell's surface. In contrast, TCRs have the ability to connect with peptide-MHC originating from

both the external and internal sources of antigens. It's imperative to highlight that CARs based on scFv generally display a heightened affinity to their respective targets compared to TCRs' affinity for peptide-MHC (D. T. Harris et al., 2018). This intense affinity can drive powerful T cell responses, but it might also precipitate early T cell weariness. Tweaking the scFv to diminish the CAR's target affinity has proven to bolster performance, especially when homing in on specific tumour-associated antigen (Ghorashian et al., 2019).

The CAR's antigen-binding component is adjoined to a transmembrane segment through a flexible hinge area, positioning the antigen-binding domain away from the membrane, which aids in its interaction with the designated tumour-associated antigen. The hinge design is paramount to ensuring unhindered antigen engagement. For instance, targeting antigens closer to the membrane might necessitate an extended, pliable hinge, but those located further might be better suited to a more abbreviated hinge (Sternier & Sternier, 2021). Predominant hinge domains are typically derived from sources like CD28, CD8, IgG1, or IgG4.

The CAR, affixed to the membrane and bridged to its internal domains through a transmembrane section, often employs the transmembrane domains of CD8a or CD28. The specific transmembrane domain chosen is pivotal, impacting both the CAR's robustness and functionality (Sternier & Sternier, 2021). Specifically, using certain transmembrane domains can lead to CAR's propensity for either homodimerization or pairing with inherent signalling components (Bridgeman et al., 2010).

CAR-T cells are classified into distinct generations based on the structure and function of their intracellular signalling domains. Each generation exhibits unique characteristics and therapeutic potential.

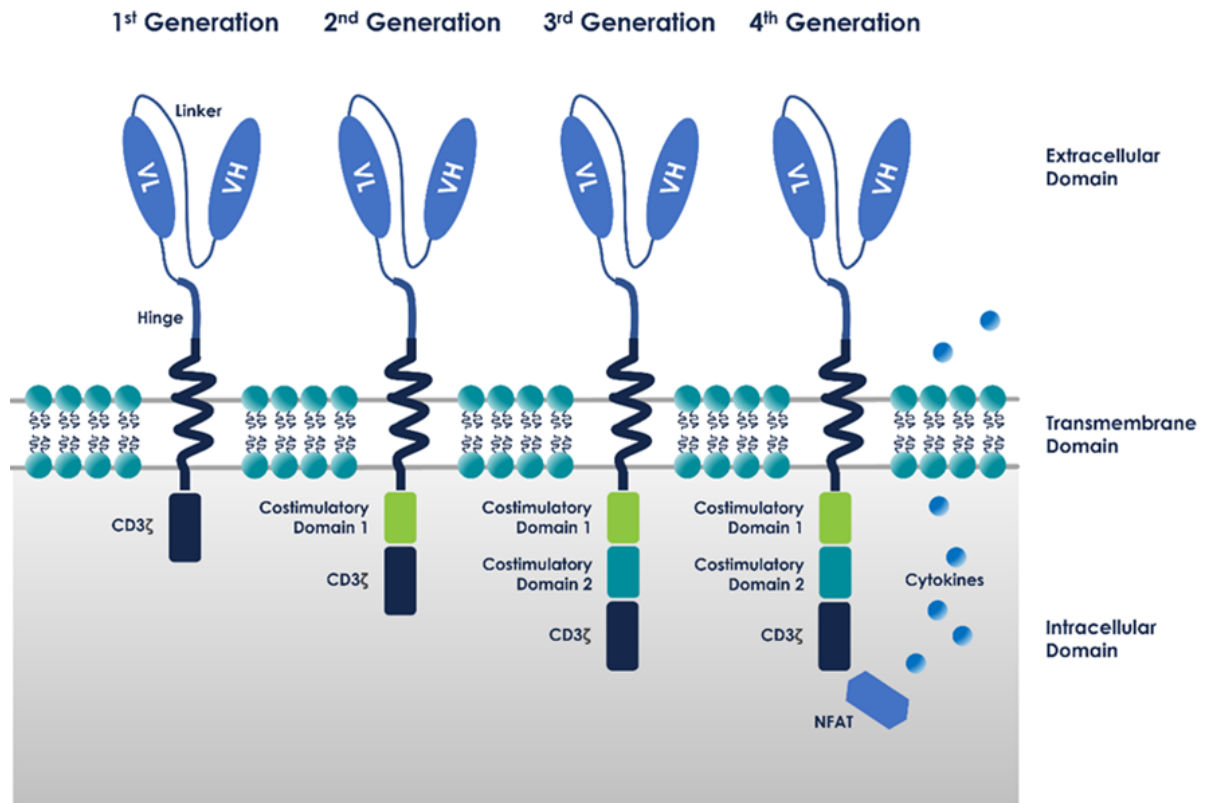
First-generation CAR-T cells consist of an extracellular antigen-recognition domain, typically derived from a single-chain variable fragment scFv of an antibody, a transmembrane domain, and an intracellular signalling domain derived from the CD3 ζ chain of the TCR (Gross et al., 1989; Kuwana et al., 1987). When first developed, these cells were shown to be capable of recognizing and binding target antigens in an MHC-independent manner (Z et al., 1993). However, their therapeutic potential was limited due to the lack of co-stimulatory signalling, leading to suboptimal T-cell activation, proliferation, and survival (Irving & Weiss, 1991).

To overcome the limitations of first-generation CAR-T cells, researchers introduced a co-stimulatory domain such as CD28 or CD137/4-1BB, into the intracellular portion of the CAR, resulting in second-generation CAR-T cells (Finney et al., 2004; Maher et al., 2002). The addition of co-stimulatory signalling significantly improved T-cell activation, proliferation, and survival, enhancing the anti-tumour efficacy of these cells (Milone et al., 2009)

Third-generation CAR-T cells were developed to further enhance the efficacy of CAR-T cell therapy by incorporating a second co-stimulatory domain in tandem with the CD3 ζ signalling domain (Carpenito et al., 2009). Commonly used co-stimulatory domains in third-generation CAR-T cells include CD28, CD137 (4-1BB), and CD134 (OX40) (Hombach et al., 2012; Kowolik et al., 2006). By including two co-stimulatory domains, these CAR-T cells exhibit improved cytokine production, T-cell proliferation, and persistence, leading to enhanced anti-tumour activity compared to second-generation CAR-T cells (Z. Zhao et al., 2015).

Fourth-generation CAR-T cells, also known as T cells Redirected for Universal Cytokine Killing (TRUCKs), were developed to further improve the potency and efficacy of CAR-T cells, particularly for solid tumours (Chmielewski & Abken, 2015). TRUCKs are engineered to secrete pro-inflammatory cytokines, such as IL-12, upon T-cell activation (Pegram et al., 2012). This is achieved by introducing a second transgene containing the cytokine coding sequence under the control of an NFAT (Nuclear Factor of Activated T-cells) inducible promoter (Koneru et al., 2015). The release of cytokines at the tumour site not only enhances the cytotoxicity of CAR-T cells but also recruits and activates other immune cells, such as CD8⁺ cytotoxic T lymphocytes and natural killer cells, to the tumour microenvironment, leading to improved anti-tumour activity (Chmielewski et al., 2014). Schematic figure depicting the different components of CAR construct and differences between generation is depicted in figure SP4.

Figure SP4: Structural components of CAR construct and generations



Structural components of CAR construct and generations

Graphical representation of CAR construct main modular component and difference between different CAR generation. Adapted from (Elahi et al., 2018)

I.8.b. Resistance to CAR-T

Despite the success of CAR-T cell therapy in CD19⁺ and B-Cell Maturation Antigen (BCMA)⁺ malignancies, primary and secondary resistance remains a challenge. Primary resistance mechanisms are described as the inadequate initial response of CAR-T cells, examples are insufficient expansion, persistence, and activity. Secondary resistance mechanisms are instead described as the one that, despite initial response, leads to CAR-T cell functional impairment and tumor relapse. Clinical trials using CAR T cells against various tumor-associated antigens across different cancer types have only shown favorable outcomes in a few cases. For instance, CAR T cells targeting Human Epidermal Growth Factor Receptor 2 (HER-2) resulted in stable disease in 4 out of 17 sarcoma patients (Ahmed et al., 2015), while GD2-specific CAR T cells led to complete remission in 3 of 11 glioblastoma patients (Louis et al., 2011). Also, Epidermal Growth Factor Receptor (EGFR)-specific CAR T cells showed a partial response in just 1 of 11 Non-Small-Cell Lung Cancer (NSCLC) patients (Feng et al., 2016). Researchers are focusing on understanding the lying mechanisms of CAR-T resistance, which can be reconducted to tumor microenvironment related multifactorial causes :

I.8.b.1. Target antigen expression

The lack of a specific, tumor associated antigen is often the main issue in the CAR-T field. In many disease context cells overexpress molecules which could be targeted by CAR constructs, but which are also naturally occurring in healthy tissue. Even if in the lymphoma setting CD19 is considered a disposable target and B cell ablation do not cause harms to the body this is not the case in other

malignancies. An example is patients treated with HER-2 CAR which experienced fatal lung toxicity due to low HER2 expressed in healthy lung cells (Feucht et al., 2019). Moreover, expression pattern of the antigen might not be conserved in patients like in the Acute Myeloid Leukemia (AML) setting described above or in Glioblastoma where target EGFRvIII mutant is inconsistently expressed across patients and its expression is subject to changes according to the treatment the patient received (Nathanson et al., 2014).

1.8.b.2 Antigen Loss

Antigen loss is recognized as a primary resistance mechanism in the context of immunotherapy. This phenomenon consists in loss of the expression of the antigen recognized by CAR-T cells in the malignant cell population (Sotillo et al., 2015). This is often observed in CD19 malignancies where after therapy the patient relapse with the disease which no longer express the antigen. This has been linked to gene mutation affecting the antigen, alternative splicing variants, or epigenetic modification (Chong et al., 2017).

1.8.b.3 CAR-T intrinsic factors

CAR cells as opposite to normal T-cells are in a constant activation status due to the chronic antigen stimulation, resulting in a rapid loss of their potency in terms of proliferation, cytotoxicity, and cytokine production, undergoing a process named Exhaustion (Turtle et al., 2016). In this scenario CAR cells modify their phenotype in terms of metabolic pathways and upregulation of checkpoint molecules leading to a premature cell death. Moreover, it is

important to underline that CAR-T cells manufacturing can play an important role in the process. It was proved that different manufacturing and ex-vivo expansion protocol can strongly impact on the exhaustion status of the final product (Ruella & June, 2016).

1.8.b.4 Tumors intrinsic pathway mutation

Due to highly plasticity of the malignant cells often activate alternative signaling pathways to escape the therapy targeted pathway impairing their effect. Once a particular pathway is targeted by the CAR cells, like a grow factor receptor, cancer cells are able to compensate by upregulating member of the same family of receptors (Chandarlapaty, 2012) or by activating a similar redounding pathway to promote survival (Q. Fan et al., 2020; J. Zhang et al., 2009). Moreover, as cytotoxic activity of CAR cell relies on cell death induction in the target cells to control the disease, tumors are able to bypass this by either upregulating anti-cell death proteins like BCL-2 (Merino et al., 2018) or by downregulate death receptor such as TRAIL and FAS (Sayers, 2011).

1.8.b.5 The tumors microenvironment

As previously discussed, the tumors microenvironment and its components can play an important role in counterattacking CAR-T cell therapy. This process can be eased by soluble mediators or by direct cell contact with different subsets of immune and stromal cells. Beyond simply reaching the right location within the body, CAR T cells need to efficiently move out of blood vessels and directly penetrate the tumor to interact with the Target Antigen and perform their roles effectively (Donnadieu et al., 2020). Navigating CAR T cells toward tumors is notably difficult due to the chaotic structure of the blood vessels and surrounding layers in these tumors. Factors promoting blood vessel growth can

weaken endothelial cells, leading to decreased levels of guiding molecules like chemokines and attachment proteins, thereby restricting T cell movement out of blood vessels (Griffioen et al., 1996). When CAR T cells do make it through the vessels, the tumor environment often stops them from reaching the cancer cells. They can get ensnared in the tumor's supporting framework, which may be due to an irregularly built ECM that impedes T cell movement. Some cancer cells are shielded by dense layers of collagen fibers, acting as physical barriers for T cells (Salmon et al., 2012). Additionally, certain cells in this supportive tumor network release chemicals, such as CXCL12, which deter T cells from entering tumor clusters (Feig et al., 2013).

Another feature of TME is hypoxia, which can play a crucial role in CAR-T impairment, especially in organs like bone marrow which naturally present a hypoxic gradient in its different areas. Hypoxic microenvironment is described as tissue area where oxygen levels are significantly lower when compared with healthy tissue. Hypoxic microenvironment can be caused by several factors like increased consumption of oxygen by fast-growing malignant cells together with an under vascularization (Marofi et al., 2022). Hypoxia have been demonstrated to have a negative impact on CAR-T activity via several mechanisms both directly and indirectly. Direct effects on CAR-T are associated with reduced infiltration in the tissue and insufficient expansion via metabolic pathway interference (Schurich et al., 2019). Indirect effect instead can be referred to the modulation of other cells in the microenvironment, like polarization of the myeloid compartment and increased expression of immunosuppressive molecules or malignant cells modulation of target antigen (Kosti et al., 2021).

Beyond just physical obstacles, the Tumor Microenvironment contains immunosuppressive chemicals and cell varieties that hinder the activity of CAR T cells. Within tumors, there's a high presence of cytokines like TGF- β , VEGF, IL-4, and IL-10 (Rodriguez-Garcia et al., 2020). These cytokines can not only directly dampen T cell actions but also attract cells with immunosuppressive qualities like tumor associated macrophages, myelo-derived suppressor (MDSC) cells, and Tregs. These cells, in turn, add to the build-up of elements that suppress immune responses.

In human cancers, tumor associated macrophages are the predominant immune cells. These are known to curb T cell activity within the Tumor Microenvironment by expressing checkpoint ligands like PD-L1, B7-H4, and VISTA, or by secreting cytokines such as TGF- β and IL-10 (Rodriguez-Garcia et al., 2020) that inhibit T cells. Additionally, they physically obstruct T cells by maintaining prolonged contact with them, preventing these T cells from interacting with cancerous cells (Peranzoni et al., 2018). In a specific melanoma mouse study, the pharmacological removal of macrophages boosted the performance of introduced tumor-targeting T cells, underscoring their significant role in dampening the function of these T cells (Peranzoni et al., 2018). In parallel, MDSCs aid tumor growth by fostering immune escape. Their prevalence in many cancer types often signals unfavorable clinical results (Q. M. Fan et al., 2014; X. Zhao et al., 2017). MDSC's suppressive actions resemble those of macrophages, with their dampening impact on CAR T cells showcased in various studies. For instance, in a liver cancer metastasis mouse model, anti-CEA CAR T cells were hampered by GM-CSF production and PD-L1 expression from liver-associated MDSCs (Burga et al., 2015). Counteracting the suppressive effects of those notably enhanced the performance of CAR T cells in this context (Long et al., 2016), spotlighting their crucial inhibitory role. In

another experiment, using all-trans retinoic acid to eliminate MDSCs restored the effectiveness of GD-2-targeted CAR T cells in a sarcoma mouse model (Long et al., 2016).

Tregs, found within human cancers, are another set of immune cells known to impede the efficiency of CAR T cells in combatting tumors. These specific CD4⁺ T cells, distinguished by the expression of FOXP3 and CD25, play a crucial role in preserving immune balance (Togashi et al., 2019). Due to their immunosuppressive actions, tumors often employ Tregs to curtail anti-tumor responses. Tregs deploy several strategies to tamp down inflammation, such as releasing immunosuppressive cytokines and restraining APC through cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expression (Togashi et al., 2019). Additionally, Tregs can stifle T cell activation by either outcompeting cytotoxic T cells for IL-2 or directly destroying cytotoxic T and NK cells (Corthay, 2009). One research identified that patients undergoing adoptive T cell therapy who didn't show positive outcomes had elevated peripheral Treg levels compared to those who benefitted (Yao et al., 2012). In a similar vein, a clinical trial utilizing EGFRvIII-specific CAR T cells for glioblastoma treatment found that the rise in Treg numbers might have played a role in treatment ineffectiveness among non-responders (O'Rourke et al., 2017).

Lastly the direct interaction between CAR-T and stromal cells have not yet been investigated.

I.8.c. Armouring CAR-T

The clinical trials for CAR T cells in solid tumours and AML reveal a varied patient response. A significant portion does not exhibit favourable outcomes; however, a minority has displayed objective responses. This, combined with a deeper comprehension of the factors behind CAR T cell inefficiency in these tumours, underscores that there's potential for improvement in current CAR T cell designs.

This understanding has sparked the creation of 'armoured' CAR T cells. These cells undergo additional genetic modifications to better their performance in the Tumour Microenvironment, often achieved using methods like internal ribosome entry site (IRES) (Renaud-Gabardos, 2015) or 2A skipping peptides (Z. Liu et al., 2017). Such mechanisms allow for generation of a polycistronic vector which results in the expression of multiple proteins from a single viral integration, enabling the CAR and the necessary armouring components to be delivered concurrently.

Addressing the challenge of antigen variability in tumours has led to the design of bi-specific CARs. These are engineered to recognize more than one antigen, adding an extra layer of control over T cell activation. For instance, some design included a so called “AND-gate” using a perpetually active synNotch-based CAR. Upon binding of the first antigen to the synNotch CAR transcription of another CAR, targeting a different antigen, is triggered and cytotoxic effect is delivered to the malignant cell (Roybal et al., 2016). NOT-gates on the other end have a different mechanism, two CAR are expressed simultaneously: one is a conventional CAR while the other have a costimulatory domain composed of signalling sequence typical of checkpoint inhibitors delivering an immunosuppressive effect. CAR-T with NOT-gate can only deliver cytotoxic

effect when the second inhibitory CAR construct and its target antigen are not bound (Fedorov et al., 2013).

Armoured CAR designs have been crafted to deal with issues like inadequate CAR T cell homing in the tumours. Often, misalignment between chemokine receptors on CAR T cells and ligands from the tumour is the culprit. Adjustments such as introducing specific CCRs can dramatically boost CAR T cell tumour infiltration, as shown with the expression of CCR2 in mesothelin-targeted CAR T cells (Moon et al., 2011) and CCR2b in GD2-targeted CAR T cells in neuroblastoma (Craddock et al., 2010).

Moreover, the instrumentalization of naturally occurring mutated protein can be employed to maximise CAR homing, recent studies demonstrated the increased antitumoral activity of CD33 CAR-T cell co-expressing a mutated form of CXCR4 which, once bound to its target CXCL12, in the bone marrow niche, is unable to unbind causing permanent homing ((Biondi et al., 2023))

Some designs tackle the tumour's ECM by incorporating enzymes that break it down, enhancing CAR T cell penetration and activity. An example is the co-expression of CAR and a membrane bound form of heparinase. This proven successful in murine models where increasing numbers of CAR were found in the tumour microenvironment (Caruana et al., 2015).

Numerous armoured CAR designs counteract the inhibitors within the tumour microenvironment. Among the major obstacles is TGF- β . By either co-expressing a dominant-negative TGF- β receptor II (dnTGF- β RII) or employing CRISPR/Cas9-mediated knock out of TGF- β RII, CAR T cells' performance has been enhanced (Kloss et al., 2018; Schuberth et al., 2013). The addition of proinflammatory cytokines like IL-12 in CAR T cells has also been fruitful (Koneru et al., 2015). Direct targeting of immunosuppressive cell types in the

microenvironment is challenging, yet some headway has been made, as with anti-CD123 CAR T cells that target tumour associated macrophages (Ruella et al., 2017).

Armoured CAR designs also focus on intrinsic T cell factors that might limit their anti-tumour efficacy. T cell exhaustion, indicated by molecules like PD-1 and CTLA-4 on the T cell's surface, is a primary concern. Various techniques, including CRISPR/Cas9, have been employed to address this issue, particularly targeting the PD-1/PD-L1 pathway (B. D. Choi et al., 2019; Hu et al., 2019; Suarez et al., 2016; Z. Wang et al., 2021; J. e. Zhou et al., 2021). Surprisingly, targeting other checkpoints like CTLA-4 (Condomines et al., 2015) and LAG-3 (Y. Zhang et al., 2017) hasn't shown the same promise.

Recent insights into the transcriptional programs governing T cell functions have revealed the role of Transcription Factors. Manipulating these, like the overexpression of AP-1 family transcription factors or the knock down of Interferon Regulatory Factors, has shown potential in boosting CAR T cell efficacy (J. Chen et al., 2019; Lynn et al., 2019; Seo et al., 2021).

Armoured CAR T cells addressing the tumour microenvironment from the metabolic restriction point of view were also attempted. Those address the effects of elevated adenosine concentrations (Poorebrahim et al., 2021) or arginine depletion, with promising results in preclinical models (Fultang et al., 2020; Pellegrino et al., 2021; T. Wang et al., 2020).

To date armoured CAR-T targeting the malignant stromal cells have been fewer. One report of CAR-T designed to target cancer associated fibroblasts via recognition of Fibroblast Activating Protein (FAP) demonstrated efficacy in a pre-clinical model of mesothelioma (Schuberth et al., 2013).

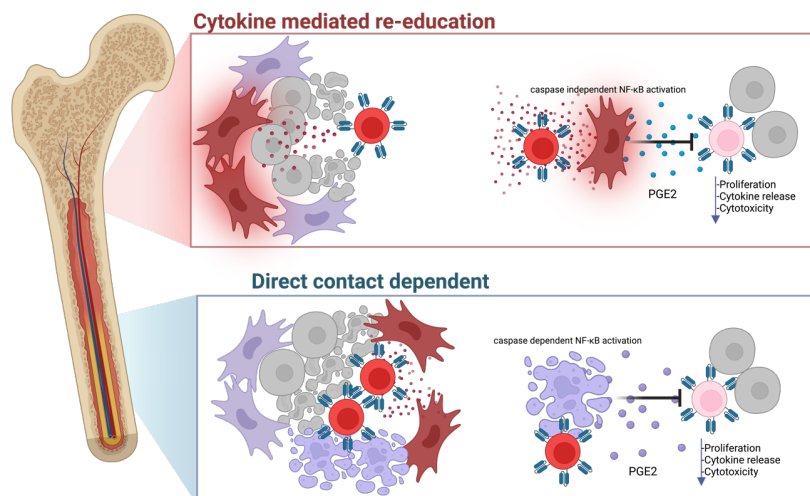
I.9 Aim and hypothesis.

This work aims to map the interaction between CAR-T cells and bone marrow stromal cells.

We have adopted a model of CAR-T targeting CD33 in an AML context using bone-marrow MSCs as stromal cells.

We hypothesize that CAR-T can interact with BM-MSCs via 2 different mechanisms: (1) a cytokine dependent re-education mechanism which relays on CAR-T cells released factors to prime MSCs towards immunosuppression. (2) a direct contact dependent mechanism by which CAR-T can induce apoptosis in MSCs. Apo MSC in return promote immunosuppression. Graphical representation in figure SP5

Figure SP5: project graphical abstract



Chapter II - Material & Methods

II.1 Cell line maintenance

KG-1 AML cell line (curtesy of Dr. Marta Serafini, Tettamanti Center, Monza, Italy) was cultured in suspension in RPMI GlutaMax (Thermo Fisher Scientific, UK) with 10% (v/v) heat inactivated-fetal bovine serum (FBS) (Gibco, UK) and 25IU/ml penicillin and 25mg/ml streptomycin (Thermo Fisher Scientific, UK), at a density of $0.3-0.5 \times 10^6$ cells per ml, splitting twice a week.

Human embryonic kidney (HEK) 293T cell line was cultured in DMEM GlutaMax (Thermo Fisher Scientific, UK) supplemented with 10% FBS in tissue culture treated flasks at a density of 2×10^4 cells/cm². Cells were harvested using Trypsine-Ethylenediaminetetraacetic acid (EDTA) (0,05%) with phenol red (Thermo Fisher Scientific, UK).

All cells were kept at 37°C, 5% CO₂ and growth monitored using a light microscope.

II.2 MSCs

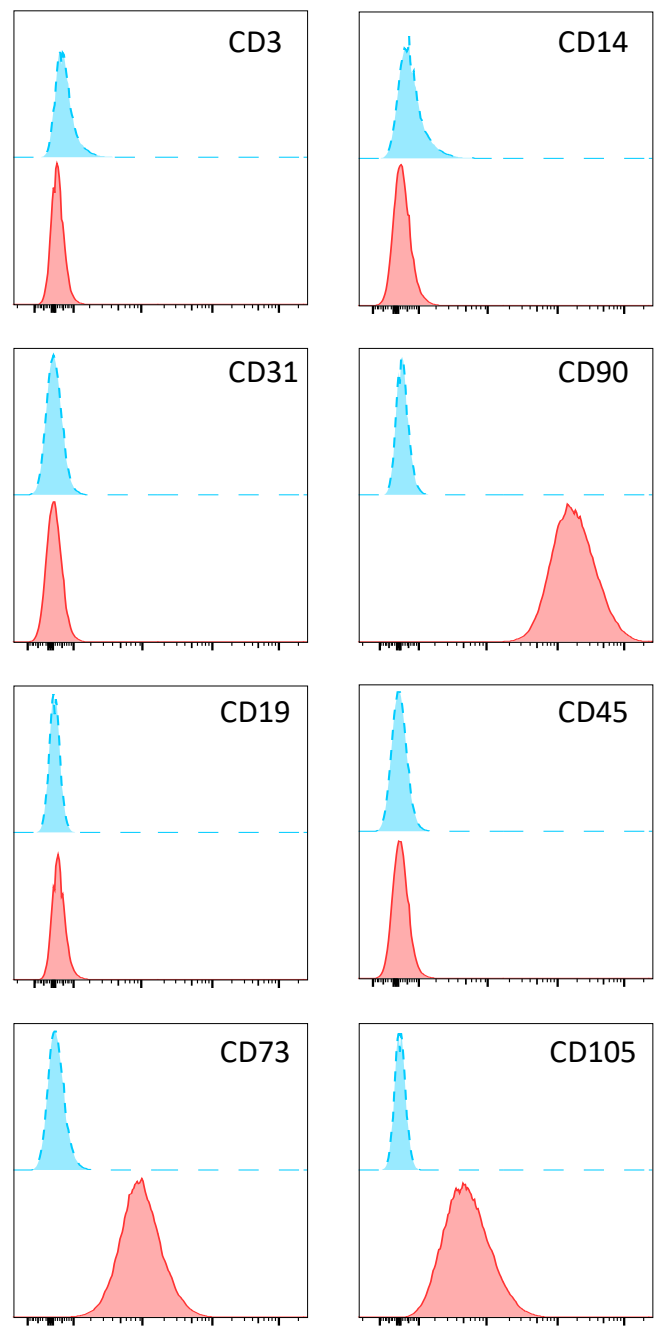
II.2.a Bone marrow derived MSC isolation

MSCs were isolated from healthy donor bone marrow aspirates after informed consent. Samples were obtained from Imperial College Healthcare Tissue Bank (ICHTB, HTA license 12275, approved by UK National Research Ethics Services to provide human material for research 12/WA/0196) donor details are reported in table 1. In brief cells were plated in tissue culture treated flasks

(Corning UK) at a density of $15-40 \times 10^3$ cells per cm^2 in alpha-minimal essential medium (α -MEM, Thermo Fisher Scientific, UK) supplemented with 5% (v/v) human platelet lysate (Cook Regentec, UK) and maintained at 37°C , 5% CO_2 . After 24h non adherent cells were removed via washing using Phosphate-buffered saline (PBS) (Thermo Fisher Scientific UK). Cells confluency was monitored and when it reached 90% cells were harvested using Trypsine-EDTA (0,05%) with phenol red (Thermo Fisher Scientific, UK). Resulting cells were either expanded or frozen in FBS supplemented with 10% DMSO and stored at -80°C for future use. Phenotype control was performed via flow cytometry and exemplificative plot is depicted in figure SP6.

MSC aliquots were thawed and cultured at a density of $1-4 \times 10^4$ cells/ cm^2 and expanded as described above for 3 to 9 passages. Different MSC batches from 4 independent donors were used for experiments.

Figure SP6: MSC phenotyping



II.3 Generation of Rel-A knock down (KD)MSCs

II.3.a Plasmid DNA preparation

Plasmids coding for 3rd generation Lentivirus and for sh-RNA targeting human Rel-A or ctrl were kindly gifted by Dr. Guido Franzoso (Imperial College London). Bacterial stocks were grown in 300ml terrific broth (Sigma-Merk, Germany) supplemented with 100µg/ml of Carbenicillin (Thermo Fisher Scientific, UK) at 37°C, 250 RPM shaking overnight. Bacteria were collected by centrifugation at 4000 RPM for 15 min and plasmid DNA was isolated using GenJet endo-free maxi prep kit (Thermo Fisher Scientific, UK) following manufacturer instruction. DNA concentration and purity was assessed using Nanodrop-100 (Thermo Fisher Scientific, UK) spectrophotometer. DNA was stored at -20°C for further use.

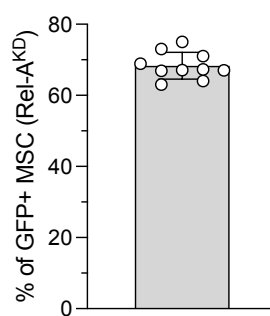
II.3.b Lentivirus production

Lentivirus encoding for Rel-A shRNA-GFP or CTRL-shRNA-GFP were generated in HEK293T cells as described (Mauro et al., 2011). In short HEK293T cells were seeded at 2.5×10^5 cells/cm² and were transfected with a mix of 4 plasmids using Polyetherimide (PEIpro) (PolyPlus, UK) at a 2:1 PEI:DNA ratio. Supernatant containing viral particles was harvested 48 and 72h after transfection.

II.3.c MSC transduction

MSCs were harvested and counted in Trypan blue (Thermo Fisher Scientific, UK) using a Neubauer hemocytometer chamber. 1×10^6 cells MSCs were resuspended in 10ml fresh viral supernatant supplemented with 0.01mg/ml of polybrene (Thermo Fisher Scientific, UK) and seeded in a T75 flask. Media was replaced after 48h, and cells were cultured for further 48h. Transduction efficiency was estimated using flow cytometry detecting Green Fluorescent Protein (GFP) positivity as shown in figure SP7.

Figure SP7: transduction level of different batches of MSCs Rel-A^{KD}

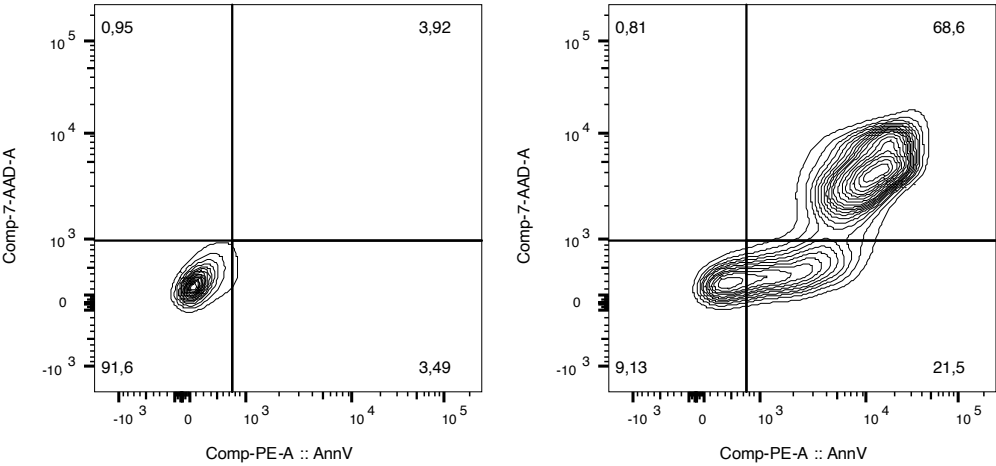


II.4 Generation of apoptotic MSCs and supernatant

5×10^6 MSCs cultured as previously described were detached using Trypsin EDTA 0.05% and treated with an activatory anti FAS antibody (Clone CH11, Merk Millipore, UK) at $10\mu\text{g/ml}$ in complete RPMI GlutaMax (Thermo Fisher Scientific, UK) for 15 minutes at 37°C 5% CO_2 . After incubation cells were washed with fresh media and centrifuged twice at 1500 RPMI for 5 minutes. Cells were resuspended at a concentration of 8×10^6 cells/ml, plated, and incubated overnight.

Apoptotic cells were harvested and collected in a tube for centrifugation. Supernatant of apoptotic MSCs was carefully collected and re spun in a pre-chilled centrifuge at 4°C for 15 minutes at 13.000 RPM to ensure debris removal. Aliquots of apoptotic supernatant were immediately stored at -80°C for further use. Apoptotic MSCs were frozen in freezing media (FBS supplemented with 10% Dimethyl sulfoxide (DMSO)) and aliquots were stored at -80°C . To assess apoptosis levels, cells were counted in trypan blue (Sigma Merk, Germany). Batches with $> 80\%$ dead cells were used for later experiments. Exemplificative FACS plot of apoptotic MSCs is depicted in figure SP8.

Figure SP8: apoMSC FACS analysis



II.5 CAR-T

II.5.a Isolation of PBMCs

Human peripheral blood mononuclear cells (PBMCs), obtained from healthy subjects upon informed consent, were isolated on a density gradient using Ficoll-Hypaque (Pharmacia LKB). Sex and age of PBMCs donor are summed in table 2.

Blood specimens were quadrupled in volume using PBS and layered on top of Ficoll-Hypaque. Following that, centrifugation was carried out at 1800 RPM for 20 minutes at 20 °C, with the brake deactivated. The middle layer, housing the mononuclear cells, was delicately transferred into a fresh tube and underwent a PBS wash at 1800 RPM for 5 minutes. Two subsequent washes at 1000 RPM for 10 minutes were conducted, and the cells were resuspended in Advanced RPMI (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS (Gibco), 2mM L-glutamine, 25IU/ml penicillin and 25mg/ml streptomycin (Lonza).

II.5.b CAR-T manufacture

CD33.CAR-T cells were generated using the non-viral Sleeping Beauty (SB) transposon system (Rotiroti et al., 2022) as depicted in figure SP9. In brief, PBMCs were activated via CD3/CD28 stimulation and nucleofected with SB transposon encoding for CD33.CAR and transposase DNA plasmids according to manufacturer's instructions using 4D-Nucleofector™ device (Lonza), before expansion for 15 days in G-Rex devices (Ludwig J et al, Methods Mol Biol, 2020;2086:165-177). To detect CAR expression, a recombinant human

sialic acid binding Ig-Like Lectin 3/Siglec-3/CD33 protein with a Fc and a 6His tag at the C-terminus (C-Fc-6His, Gentaur, UK) was employed, before proceeding to secondary staining with an anti-human IgG Fc conjugated mAb (Biolegends, USA). Batches with transduction rate ranging from 27% to 89% were used for experiments. CAR-T cells were frozen in FBS (Gibco, USA) supplemented with 10% (v/v) DMSO (Sigma-Merk, Germany) and stored in liquid nitrogen for later use. CAR-T production was conducted by Dr. Alice Pievani and Dr. Sarah Tettamanti from Dr. Marta Serafini Lab (Tettamanti Center, Monza, Italy) as part of collaboration. Representative FACS plot of CAR phenotype and transduction levels are depicted in figure SP10.

Figure SP9: representation of CAR construct

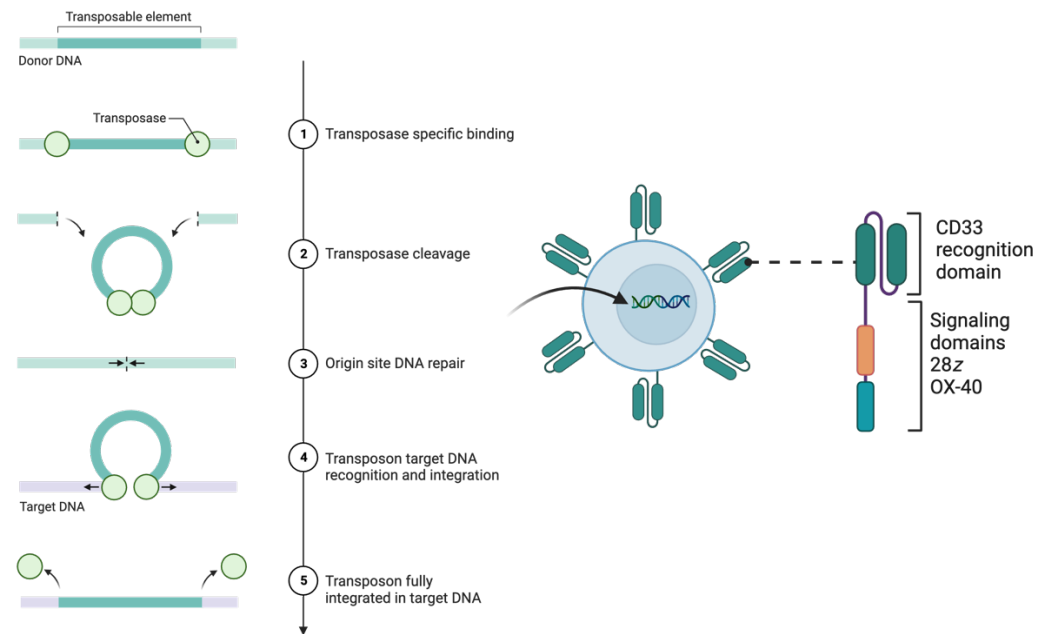
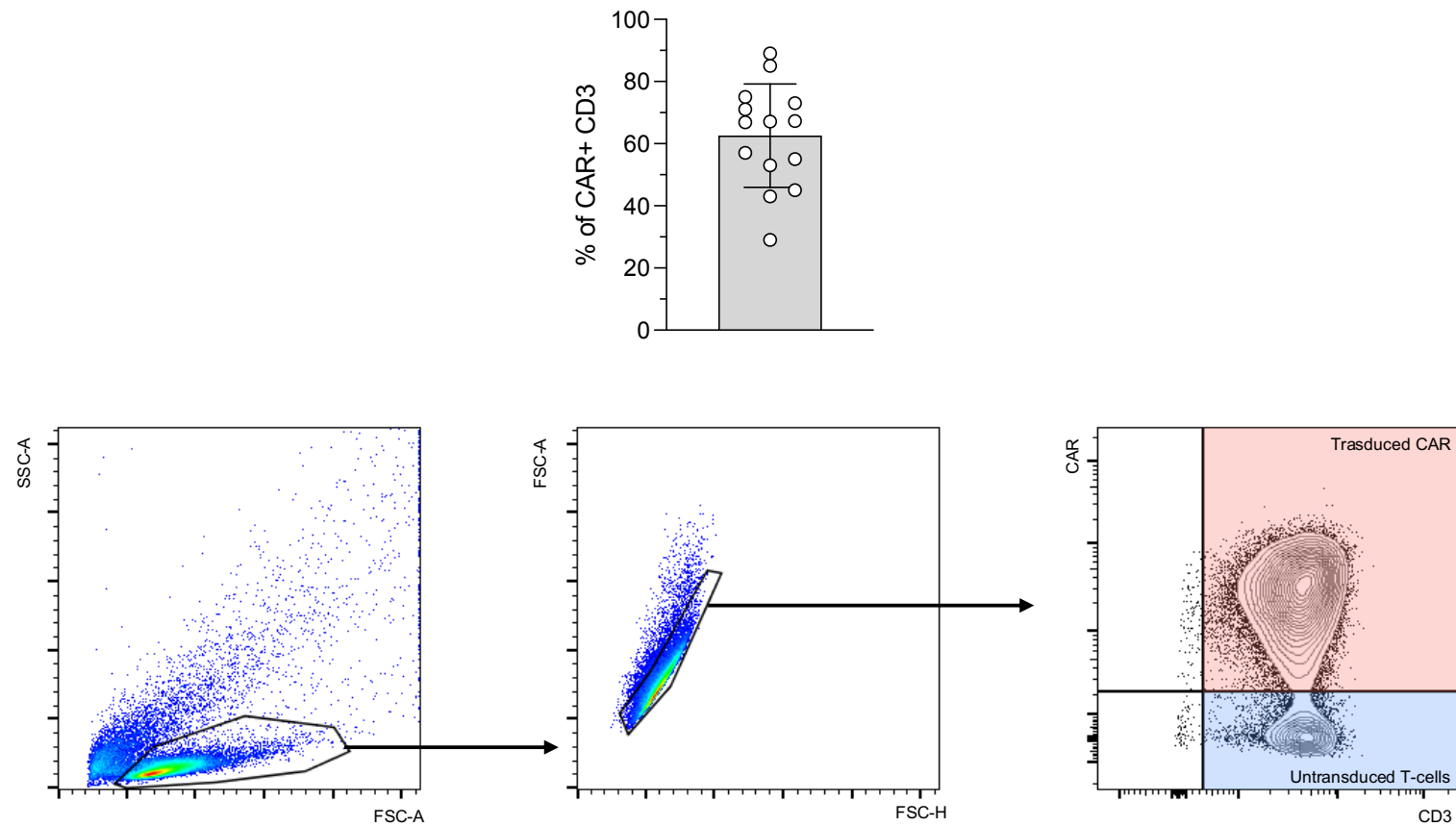


Figure SP10 : Representative batch transduction levels and gating strategy of CAR-T.



II.5.c T cell activation using recombinant CD33-Fc-6His protein

Frozen aliquots of CAR-T cells were thawed and plated at 1×10^6 cells/ml in complete Advanced with 50 U/ml of recombinant human IL-2 (Peprotech, UK).

Recombinant human CD33-Fc-6His protein (Gentaur, UK) was used to coat tissue culture 48-well plates. CD33-Fc-6His protein was brought to a concentration of $10 \mu\text{g/mL}$ in TRIS-HCl buffer (Sigma-Merk, Germany) at pH 9.2, and $160 \mu\text{L}$ were plated for each well and incubated overnight at 4°C . Wells were then washed 3 times using complete Advanced RPMI. To activate CAR-T cells, 2.5×10^6 cells were plated in each coated well and incubated overnight without IL-2 in $250 \mu\text{L}$ of complete Advanced RPMI. Resting CAR-T cells were plated in wells without coating and maintained in culture for the same time. Cells were then harvested and collected via centrifugation. Supernatants were stored at -80°C for future use while cells were used in co culture assays.

II.6 MSC re-education

MSCs have been cultured as above described. 2.5×10^4 /well were seeded in a 48-well plate (Corning, UK) and left to adhere for at least 4h. Media was aspirated and substituted with the supernatant from activated or resting CAR-T, produced as above described. Complete Advanced RPMI was used as basal control. Cells were kept for 24h at 37°C 5% CO_2 before supernatant sampling and/or being used in experiments. Supernatant samples were spun twice for 5 minutes at 2000 RPM and 15 minutes at 13000 RPM in pre-cooled centrifuge at 4°C to ensure cell debris removal and stored at -80°C for further analysis.

II.6.a RNA extraction

MSC monolayers after re-education were rinsed three times with PBS (Thermo Fisher Scientific, UK) and directly lysed using TCL lysis buffer (Qiagen, USA). Total RNA was purified using Norgen Single Cell RNA purification kit (Norgen, Canada) following manufacturer instruction. RNA purity and yield have been measured using Nanodrop-100 instrument before storage at -80°C.

II.6.b RNA sequencing and data analysis

Bulk RNA sequencing (RNAseq) was performed by Genome Scan Ltd. (Netherlands). In short library have been built using NEBNext Ultra II Directional RNA Library Prep Kit with poly(A) capture. RNAseq analysis is summarized in figure SP11. Library was sequences with NovaSeq 6000 PE151 using standard Illumina adapters, no trimming was performed. Raw sequencing reads were initially analyzed with FastQC 0.11.8 for quality control. Low quality bases ($Q \leq 20$) were turned into N by SeqTK. Adapters and reads with more than 10% of N bases were then removed by cutadapt 2.5 (-O 6 -e 0.1 -m 40). Reads were then mapped to the human genome reference GRCh38 (Version 32, Ensembl 98) by STAR 2.7.9a and transcripts were counts by RSEM 1.3.3 with default settings. DE inferences were performed by edgeR with a cut-off of $FDR < 0.05$. GO and KEGG analyses were performed by edgeR. All other calculations and plots were performed in R with base and tidyverse functions. Bioinformatics analyses were performed by Dr. Desmond Choi and Dr. Nicolas Sompairac from Dr. Kordasti SCI LAB in King's College London as part of collaboration. Intermediate graphs generated in the analysis are reported in figure SP12.

Figure SP11: Graphical representation of analysis pipeline

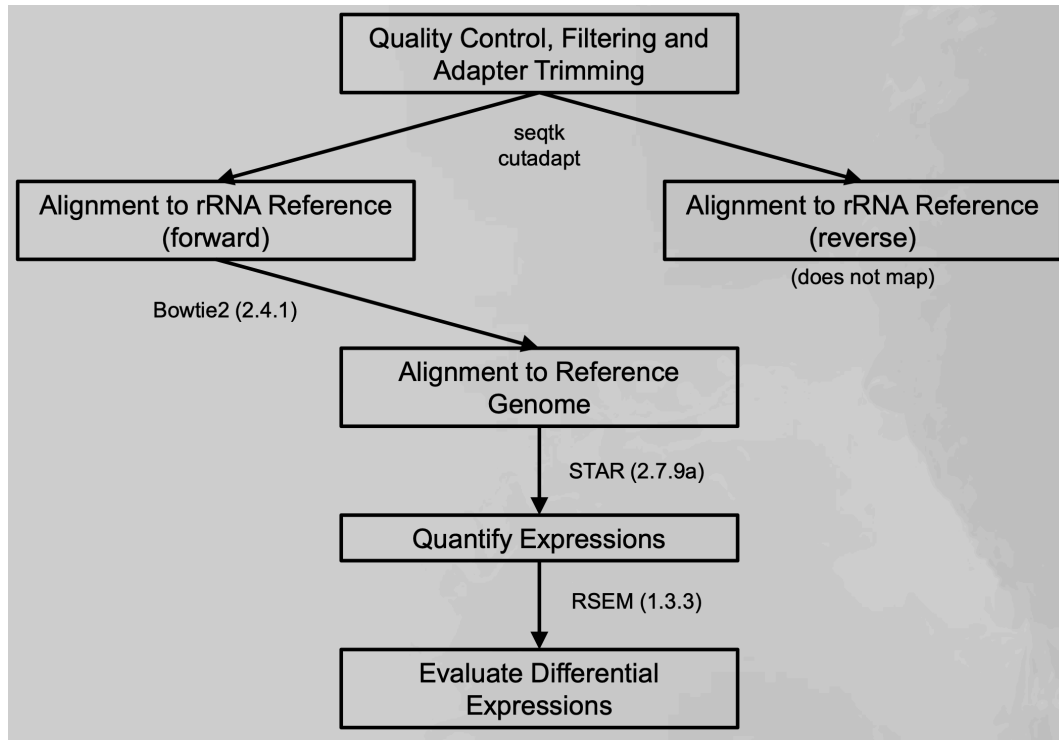
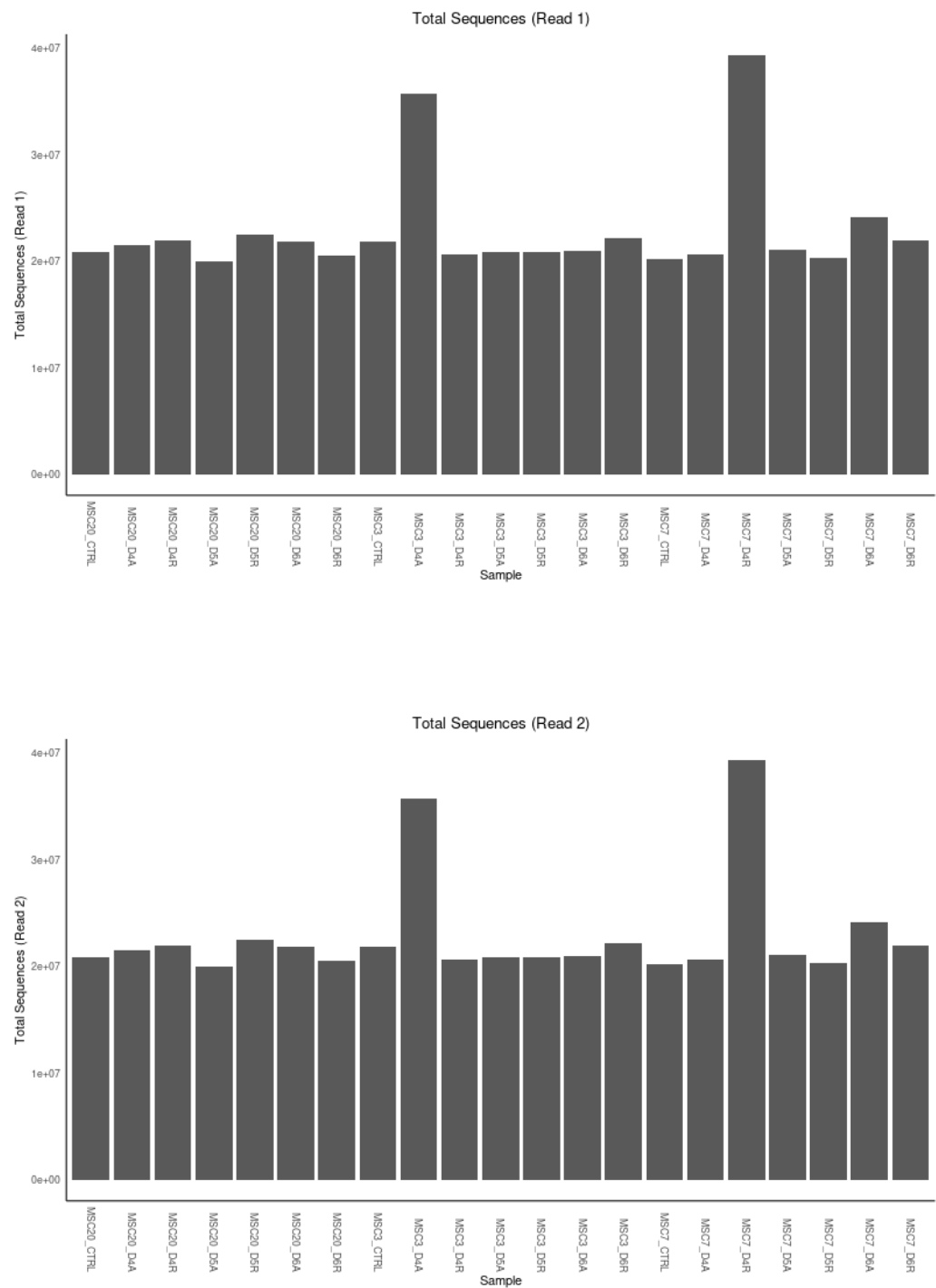
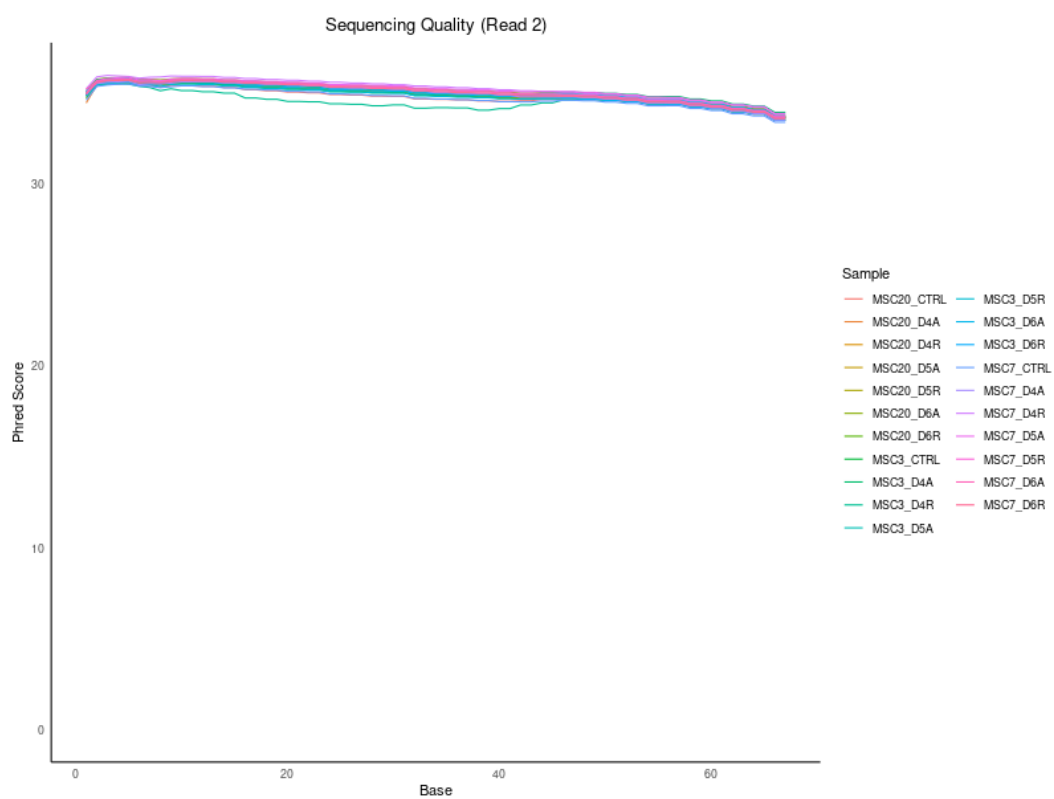
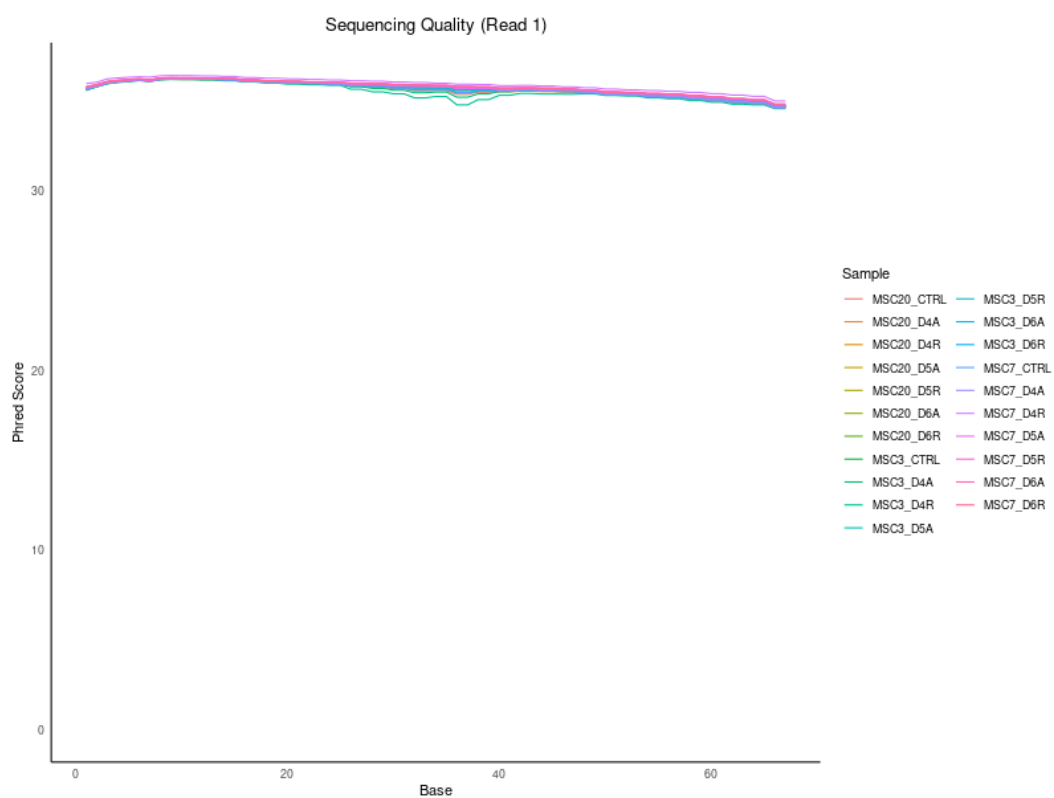
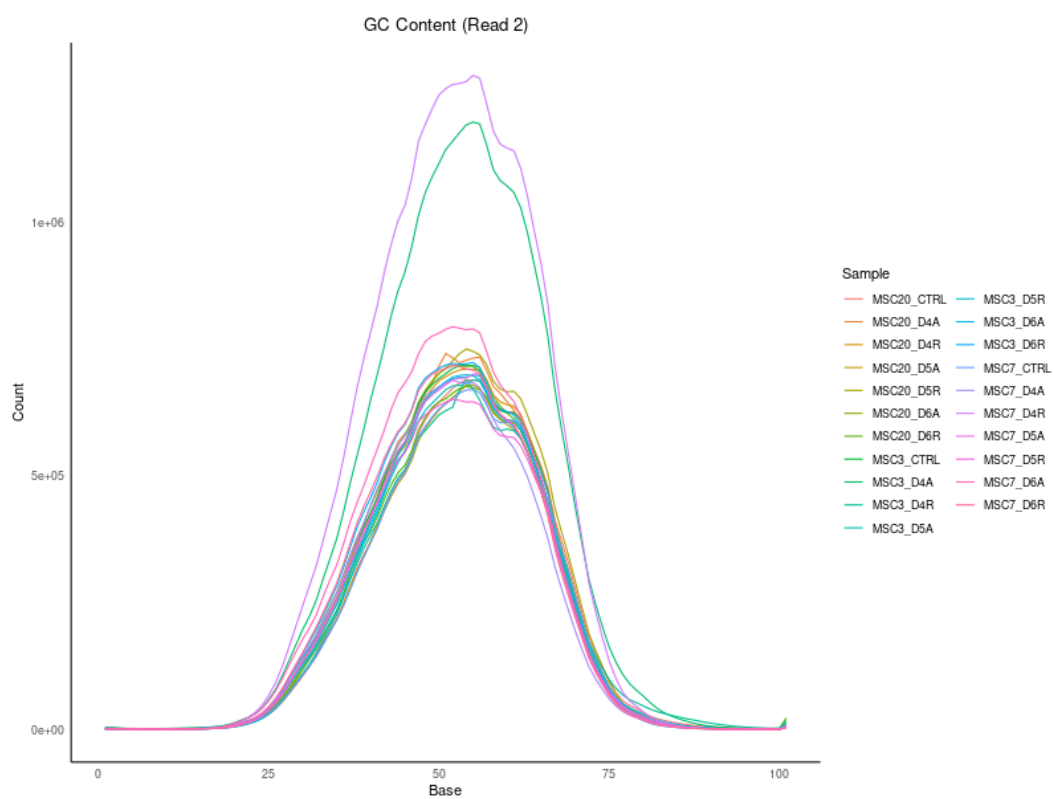
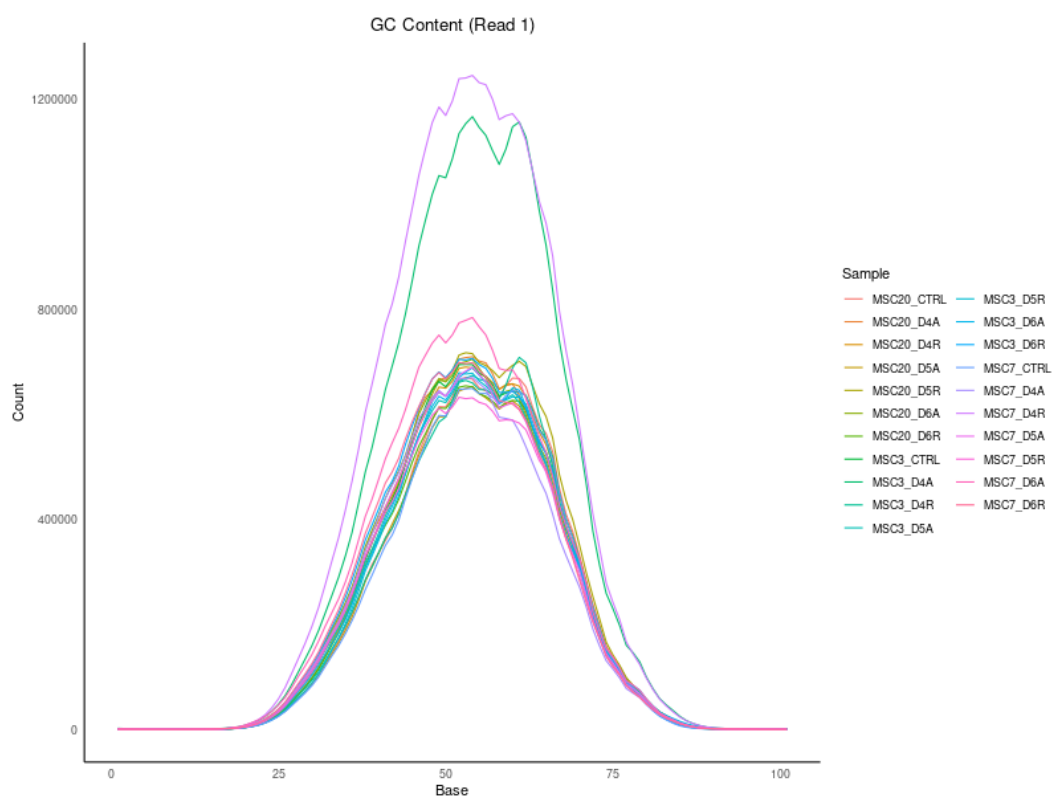
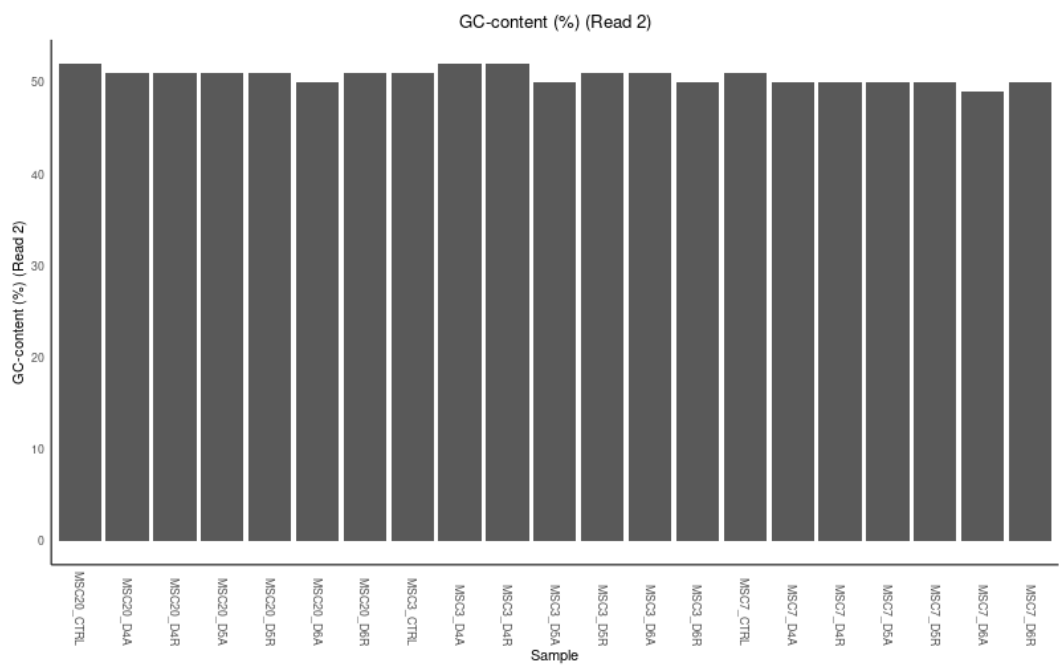
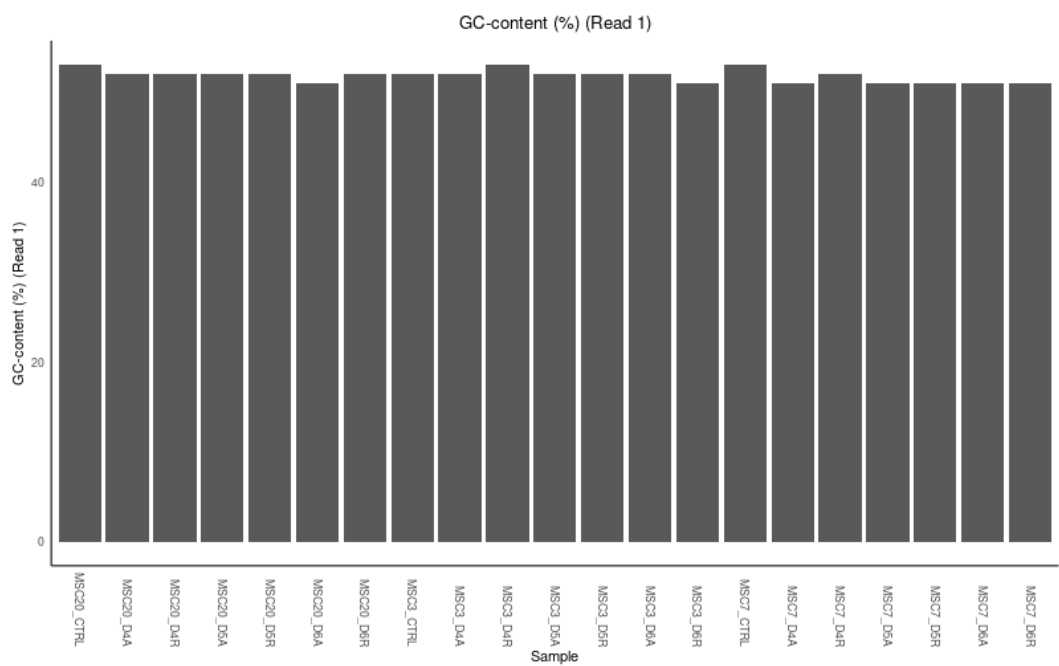


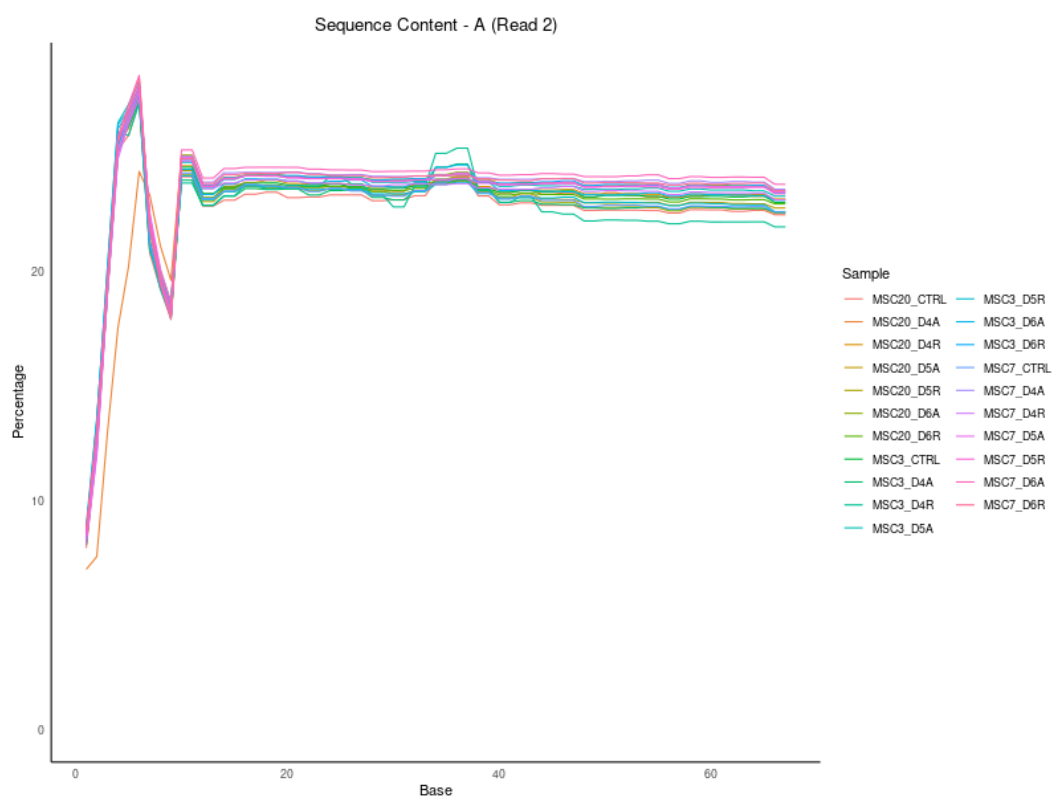
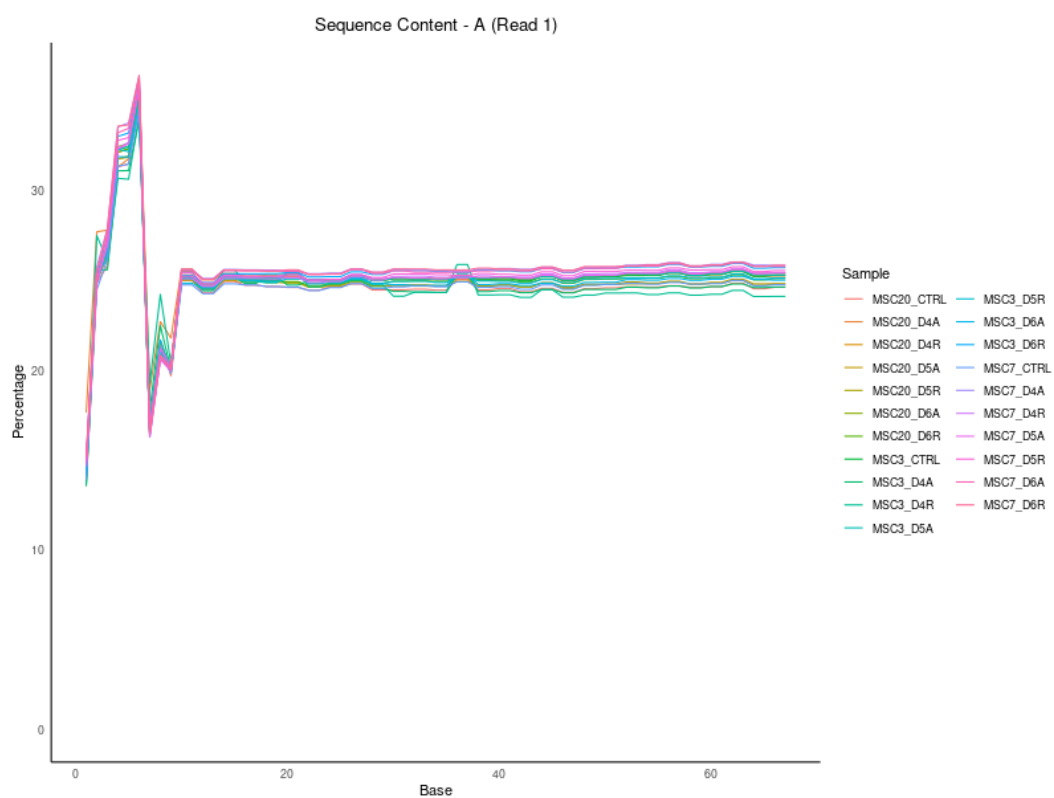
Figure SP12: RNAseq analysis step-by-step results

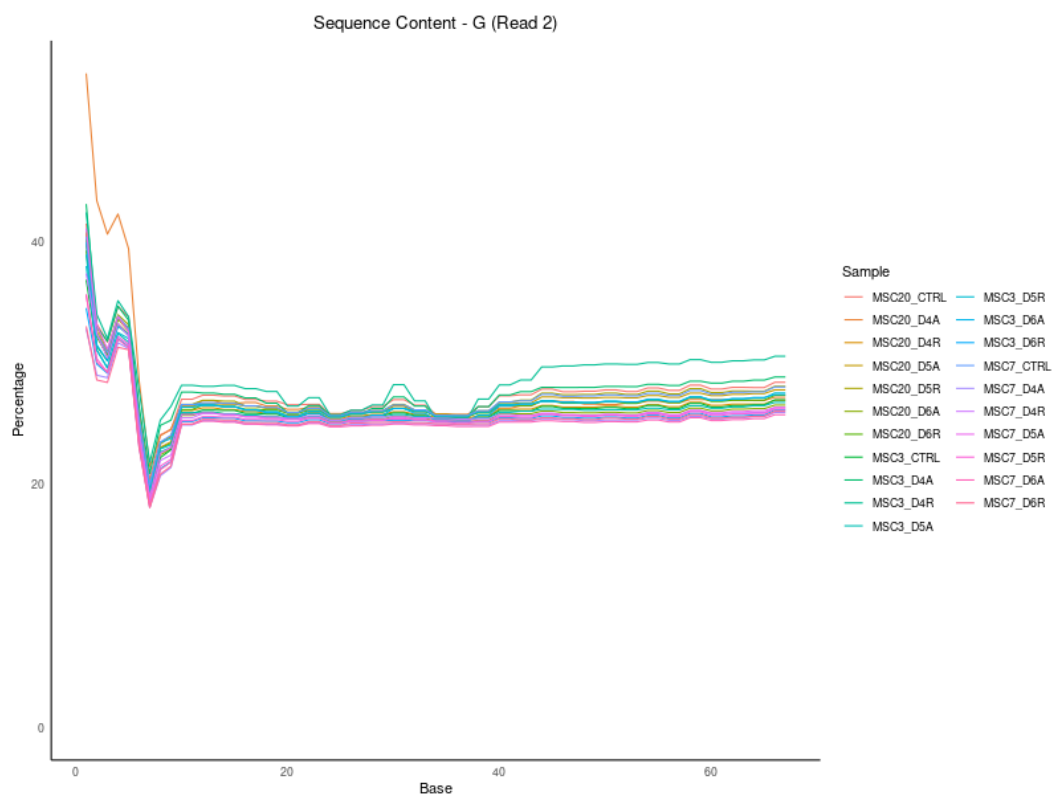
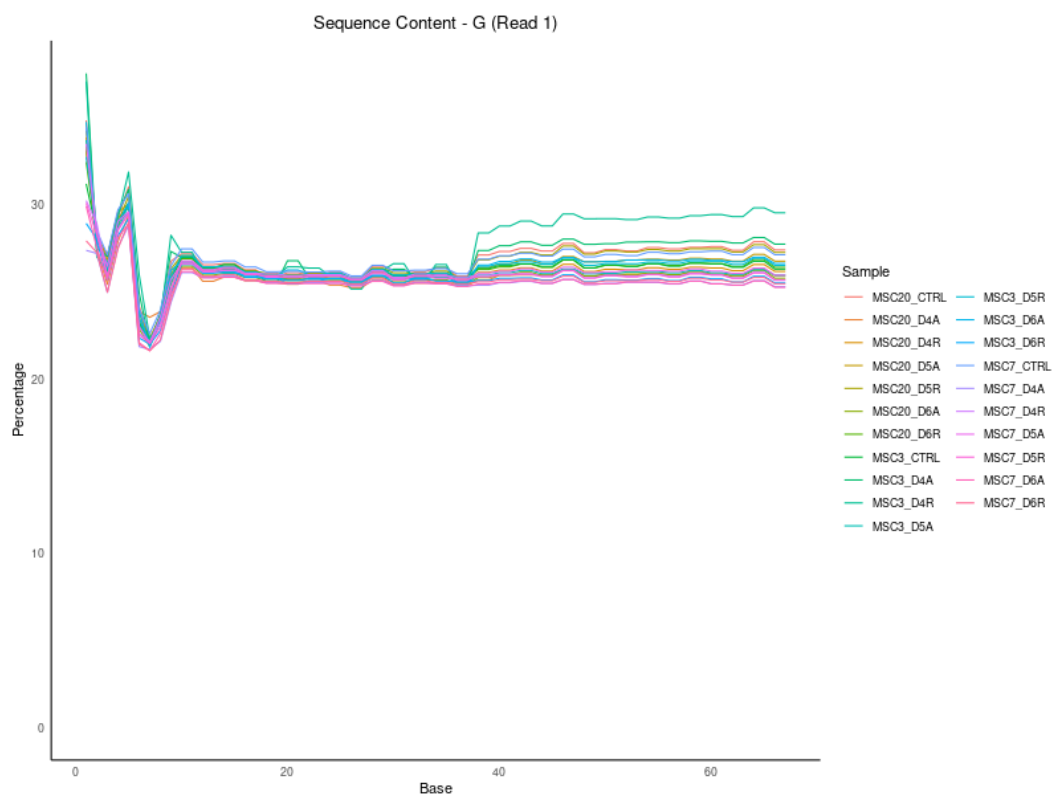


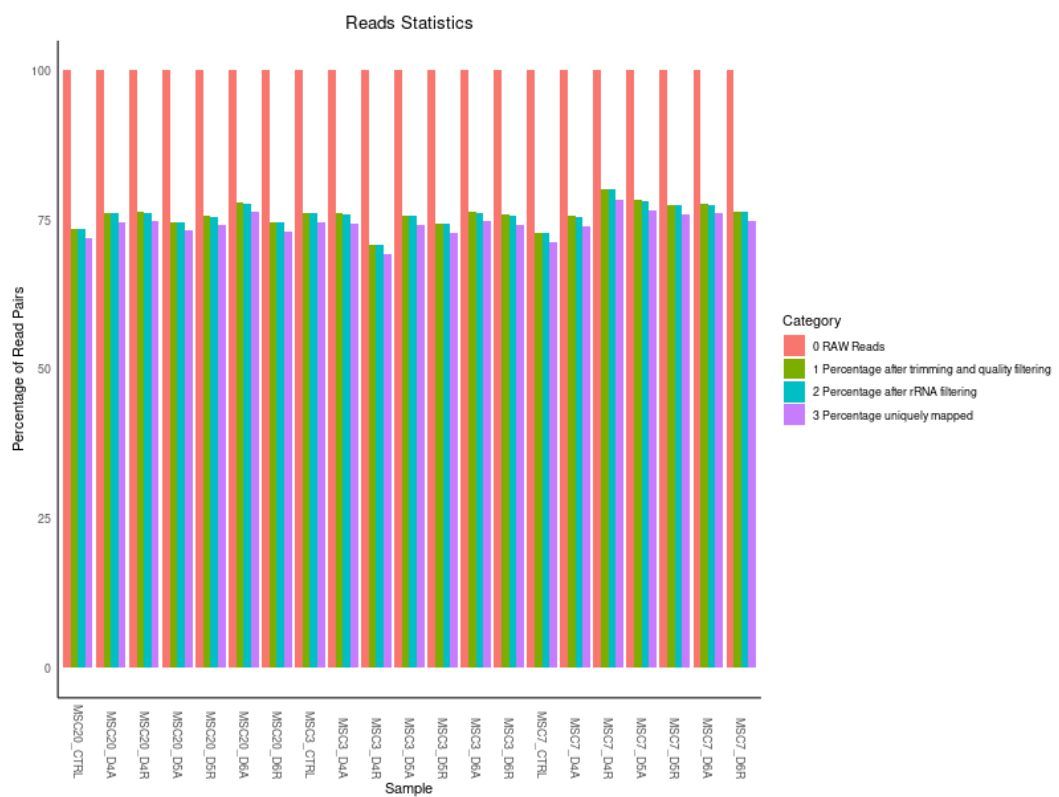
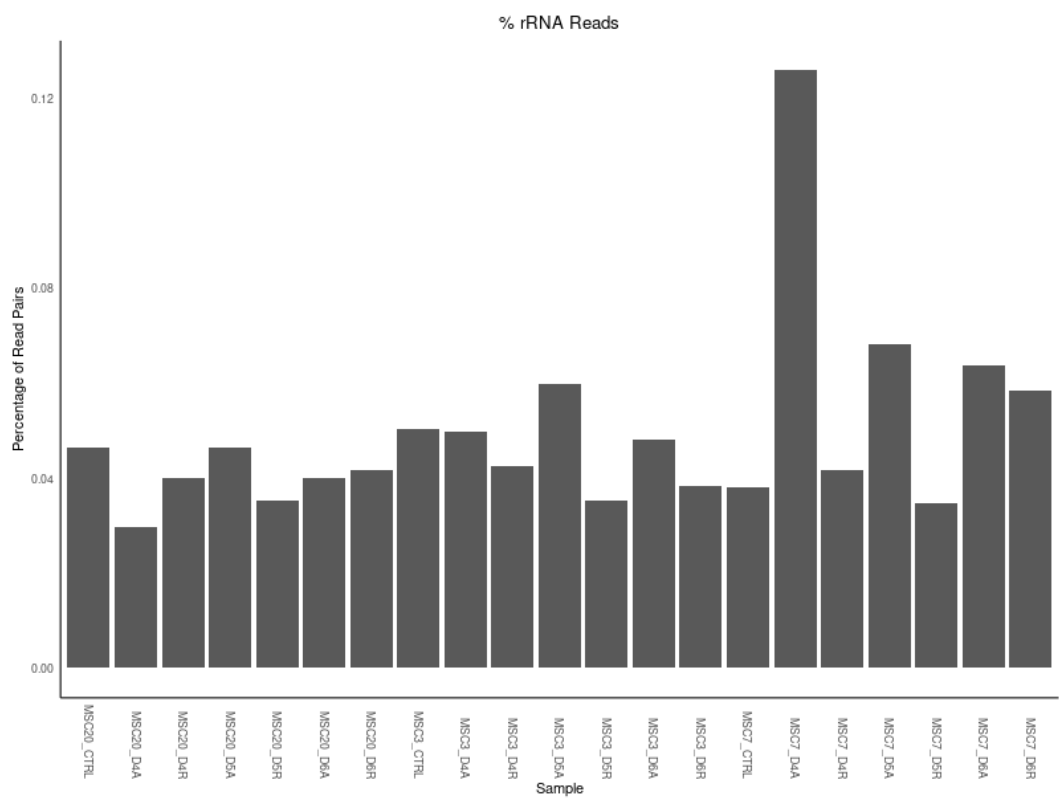


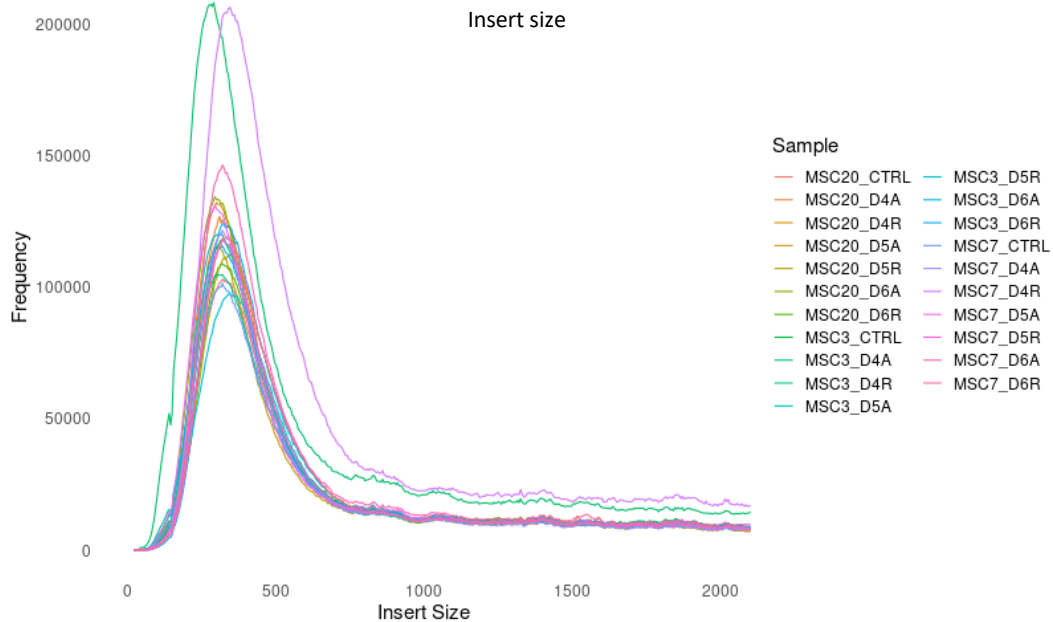












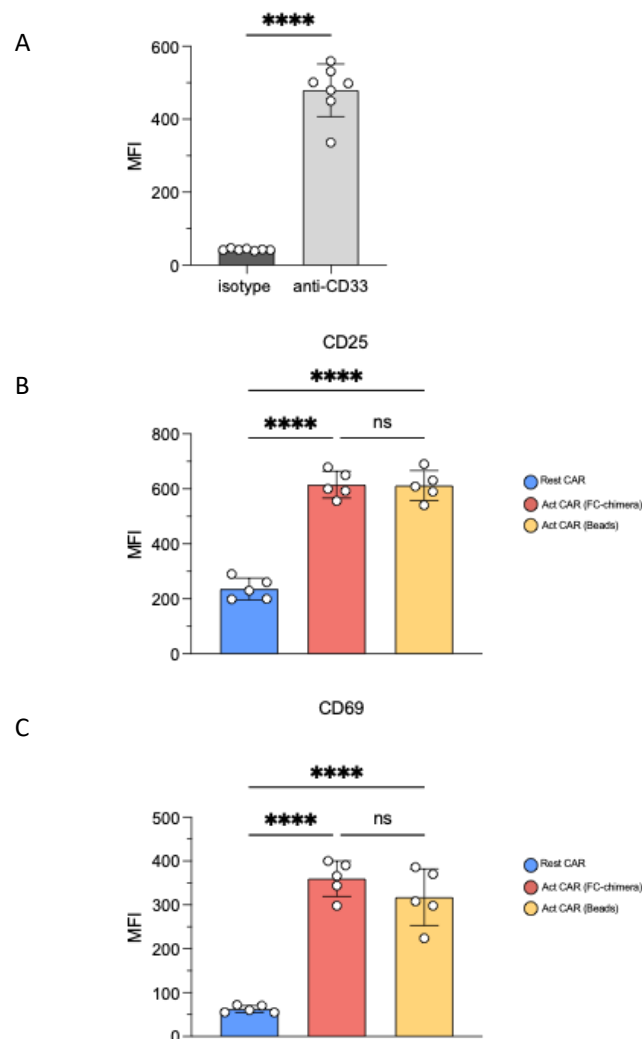
II.6.c Proliferation assay with re-educated MSCs

The proliferation ability of CAR-T was evaluated after co-culture with re-educated MSCs plated in monolayer in a 48-well plate as above described. Dynabeads M-450 Tosylactivated (Thermo Fisher Scientifics) were coated with CD33-Fc-6His protein following manufacturer instructions. Briefly, 250 μ L of beads were resuspended in 0.1 M Na₂PO₄ pH 9.5 with 10 μ g of CD33-Fc-6His protein and incubated overnight on rollers at room temperature. After incubation, beads were washed 3 times and conjugation was estimated by flow cytometry using anti CD33 antibody or isotype control (BD Bioscience, Belgium) as shown in figure SP13. CAR-T cells were maintained without IL-2 overnight and stained with CellTrace Violet (CTV) (Thermo Fisher Scientifics, UK) following manufacturer recommendation, and plated on re-educated MSCs at

a ratio of 5:1. Freshly conjugated beads were added at a 1:1 ratio to CAR-T. After 72 hours of co-cultures, cells were harvested, and CAR-T cell proliferation was analyzed by flow cytometry measuring the CellTrace Violet dilution. Proliferation assay with apoptotic MSC conditioned medium.

The effect of apoptotic MSC conditioned medium on CAR-T proliferation was evaluated as described in the previous paragraph. CAR-T cells were maintained in culture without IL-2 overnight and seeded in a 96-well plate previously coated with CD33-Fc-6His protein (10.000 cells/well) in 100 μ L of conditioned media formed by 75% complete Advanced RPMI and 25% apoptotic or live MSC supernatant. For experiments with PGE2 blocking, anti PGE2 antibody (Cayman Scientifics, USA) was added at 0.05mg/mL and pre incubated in conditioned media for 30 min prior to cell addition. Example gating strategy is represented in figure SP14.

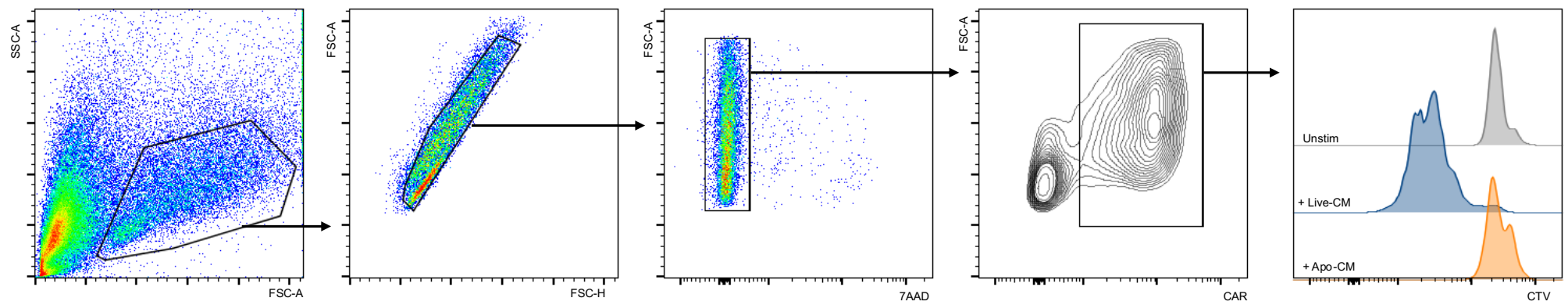
Figure SP13: CD33-FC protein coating beads and efficacy test to activate CAR-T



CD33-FC protein coating beads and efficacy test to activate CAR-T

(A) Conjugated beads were stained with anti-CD33 (APC) or isotype control (APC). Mean Fluorescent Intensity was calculated. Data are expressed as mean $\pm$ SD from n=7 experiments ****P<0.0001, paired t-test. **(B)** Resting or activated by beads or CD33-FC coated plates, CD33.CAR-T were stained with anti-CD25 (PE-cy7) and anti-CD69 (APC-cy7). Mean Fluorescent Intensity of live single cell population was calculated and plotted. Data are expressed as mean $\pm$ SD from n=5 experiments. ****P<0.0001, one-way Anova.

Figure SP14: Example of CAR-T gating strategy for proliferation assay (Apo-CM samples)



II.7 Cytokine level assessment

II.7.a ELISA for PGE2

PGE2 content of samples was determined using enzyme linked immunosorbent assay (ELISA) (Cayman, USA) following manufacturer instructions.

II.7.b Cytokine quantification via Luminex assay

Cytokines were measured in CAR-T supernatants by bead-based multiplex assay applying the Luminex technology using a 18-parameters customized panel from Milliplex encompassing GM-CSF, IFN- γ , IL-10, IL-12p70, IL-13, IL-15, IL-17a, IL-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, TNF- α , TNF- β (Merk Millipore, Germany). Supernatant samples collected from proliferation assays were thawed and diluted 1:2 before performing the assay following manufacturer instructions. Measurements and data analysis of all assays were performed using the Luminex analyzer (MAGPIX) in combination with the xPONENT 4.2 software.

II.8 MSC-CAR T co-cultures

II.8.a Apoptotic assay

MSCs were labeled with CTV (Thermo Fisher Scientific, UK) and plated at 2.5×10^4 cells/well in a 48-well plate overnight with a total volume of 200 μ l. The following day, the activated or resting CAR-T were added to the MSC layer at a CAR-T: MSC ratio of 5:1 for a period of 4 hours. In selected experiments, a transwell insert with pore size 0.4 μ m (Corning, UK) was utilized to prevent direct physical interaction between the CAR-T and MSCs. MSC apoptosis was measured by flow cytometry, after staining with PE-Annexin-V and 7-Aminoactinomycin D (7-AAD) kit (BD Biosciences, UK). The percentage of apoptotic MSCs was determined adding the percentage of Violet⁺/Annexin V⁺/7-AAD⁻ cells to that of Violet⁺/Annexin V⁺/7-AAD⁺ cells in co-culture with CAR-T compared to target cells alone. Figure SP15 depict an example of the used gating strategy.

II.8.b Live cell imaging

Time-dependent apoptosis mediated by CAR-T cells against MSCs was evaluated using Operetta CLS (Perkin Elmer, USA). Monolayer of MSCs seeded in imaging glass bottom plates (Generon, UK) were stained with CellMask Deep Red Actin Stain (ThermoFisher Scientifics, UK) and Hoechst (Perkin Elmer, USA) following manufacturer instruction. CAR-T cells were activated as above described and, after dead cells removal using MojoSort human dead cells removal kit (Biolegends, USA), labeled with CellTracker Orange CMRA (ThermoFisher Scientific, UK) following manufacturer protocol. CAR-T were co-

cultured for 8 hours with MSCs at an E:T ratio of 5:1 and images acquisition were performed every 5 minutes using the 20X water immersion objective with the help of Dr. Lazarous Fotopoulos and Dr. Thomas Williams at King's College London Stem Cell Hotel. To quantify cell death, percentage number of MSC nuclei were estimated during the 6h imaging time with Harmony V4.9 software (Perkin Elmer, USA), analysis pipeline is depicted in figure SP16.

Figure SP15: Gating strategy examples of MSC-CAR co culture after 4h to identify apoptosis in MSCs recorded by Annexin-V/7AAD staining and Active Caspase 3/7

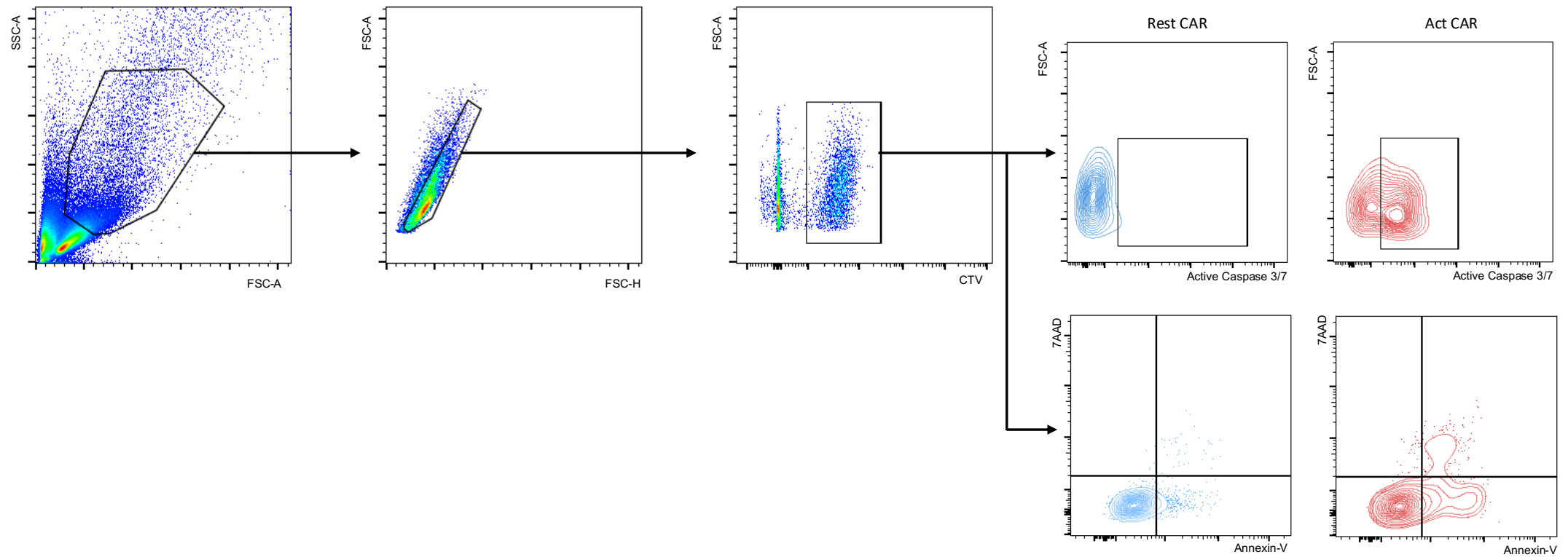


Figure SP16: Live cell imaging analysis pipeline

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20220802 Test Analysis For Cytenia Cytotox v3

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Find Nuclei (2)	Input	Method	Output

II.8.c Blockade assays

For conducting blocking experiments, various amounts of specific inhibitors or neutralizing antibodies were added to MSC: CAR-T mixed cultures, as listed in Table 3 and Table 4. Ethylene glycol-bis-(beta-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), Z-VAD-FMK, NEC-1, Z-AAD-CMK, Chloroquine, Bafilomycin or anti-CD33 anti-HLA-DR and anti-HLA-ABC were mixed with MSCs one hour prior to the co-culture with CAR-T. The neutralizing anti-FAS, anti-TRAIL or anti-NKG2D were incubated with effector cells two hours before their co-culture with MSCs. In all instances, the concentration of the respective inhibitor was sustained throughout the cytotoxicity assay. The selected concentrations were based on prior titration experiments and information from published studies under analogous experimental conditions.

II.9 Cytotoxicity assays of AML cells

To evaluate the killing ability of CAR-T cells pre-exposed to apoptotic MSC conditioned medium, they were co-cultured with CD33⁺ KG-1 AML target cells.

CAR-T cells were allowed to proliferate as previously described. At proliferation endpoint, cells were collected by centrifugation at 1800 RPMI for 10 minutes. Media were discarded and cells were resuspended in fresh media. KG-1 cells were labeled with CellTrace Violet (Invitrogen) and added to CAR-T cells at an Effector to Target (E:T) ratio of 1:10, calculated on the initial number of CAR-T cells seeded for the proliferation. Plates were incubated at 37°C, 5%CO₂ for 24h. Cells were then collected by centrifugation and stained with Annexin V/7-AAD apoptosis detection kit (BD Bioscience, Belgium) according to the

manufacturer's instructions. Target cells killing was measured by flow cytometry. The percentage of killed cells was determined adding the percentage of Violet⁺/Annexin V⁺/7-AAD⁻ cells to that of Violet⁺/Annexin V⁺/7-AAD⁺ cells in co-culture with the effectors compared to target cells alone. Gating strategy used in the analysis is represented in figure SP17.

II.10 Flow cytometry analysis

All flow cytometry experiments were conducted using Fortessa-X20 or Canto-II instruments (BD Bioscience, Belgium). For intracellular staining BD Fix/Perm kit was used (BD Bioscience, Belgium), representative gating can be found in figure SP18 Application setting were set to ensure coherence between experiments. Compensation was generated using Comp-Beads Plus (BD Bioscience Belgium). Antibodies and dyes used are summed in Table 5 and 6. FCS files were analyzed using FlowJo software version 10.4 (FlowJo, USA).

Figure SP17: example of gating strategy used to analyze cytotoxic assay after CAR and KG-1 co cloture.

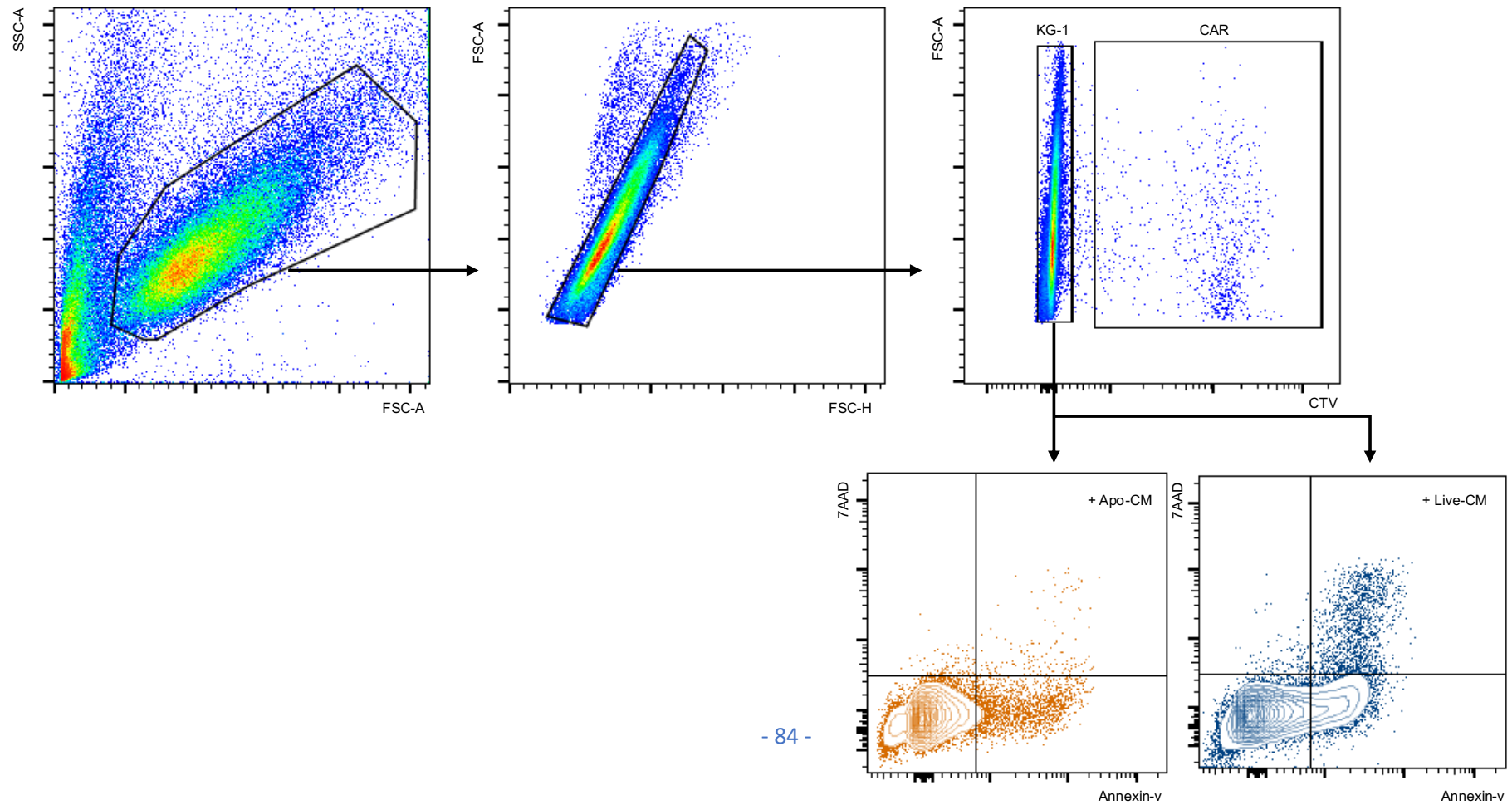
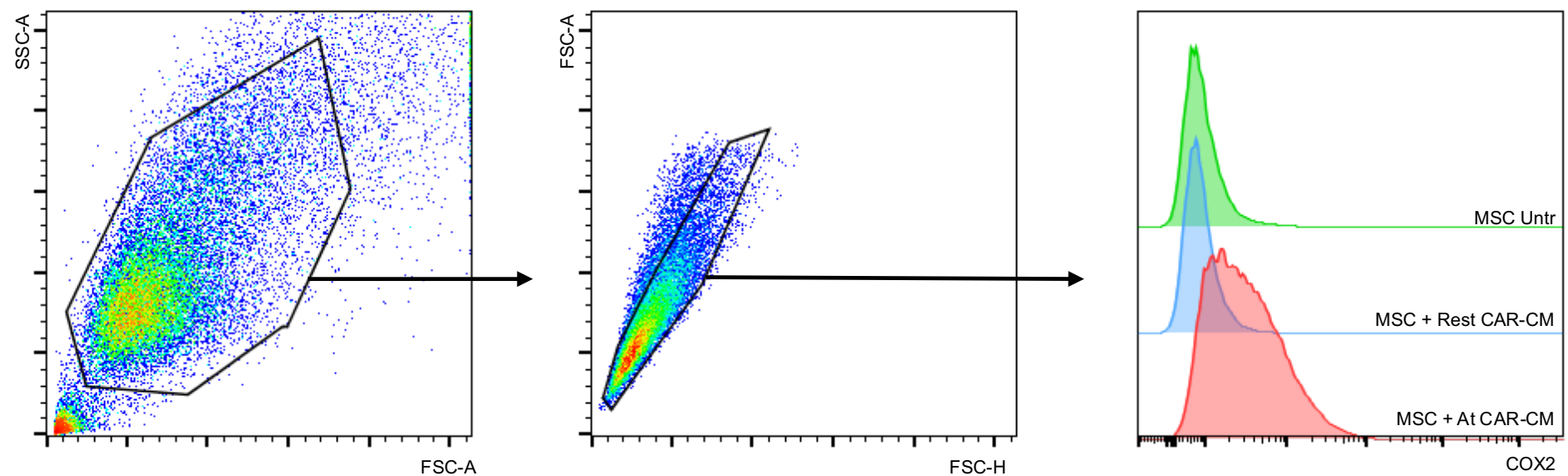


Figure SP18: Example of gating strategy used to analyze intracellular level of COX2 in MSCs after CAR-CM re-education.



II.11 In-vivo experiments.

Experimental protocols for animal studies were approved by the Italian Ministry of Health (permit number 178/2023-PR).

Six-week-old male NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were used (Charles River Laboratories). A leukemia xenograft model was established by intravenously injecting CD33⁺ KG-1 AML cells into NSG mice (5x10⁶/mouse). Fourteen days later, mice were left untreated or infused with CD33.CAR-T (10x10⁶/mouse) alone or together with APO-MSCs (2x10⁶/mouse). Peripheral blood was sampled from the tail vein weekly to evaluate percentage of CD33⁺ AML cells and CD3⁺ CAR-T cells. Mice were brought to survival and euthanized when body weight dropped of 20% or when the limbs were paralyzed.

Human grafts in mice were evaluated using anti-human CD45 (clone 30-F11) (Life Technologies), CD3, CD33 (clone P67.6) (BD Biosciences) and anti-mouse CD45 (clone 30-F11) mAbs (BioLegend). The absolute number of CAR-Clks and CD33⁺ KG-1 cells was quantified by flow cytometry, after adding 10µl of CountBright Absolute Counting Beads (Thermo Fisher Scientific) per tube. Representative example of gating strategy is reported in figure SP19.

II.12 Statistical analysis

Data were analyzed using GraphPad PRISM version 9.3 software (GraphPad, USA). The experimental findings are represented as the mean $\pm$ SD, based on a minimum of three biological replicates often represented as the average of 3 technical replicates. To determine the average discrepancies when there were more than two samples, a one-way ANOVA and subsequent Tukey test were employed. For comparing average differences across groups, a two-way ANOVA and post-hoc Tukey test were used. For contrasting differences between two individual samples, both unpaired and paired Student's t-tests, depending on the experimental context, were conducted. The Kaplan-Meier method was applied to estimate survival curves, while the log-rank test was used for comparisons. The tests were performed two-sided. and the statistical significance was attributed to p-values less than 0.05.

Figure SP19: example of gating strategy used to analyze samples of murine peripheral blood.

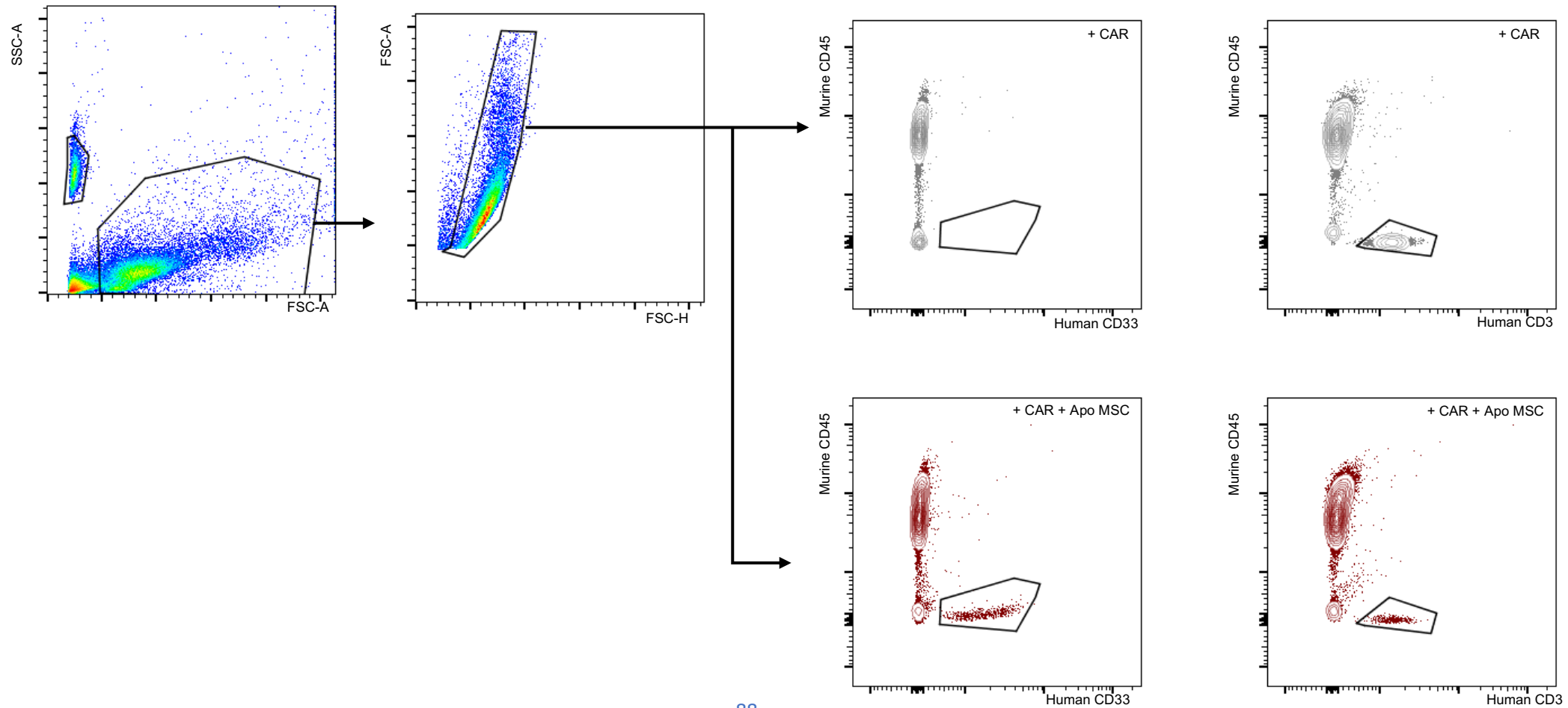


Table 1 : MSC donor sex and age

Identifier	Sex	Age
1	Female	42
3	Male	19
5	Male	18
20	Male	47
Guba	Female	24
Monz	Female	39

Table 2 : PBMC donor sex and age

Identifier	Sex	Age
1	Male	19
2	Male	42
3	Male	37
4	Female	26
5	Male	29
6	Female	33
7	Male	31
8	Male	42
64	Female	48
65	Female	36
66	Male	22
71	Male	25
73	Male	45
77	Female	31

Table 3: Blocking and Activating antibody.

Blocking / activatory antibodies					
Recognized epitope	Species reactivity	Origine species	Clone	Final concentration	Manufacturer
FAS (activating)	human	mouse	CH11	10 µg/ml	Millipore
PGE2	human	mouse	2B5	0,05 mg/ml	Caymann
MHC-A,B,C	human	mouse	W6/32	100 µg/ml	Biolegends
MHC-DR	human	mouse	LN3	50 µg/ml	Biolegends
CD33	human	mouse	S21002A	100 µg/ml	Biolegends
NKG2D	human	mouse	1D11	100 µg/ml	Biolegends
FAS (blocking)	human	mouse	DX2	100 µg/ml	Biolegends
TRAIL	human	mouse	71903	75µg/ml	R&D

Table 4: Inhibitors and small molecules

Inhibitors and small molecule			
Reagent	Target	Final concentration	Manufacturer
EGTA	Ca ions	4 mM	Sigma-Merk
Z-AAD-CMK	Granzyme b	100 µM	Merk-Millipore
NEC-1	RIPK3	50 µM	Cayman
Z-VAD-FMK	Pan-caspase	50 µM	R&D System
Cloraquine	Endosome	100 µM	Sigma-Merk
Bafilomycin	Autolysosome	50 µM	ThermoFisher

Table 5: Flow cytometry antibody

Flow cytometry antibody						
Recognized epitope	Species reactivity	Origine species	Clone	Dilution	Fluorophore	Manufacturer
FC	human	mouse	M1310G05	1 : 100	PE	Biolegends
CD33	human	mouse	P67.7	1 : 40	PE-Cy 7 /APC	BD bioscience
COX2	human	mouse	AS67	1 : 50	PE	BD bioscience
CD45	human	mouse	HI30	1 : 200	Pacific Orange	Life Technology
CD45	mouse	rat	30-F11	1 : 50	PE	Biolegends
CD3	human	mouse	OKT3	1 : 40	Per-CP Cy 5.5	Biolegends

Table 6: Fluorescent dyes used in flow cytometry.

Flow cytometry reagents			
Reagent name	Staining target	Emission (nm)	Manufacturer
Cell Trace Violet	cytoplasm	450	Thermofisher
Cell Trace Orange CMRA	cytoplasm	576	Thermofisher
Cell Mask Live Actine Deep Red	actine filaments	669	Thermofisher
7AAD	nucleus	647	BD bioscience
Hoechst 34580	nucleus	461	Thermofisher
CellEvent Caspase 3/7	cytoplasm	523	Thermofisher
Annexin-V	exposed phosphatidil serine	523 or 574	BD bioscience

Chapter III – Results:

CAR-T released soluble factors re-educate MSCs towards an immunosuppressive phenotype.

III.1 introduction

As previously described, MSCs are very plastic cells, which can be strongly influenced by the surrounding microenvironment. Examples are several, like TLR4 activation which induces MSCs to produce pro-inflammatory mediators and chemoattract inflammatory cells (Romieu-Mourez et al., 2009). On the opposite side of the spectrum, MSCs “licensing” by an inflammatory microenvironment to acquire an immunosuppression-promoting phenotype has been described (Krampera et al., 2006).

Moreover, MSCs can interact with the immune system by several means such as soluble factors (Nasef et al., 2007; Nicola et al., 2002), membrane bound factors (Giuliani et al., 2011; Sheng et al., 2008), metabolic reprogramming (François et al., 2012; Ryan et al., 2007) and by releasing chemokine to attract immune cells in the microenvironment (Sala et al., 2015; Selmani et al., 2008).

Considering that information, in this chapter we aim to shed light on the phenotypic changes that MSCs can undergo in a microenvironment characterized by the presence of activated CAR-T derived soluble factors, and to understand the key molecular pathways driving this change and its impact on the CAR-T cell functionality.

III.2 Soluble factor released by activated CAR-T cells induce MSCs re-education towards an immunosuppressive phenotype.

CD33.CAR-T were activated for 24h on plates coated with human recombinant CD33-Fc chimera protein as depicted in the experimental schematics in figure 1A. To verify the CAR-T activation, staining with anti CD25 and CD69 was performed (figure 1B). CD25 is a subunit of the IL-2 receptor and is upregulated upon Tcell activation granting them the ability to expand, its upregulation is sustained after activation, CD69 on the other hand is known to be an early activation marker and it is rapidly upregulated upon stimulation. After stimulation CD33.CAR-T show a 2- and 3-fold increase of CD69 and CD25 expression respectively.

WT MSCs were exposed for 24 hours to the media conditioned by activated or resting CAR-T before performing bulk RNA-seq analysis. MSCs at steady state, re-educated by resting or activated CAR-T were clustered by Principal Component (PC) as depicted in figure 1C. Different MSC donors were spread across PC2 (12% of variance) and this can be reconducted to differences in isolation method and donor sex and age (Mets & Verdonk, 1981; Yang et al., 2018). More importantly across PC1 (57.7% of variance) we can see that steady state MSCs and MSCs re-educated by resting CAR-T supernatants clustered close to each other, and far from the ones re-educated with activated CAR-T supernatants. Differentially expressed genes (DEG) between MSCs re-educated by resting or activated CAR-T were identified using EdgeR pipeline, 11447 genes were significantly differentially expressed, around 70% of which were upregulated. DEGs are represented in the volcano plot (figure 1D). DEGs were further represented in the heatmap (figure 1E) where they were divided into 4 different groups by their role: metabolic, soluble mediators, chemokines and

membrane bound factors. Many well-established immunosuppressive molecules such as CD274, IDO1, STC1 and CEACAM1, which together have already demonstrated their role in maintaining a “cold” TME (Bonavita et al., 2020; Gong et al., 2023; Pelly et al., 2021)(figure 1E), are specifically upregulated in MSCs re-educated by activated CAR-T. Notably, the PTGS2 (COX2)- Prostaglandin E2 synthetase (PTGES) axis, reported as a significant mediator of MSC-mediated immune regulation, is upregulated in MSCs exposed to the supernatant of activated CAR-T compared to those at steady state or exposed to resting CAR-T.

Furthermore, to identify a key regulator responsible for the phenotypic changes observed in MSCs re-educated by activated CAR-T, the top 500 upregulated genes were used as input in Enrichr pipeline. The top 2 terms from relevant databases were selected and visualised in a lollipop plot (Figure 1F). This analysis clearly showed an enrichment in genes related to interferon gamma signalling and a transcriptomic profile which could be linked to NF- κ B activation, giving us possible trigger and executor candidates for the phenotypic switch of the MSCs after exposure to factors released by activated CAR-T.

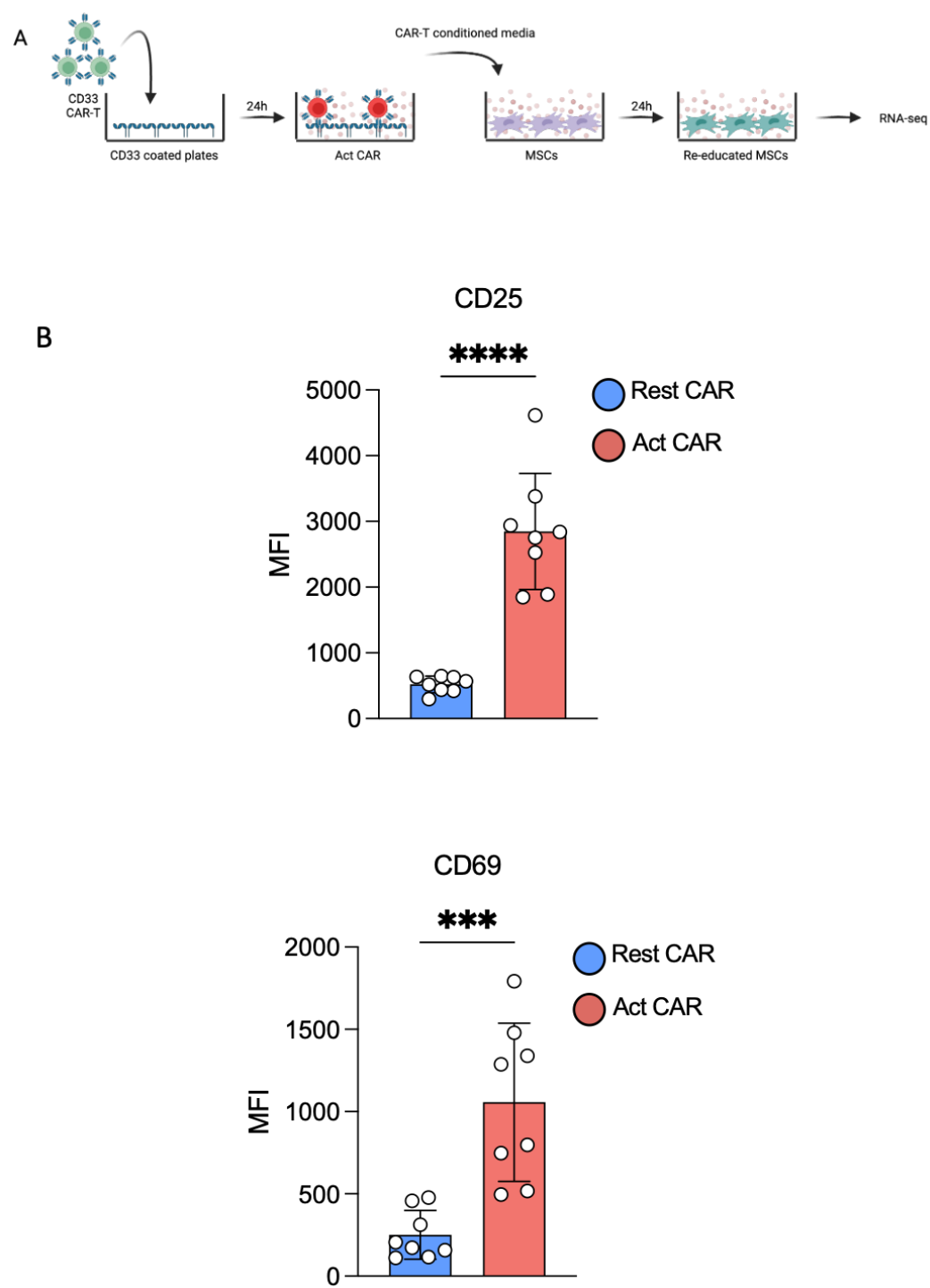
III.3 Re-educated MSCs express increasing level of COX2 and PGE2 in a NF- κ B dependent fashion

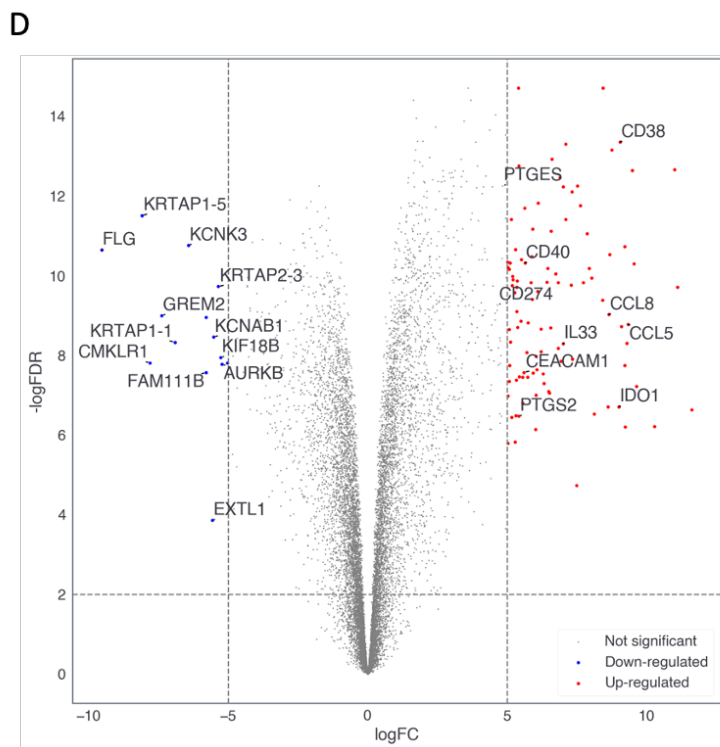
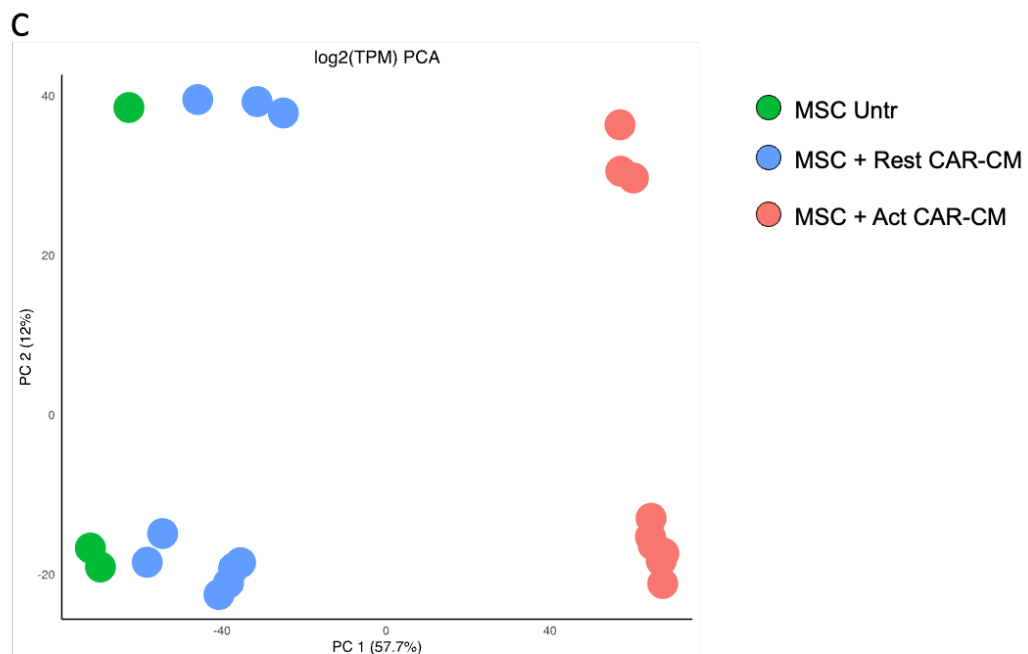
To validate RNA-seq data, flow cytometry was employed to measure the level of expression of COX-2 enzyme in MSCs after re-education by activated CAR-T. As shown in figure 1G, at steady state only around 7% of the MSC population expresses the enzyme, percentage that increases to 40% after re-education. Interestingly, only activated CAR-T induce the up-regulation of COX-2 expression in MSCs.

For investigating the role of NF- κ B complex in this up-regulation, their subunit Rel-A was knocked down in MSCs via lentiviral transduction with an shRNA construct kindly gifted by Dr. Guido Franzoso (Imperial College London). Transduction efficiency was measured by GFP expression and assessed by flow cytometry. MSCs batches with > 40% transduction efficiency were used for experiments due to the low tolerability of MSCs to be sorted and expand after lentiviral transduction.

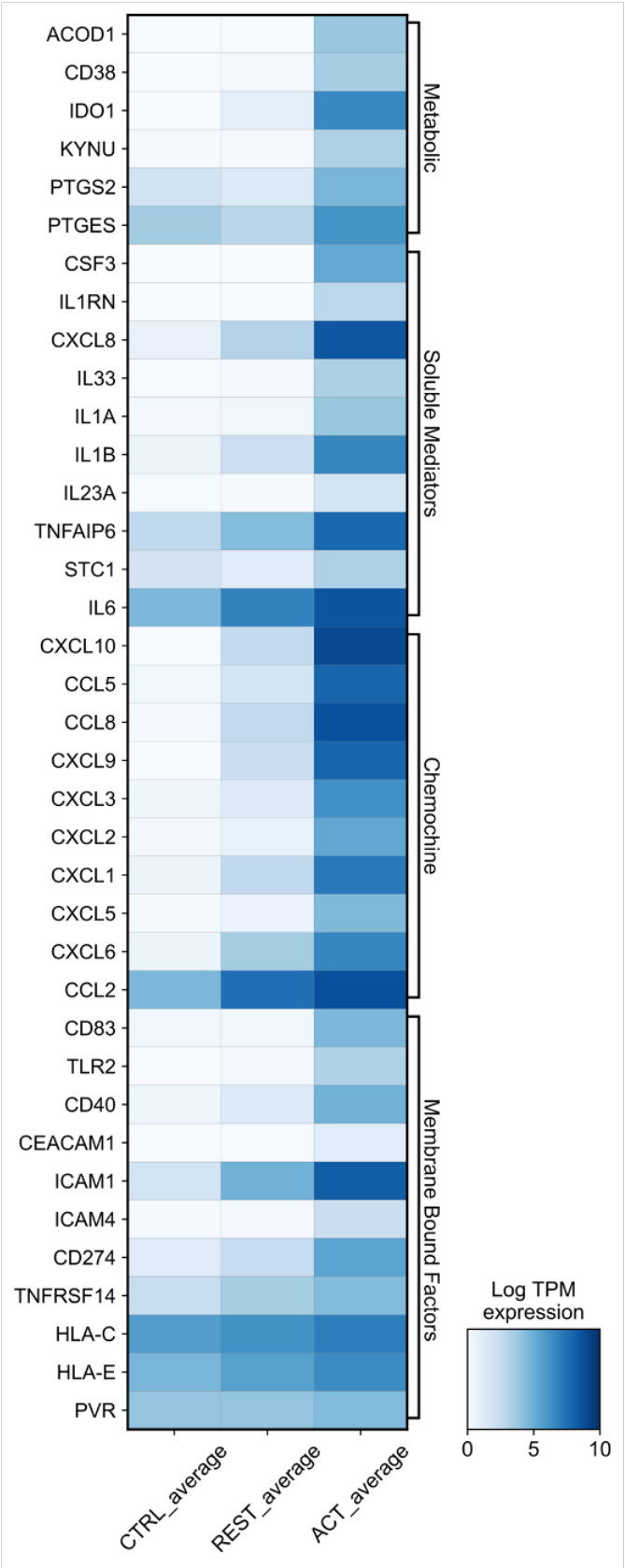
Rel-A^{KD} in MSCs (hereafter named REL-A^{KD} MSCs) significantly inhibit the COX-2 upregulation after exposure to supernatant of activated CAR-T (Figure 1G) when compared to MSC transduced with scrambled shRNA control (hereafter named MSC). Moreover, we investigated the enzymatic activity of COX-2 by measuring the levels of PGE2, the final product of the enzymatic pathways of arachnoid acid catabolism. For this aim ELISA assay was used and, as we can see in figure 1H, MSCs re-educated by activated CAR-T release in the culture medium a significantly increased amount of PGE2 compared to those exposed to resting CAR-T. This increase was strongly reduced when Rel-A^{KD} MSCs were used, enforcing the NF- κ B dependency of COX-2 pathway activation (figure 1I).

Figure 1: Activated CAR T cells re-educate MSCs to acquire an immunosuppressive phenotype.

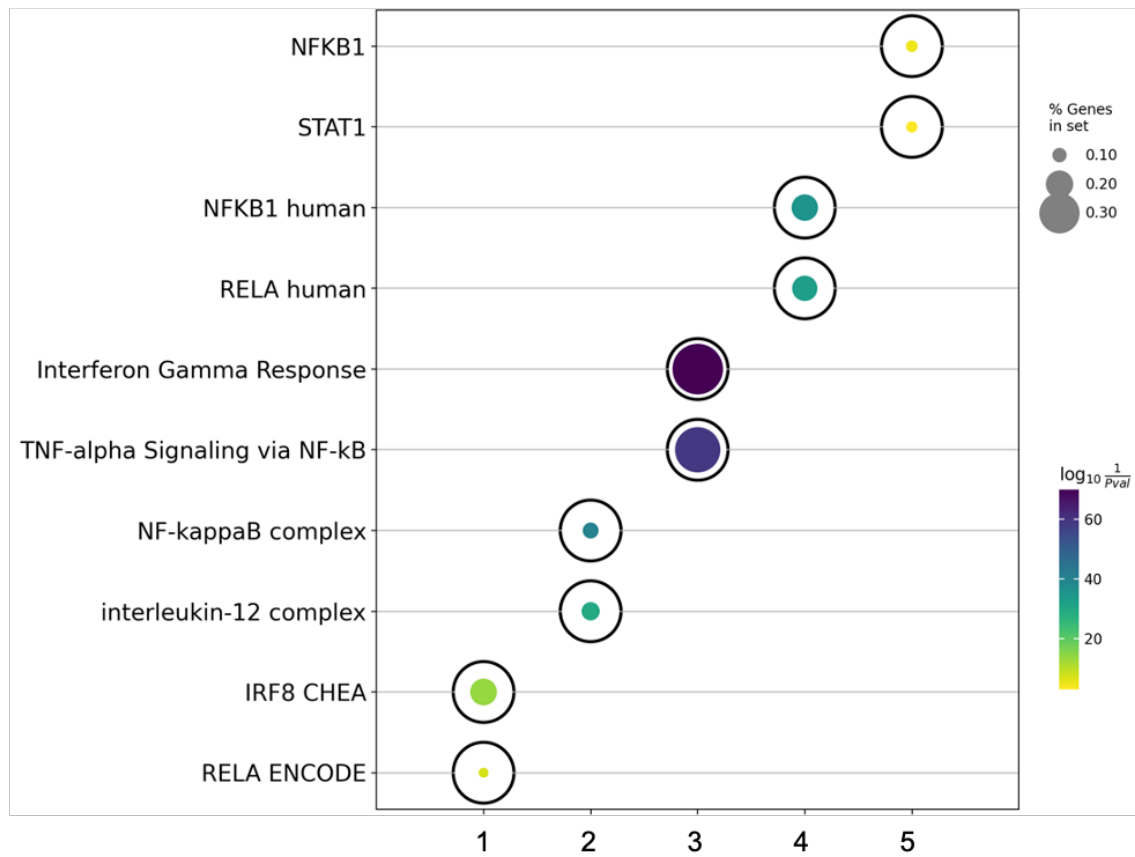




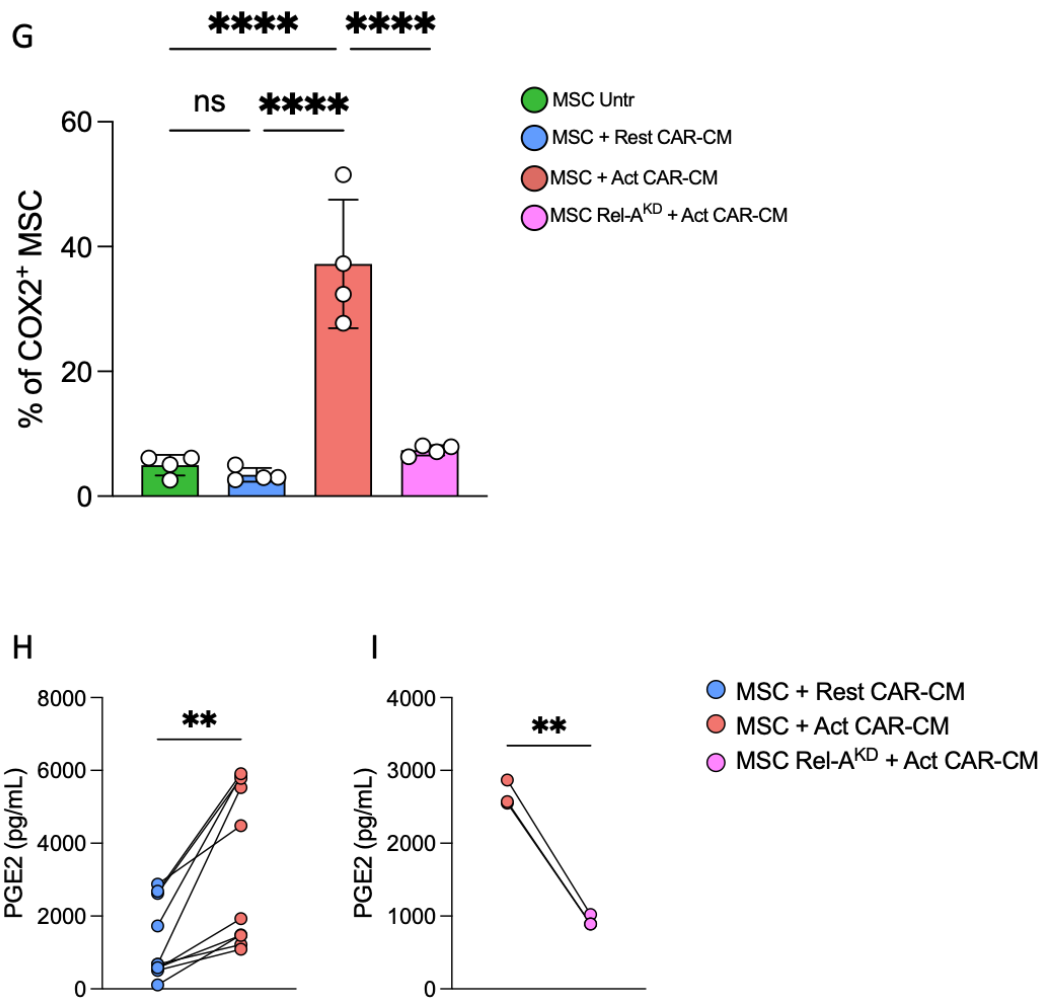
E



F



1 Encode and ChEA consensus TFs from Chip-X
 2 Jansen Compartments
 3 Msig DB hallmarks 2020
 4 TRRUST trascription factors 2019
 5 Transcription factor PPIs



Activated CAR T cells re-educate MSCs to acquire an immunosuppressive phenotype.

(A) Experimental design for CAR-T activation and MSCs re-education. CD33.CAR-T cells were seeded for 24h onto plates coated with human recombinant CD33-Fc-6His protein. Conditioned media was collected and used to stimulate MSC monolayers for 24 h. Treated MSCs were analyzed by RNAseq. MSCs generated from 3 different donors were exposed to the

supernatant of 3 independent CAR-T preparations. MSCs untreated or exposed to the supernatant of resting CD33.CAR-T cells were used as control. (B) Resting or activated CD33.CAR-T were stained with anti-CD25 (PE-cy7) and anti-CD69 (APC-cy7). Mean Fluorescent Intensity of live single cell population was calculated and plotted. Data are expressed as mean $\pm$ SD from n=8 experiments. ***P<0.001, ****P<0.0001, paired t-test. (C) PCA clustering of MSCs untreated (green) or re-educated by resting (blue) or activated (red) CAR-T milieu. (D) Volcano plot comparing the DEG of MSC re-educated by resting versus activated CAR-T. Thresholds for upregulated genes are LogFC > 5 and FDR < 0.01, while for downregulated genes are LogFC < -5 and FDR < 0.01. (E) Heatmap of the log TMP expression of selected genes for MSC untreated and re-educated by resting or activated CAR-T. Marker genes were divided in 4 groups: metabolic, soluble factors, chemokine and membrane bound factor. (F) Enrichment results of the top 500 upregulated genes, ranked by LogFC and with adjusted p value < 0.005, using Enrichr gene set through GSEAPy package. Only top 2 enrichment hits are shown for each pertinent database. (G) Expression of COX-2 on either scrambled shRNA ctrl or REL-A^{KD} MSCs re-educated by activated CAR-T assessed by flow cytometry. Data are represented as mean $\pm$ SD from 4 independent experiments using 2 different MSC strains and CAR-T generated from 2 different donors. ****p<0.0001 by one-way ANOVA. (H-I) ELISA assay for PGE2 in culture media of MSCs re-educated by CAR-T. Data are represented as mean $\pm$ SD from 3 independent experiments. **p<0.005 by paired t-test.

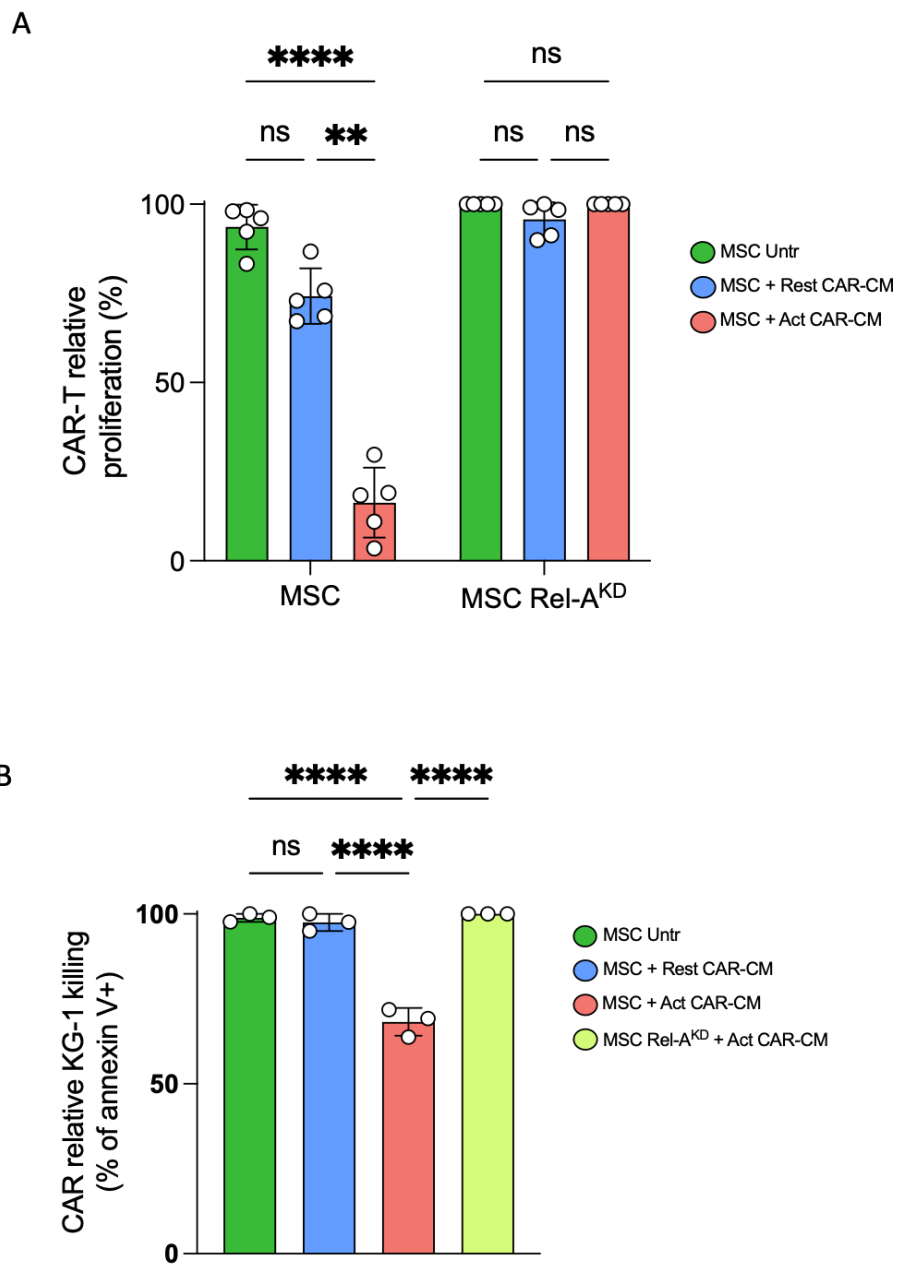
III.4 CAR-re-educated MSCs are capable to inhibit CAR-T cell proliferation, cytokine release and cytotoxicity by a NF- κ B dependent mechanism.

To investigate the potential inhibitory effect of re-educated MSCs on the CAR-T efficacy, functional assays were performed. We tested proliferation, cytokine production and cytotoxicity of CD33.CAR-T cells incubated for 72 hours with CD33 chimeric protein, in the presence or absence of re-educated MSCs. As shown in figure 2A, re-educated MSCs were able to inhibit CD33.CAR-T proliferation in response to CD33 antigen by 80%. This effect was reversed when re-educated MSCs Rel-A^{KD} were used. Notably, MSCs at steady state or re-educated by resting CAR-T cell supernatant do not alter the antigen-dependent proliferation of CAR-T cells.

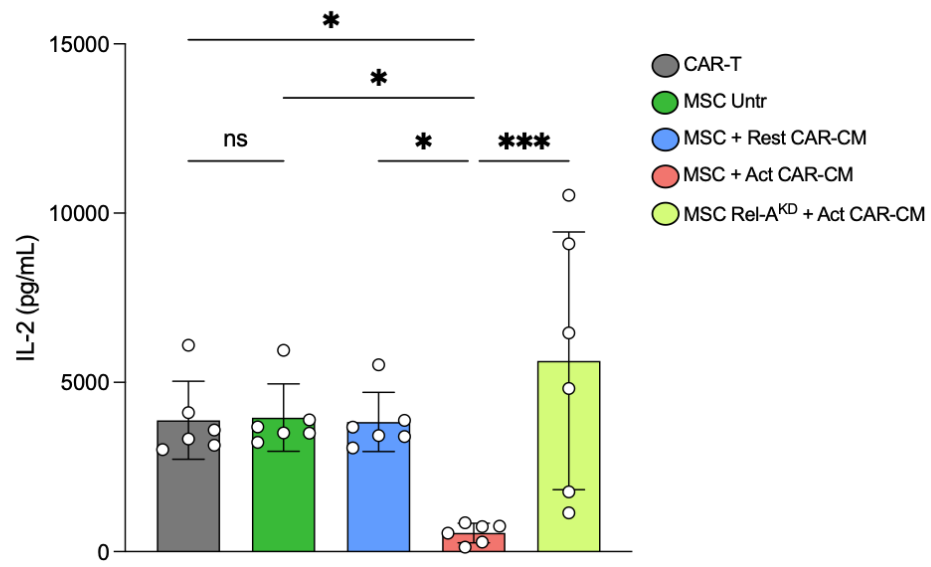
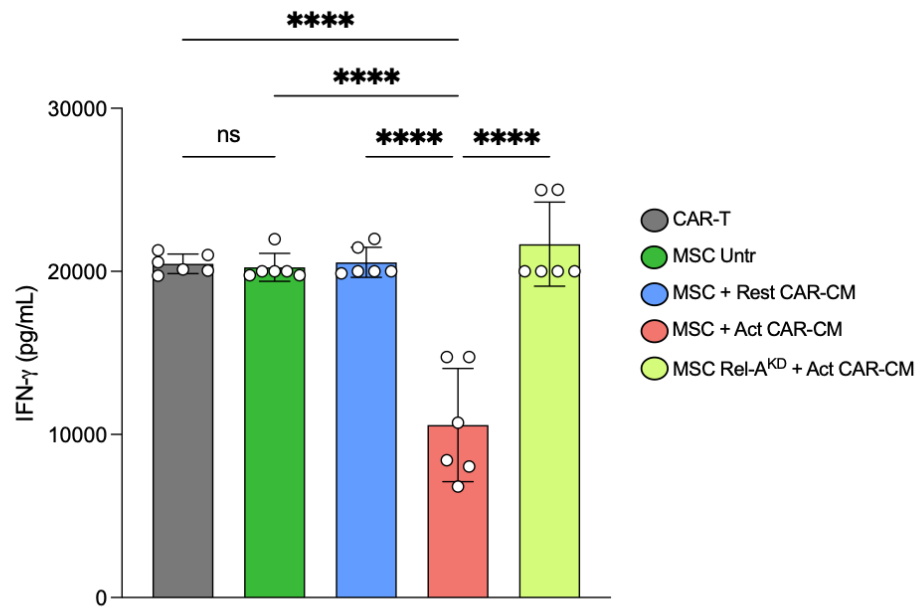
To assess the cytotoxic activity of CD33.CAR-T exposed for 72h to CD33 antigen in the presence or absence of re-educated MSCs, they were harvested and co-cultured for 24h with CD33⁺ KG-1 AML cell line at an effector to target ratio of 1:10. The viability of target cells was determined by annexin V and 7AAD staining and expressed as relative killing compared to CD33.CAR-T cells not exposed to MSCs. As shown in figure 2B, CAR-T cells which have been co-cultured with re-educated MSCs display a 30% reduction in their killing activity and once again this inhibitory effect is reverted when Rel-A^{KD} cells were used.

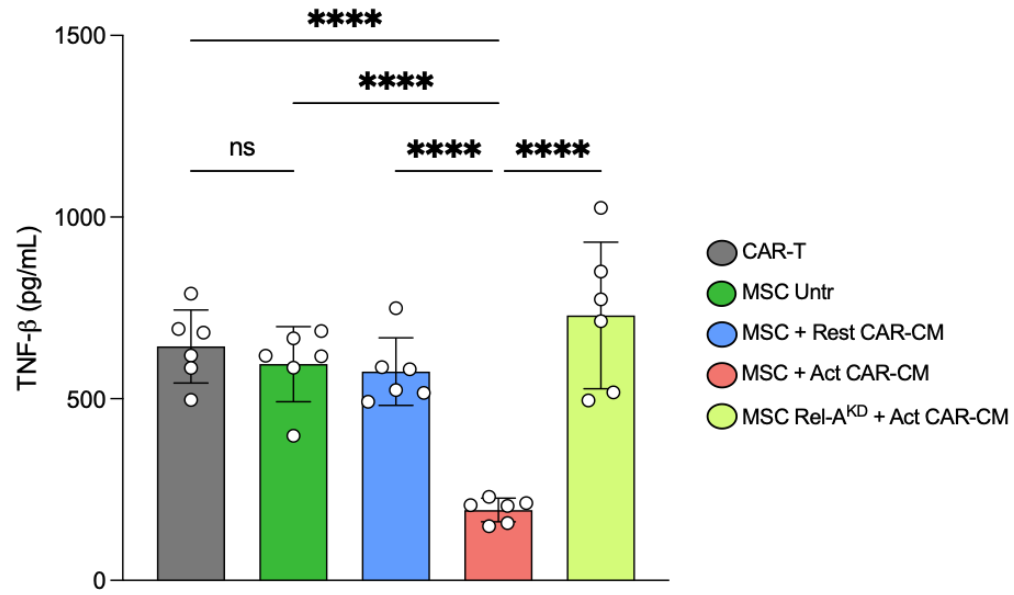
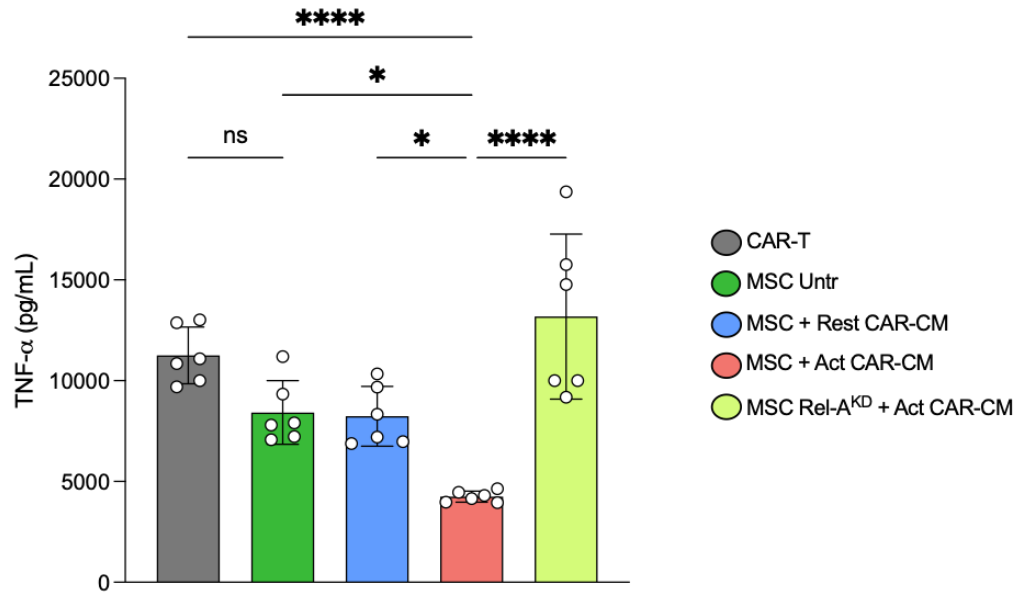
Supernatants of CD33.CAR-T exposed for 72h to CD33 antigen in the presence or absence of re-educated MSCs were analysed by Luminex assay to measure levels of key cytokines typically released by CAR-T upon activation (Figure 2C). Amounts of IFN- γ , IL-2, TNF- α and TNF- β were significantly reduced when CD33.CAR-T cells exposed to CD33 antigen were co-cultured with MSCs re-educated by activated CAR-T compared to steady state MSCs. It is important to appreciate how this effect was reverted when MSCs Rel-A^{KD} were used instead of MSCs scrambled shRNA control. CAR-T viability was not impacted as shown in figure 2D.

Figure 2: Re-educated MSCs impact on CAR-T cells later functions through an NF- κ B dependent mechanism.

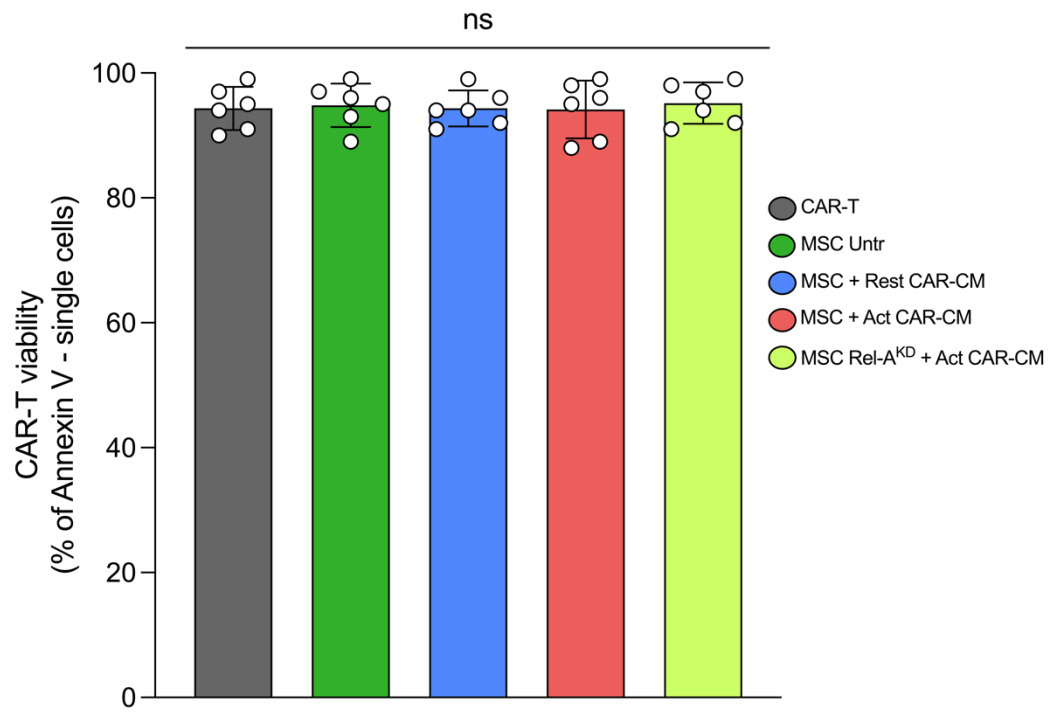


C





D



Re-educated MSCs impact on CAR-T cells later functions through an NF- κ B dependent mechanism.

(A) Proliferation of CAR-T cells in response to CD33 stimulation for 72 h in the presence or absence of MSC untreated or re-educated by resting or activated CAR-T. Both MSCs wild type and Rel-A^{KD} were used. Dilution of CellTrace Violet in CAR-T cells was measured by flow cytometry to evaluate proliferation. Proliferation was expressed as relative to control represented by CAR-T pre-stimulated without MSCs. Results are expressed as mean $\pm$ SD from 5 independent experiments. ** $p < 0.005$ and **** $p < 0.0001$ by two-way ANOVA. (B) Killing activity of CAR-T upon antigen stimulation for 72 h in the presence or absence of MSCs re-educated by resting or activated CAR-T. Co-cultures with KG-1 cells were conducted for 24h at E:T ratio of 1:10. Killing was expressed as relative to control represented by CAR-T pre-stimulated without MSCs. Results are expressed as mean $\pm$ SD from 3 independent experiments. **** $p < 0.0001$ by one-way ANOVA. (C) Supernatants from proliferation experiments were harvested and analysed for key cytokines (IFN- γ , IL-2, TNF- α and TNF- β). Results are expressed as mean $\pm$ SD from 6 independent experiments. * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA. (D) Viability of CAR-T measured as annexin V – cells in the co culture. Results are expressed as mean $\pm$ SD from 6 independent experiments.

III.5 Discussion

In this chapter we characterised the functional phenotype of stromal cells exposed to soluble factors released by activated CAR-T cells. We employed CD33.CAR-T cells as working model and we chose to use a tumour-free system to avoid any possible bias of malignant cells on MSCs.

The results of this transcriptomic analysis revealed that MSCs exposed to the supernatant of activated CAR-T cells strongly upregulate immunosuppressive molecules as well as chemokines. Among the very well-established mechanisms of MSC-mediated immunosuppression, such as PD1-PDL1, TIM3-CEACAM1, IL6 and IDO1 already described in depth by the literature, we decided to focus on the COX2-PGE2 axis. Moreover, IL-33, one of the top upregulated genes in our analysis, have been reported to play a crucial role in MDSCs development and immunosuppression in the tumour context by promoting their expansion, and production of immunosuppressive factors such as Arginase-1 and iNOS(Xiao et al., 2016; Yeoh et al., 2022) .

Gene ontology analysis of the upregulated genes in MSCs exposed to factors released by activated CAR-T revealed a strong enrichment for IFN- γ signalling (Figure 1F) as testified by up-regulation of STAT 1 and IRF8 pathways. Moreover, a clear and strong signature of NF- κ B also emerged from this analysis making it a good candidate as the master regulator of the phenotypic change in MSCs. In this work it was not investigate whether IFN- γ was the actual trigger in the activated CAR-T driven re-education of MSCs, and there are no reports of IFN- γ directly activating NF- κ B. We can speculate that IFN- γ induced genes such as CD40 and IL-1 β can sustain the activation of NF- κ B in an autocrine fashion synergizing with TNF- α secreted by activated CAR-T cells. It

is also important to underline how the interaction between CD40 and CD40L was associated with an increasing risk of cytokine release syndrome (CRS), one of the major adverse effects of CAR-T cell therapy (Gong et al., 2023)

Among the other already discussed immunomodulatory molecules it is also important to highlight the presence of many chemokines. Those are responsible not only to attract CAR-T cells but also other cells such as monocytes and neutrophils in the microenvironment, with a potential impact converting them into immunosuppressive mode. For example, neutrophils primed by PGE2 have been shown to be pro-tumorigenic and pro-metastatic in models of breast cancer and lung metastasis.

PGE2 effect on bone marrow microenvironment have also been described in the context of hematopoietic stem cell transplantation. It have been shown that PGE2 enhance HSCs mobilization from the bone marrow, which in the malignant context can coincide with the formation of metastasis (Patterson et al., 2021). Moreover the immunosuppressive effect of this metabolite have been linked with transplanted HSCs survival and proliferation post-transplant via immune reaction mitigation (Hoggatt et al., 2009).

To further investigate the role of NF- κ B in the upregulation of COX2, the subunit REL-A was knocked down in different batches of MSCs. As shown in figure 2C-E this significantly reduced the population of MSCs expressing COX2 as well as the levels of PGE2 released after re-education mediated by activated CAR-T cells.

Functionally, as depicted in figure 2, re-educated MSCs can strongly inhibit CAR-T effector functions represented by proliferation, cytokine release and cytotoxicity.

Regarding cytokine release we can appreciate a strong reduction of pro-mitotic IL-2 as well as a decrease in IFN- γ , TNF- α and TNF- β , which can be reconducted of a less cytotoxic CAR-T phenotype. All these CAR-T effector functions are not inhibited when MSC REL-A knock down were tested.

Together these findings shed light on one of the two mechanisms described in this thesis by which MSCs can counteract the beneficial effects of CAR-T in the bone marrow niche. Moreover, it also proposes a possible pharmacological target to augment CAR-T cell therapy efficacy.

Chapter IV - Results :

CAR-T cells are able to induce caspase dependent apoptosis in MSCs in an antigen independent manner.

IV.1 introduction

This chapter is aimed to dissect the series of events triggered by CAR-T which directly came into contact with MSCs.

MSCs have been infused in patients with different diseases such as like GVHD, IBD and SLE in several pre-clinical and clinical setting to control inflammation (Y. Shi et al., 2018) . Partial response was observed, despite efficacy, no MSC engraftment was observed in any of those settings (Fitzsimmons et al., 2018).

Galleu et al., in 2017 showed that MSCs can successfully deliver a strong immunosuppressive signal in-vivo only when in contact with activated

cytotoxic cells which induce caspase dependent apoptosis in the stromal cells (Galleu et al., 2017).

Apoptosis, as previously described is a programmed cell death pathways characterized by caspase activation and a cascade of events which leads to the release of many soluble mediators as well as cell debris (Galluzzi et al., 2018a). Moreover, a consistent amount of studies suggests that cell undergoing apoptosis can promote an immunosuppressive microenvironment by release of soluble mediators (W. J. Chen et al., 2001; Kazama et al., 2008; Ricci et al., 2004; White et al., 2014) and well as by interaction of the apoptotic bodies with professional phagocytic cells such as macrophages (Byrne & Reen, 2002; Fadok et al., 1998; Tassiulas et al., 2007; Voll et al., 1997).

Hereafter we aim to demonstrate the consequence of CAR-T and MSC direct interaction, showing the outcome of this contact on both parties characterizing the immunosuppressive outcome.

IV.2 CAR-T cells induce caspase activation and consequent cell death in MSCs in a contact dependent fashion.

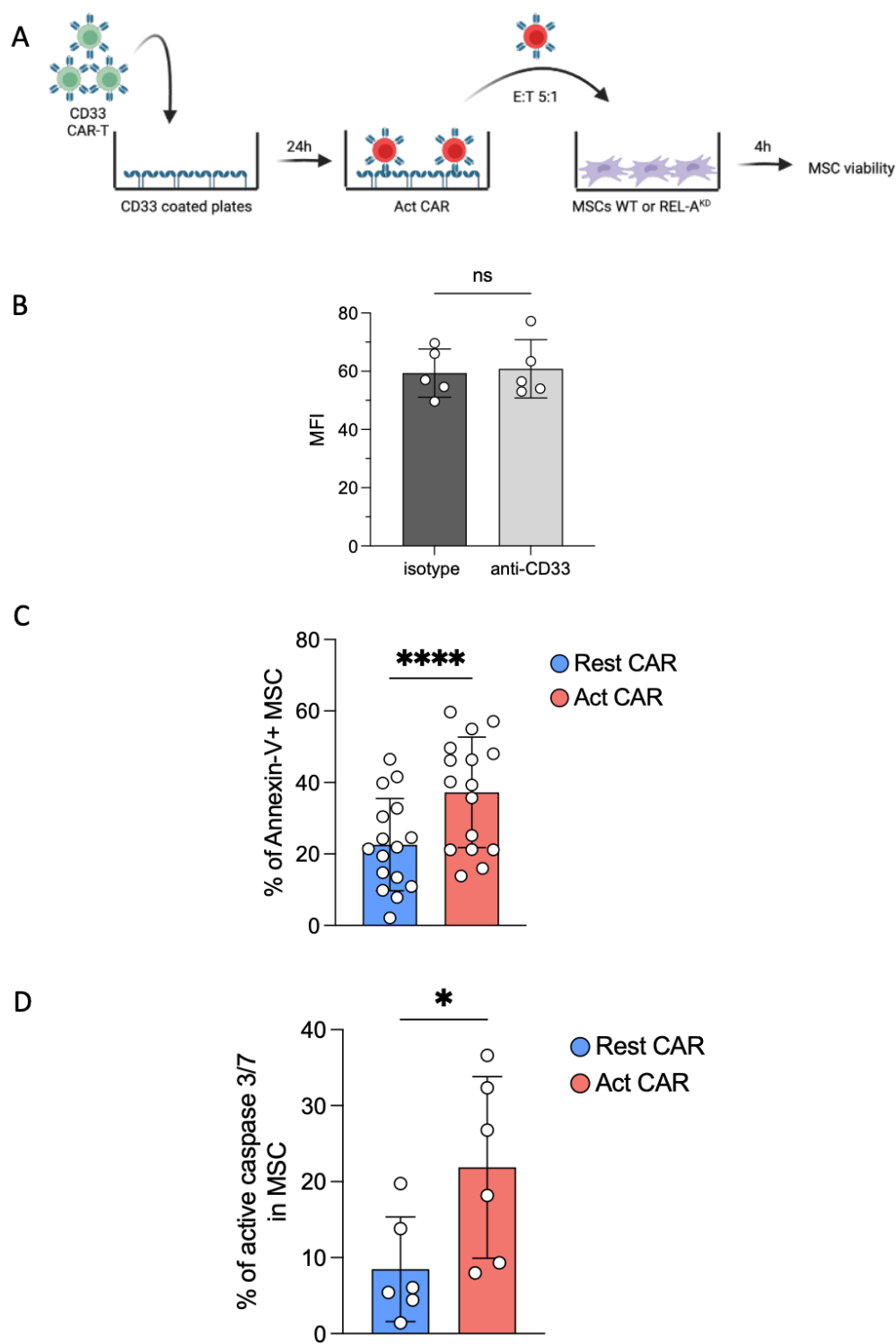
To investigate the capacity of activated CAR-T cells to induce apoptosis in MSCs, CD33.CAR-T cells were activated in tumour free condition as described before, and co-cultured with MSCs for 4h at an effector-to-target ratio of 5:1, before flow cytometric assessment of cell death (figure 3A). MSC do not express CD33 target antigen (figure 3B).

As depicted in figure 3 C-D both resting and activated CAR-T cells were able to induce cell death and caspase activation in MSCs, but levels are significantly higher when CAR-T are in the activated status.

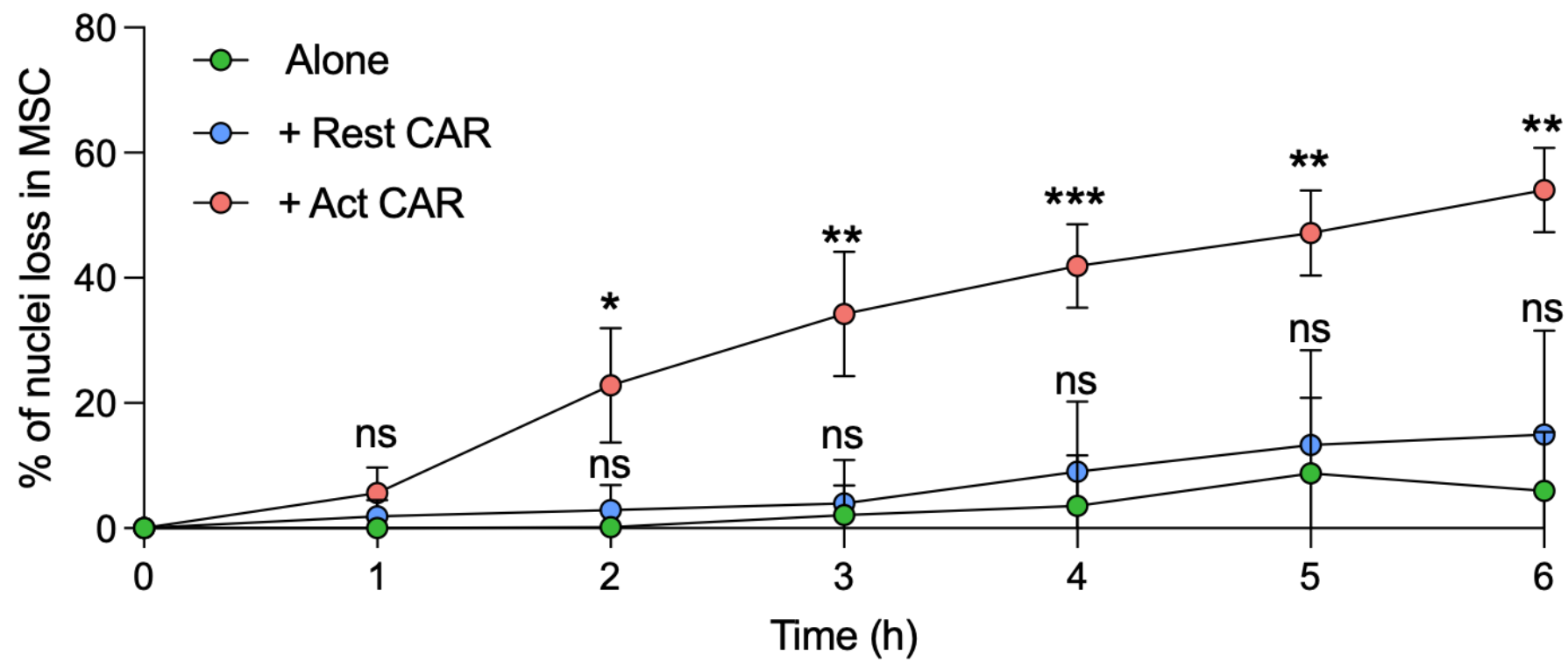
To further validate these results, we performed live cell imaging on co-cultures between MSCs and CAR-T cells using Operetta imaging system. Given the size difference between CAR-T and MSCs it was easy to estimate the amount of dying MSCs in the co-culture via nuclear destruction and cytoskeleton loss (figure 3E). Representative figure of beginning and end of cloture from the imaging are depicted in figure 3F.

Finally, Rel-A^{KD} had no effect on MSCs susceptibility to undergo apoptosis when in contact with CAR-T (figure 3G)

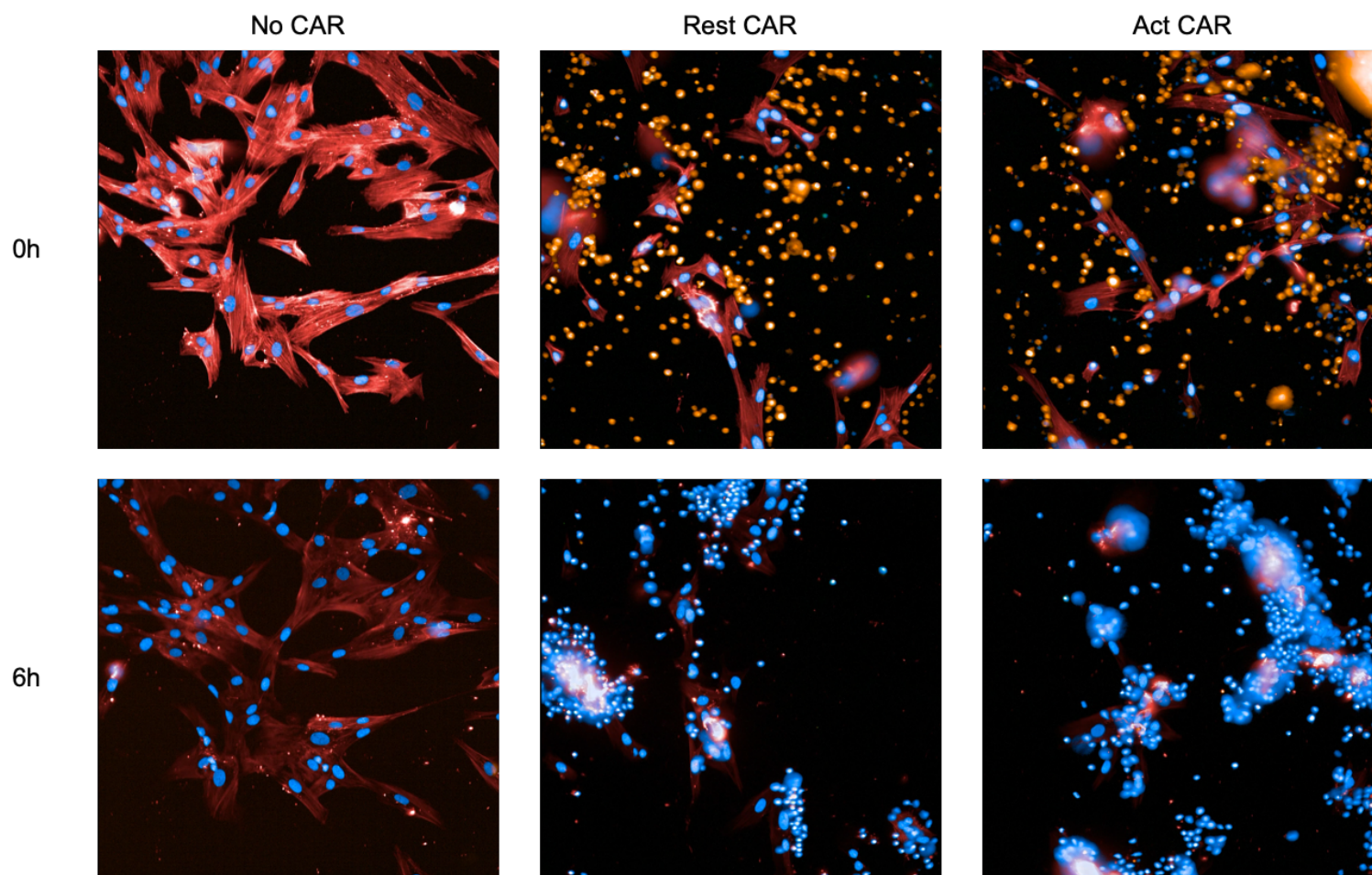
Figure 3: MSCs undergo apoptosis when in contact with activated CAR-T cells.



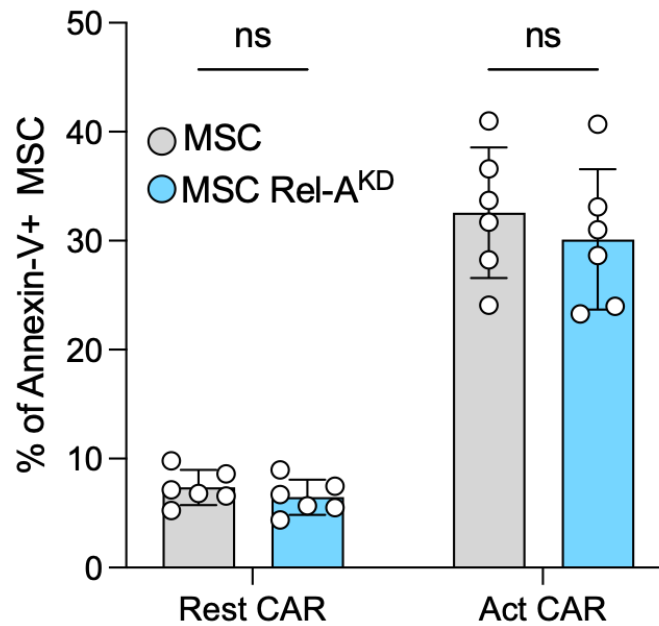
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MSCs undergo apoptosis when in contact with activated CAR-T cells.

(A) Experimental design schematics depicting the 4h co-culture of activated CD33.CAR-T with MSCs at E:T ratio of 5:1. Both MSC wild type and MSC Rel-A^{KD} were used as target cells. (B) Flow cytometric assessment of CD33 expression on MSCs. Results are expressed as mean ± SD from 5 independent experiments. ****p<0.0001 by paired t-test. (C) Flow cytometric evaluation of Annexin V positivity on MSCs after co-culture with resting or activated CAR-T cells. Results are expressed as mean ± SD from 16 independent experiments. ****p<0.0001 by paired t-test. (D) Evaluation of caspase 3/7 activation on MSCs after co-cultures. Results are expressed as mean ± SD from 6 independent experiments. *p<0.05 by paired t-test. (E) Time-course analysis of apoptosis mediated by activated CAR-T on MSCs was tested in a 6-hour co-culture (E:T ratio 5:1) using Operetta CLS. Quantification of MSC death was obtained by image analysis to

extract the nuclear distraction. Results for each experiment are calculated as the average of 3 wells per condition and 16 fields each well. Results are expressed as mean $\pm$ SD from 3 independent. **p<0.005, ***p<0.001 by one-way ANOVA at final timepoint. (F) Representative images show co-culture of MSCs without or with resting or activated CAR-T. First row represent frame took at time 0, while second row reports frame took at the 6-hours end point. (G) Flow cytometric evaluation of Annexin V positivity on MSCs or MSC Rel-A^{KD} after co-culture with resting or activated CAR-T cells. Results are expressed as mean $\pm$ SD from 6 independent experiments.

IV.3 CAR-T induced cell death is a “bystander effect”.

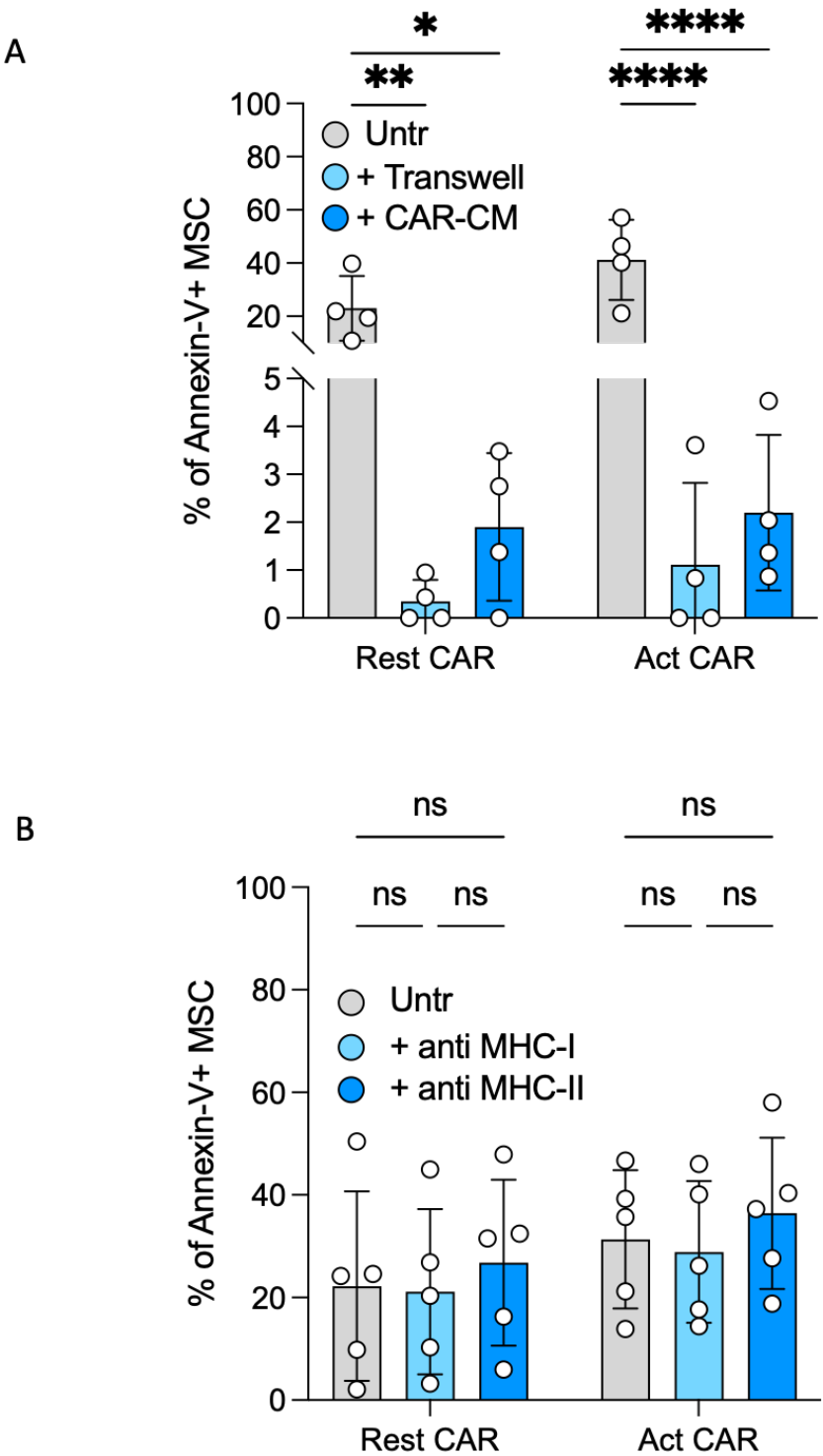
To evaluate the mechanisms by which CAR-T cause MSC death several inhibitors were tested. As expected, MSCs did not express CD33, the antigen recognised by the specific CAR. To prove contact dependency MSCs were co-cultured with both CAR supernatants and CAR-T cells in traswell (figure 4A). In order to exclude an allogenic recognition, both MHC-I and MHC-II were blocked using specific antibodies but in both conditions no inhibition of cell death was observed (Figure 4B). Moreover, CD33, despite not being detectable by flow cytometry, was blocked with an antibody to exclude the antigen specificity once again, but no difference in the percentage of dying MSC was observed (Figure 4C).

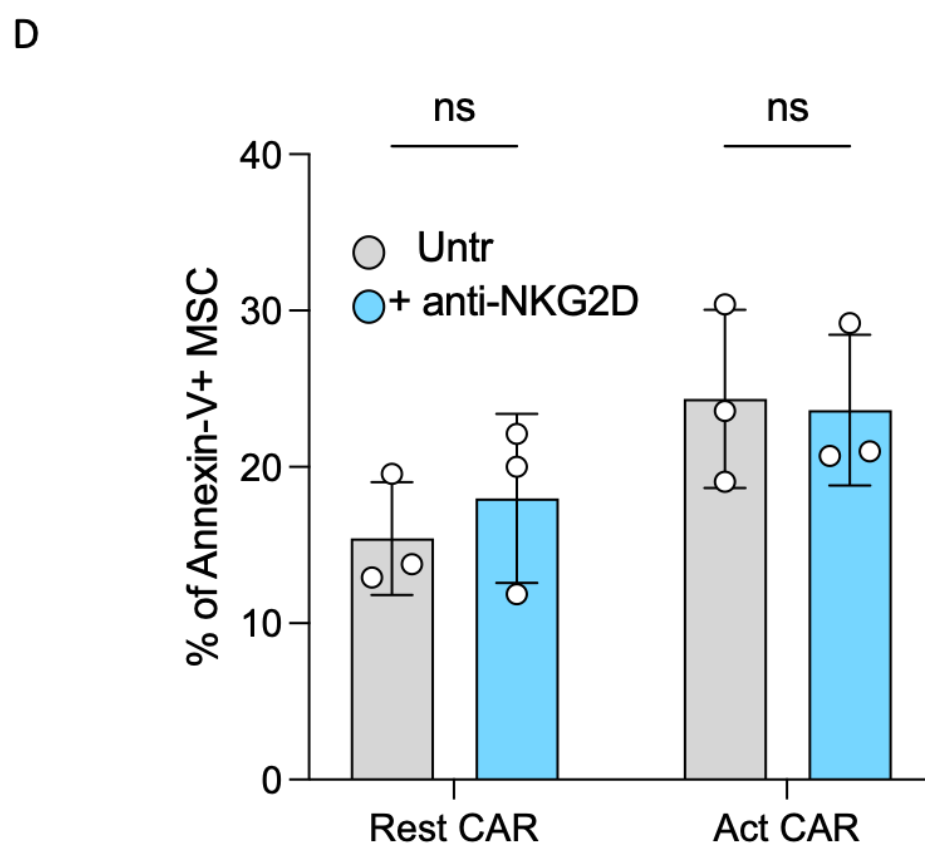
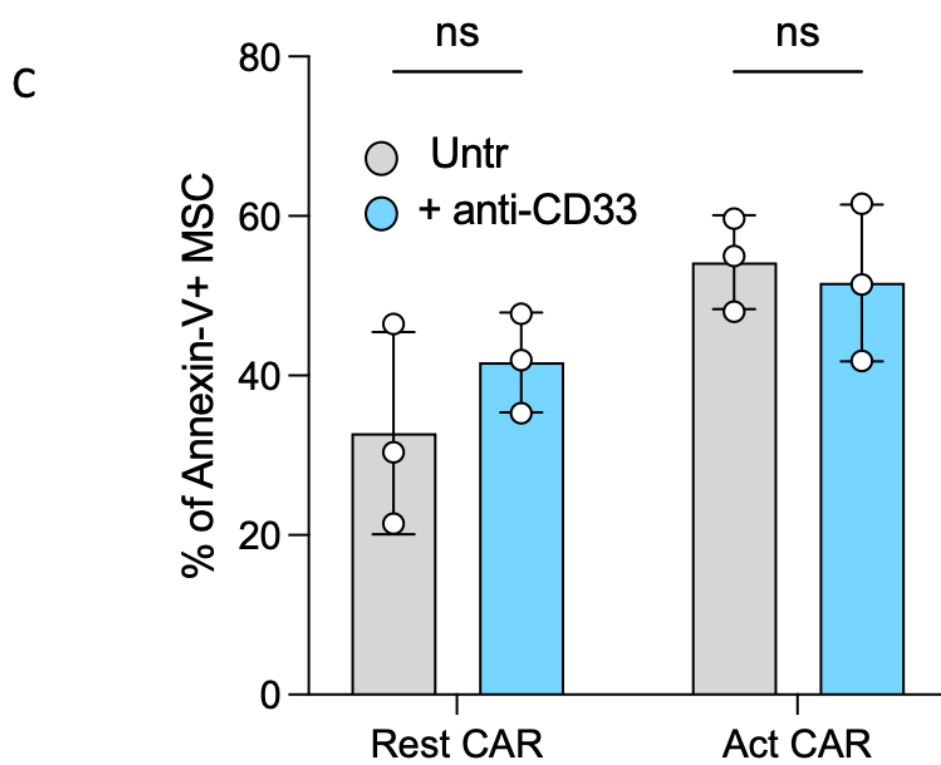
To understand the mediator of cell death process common stimuli were blocked. NKG2D (Figure 4D), FAS (Figure 4E) and TRAIL (Figure 4F) were inhibited with specific antibody. In none of the cases this had any inhibitory effect on the CAR-T mediated cytotoxicity against MSC.

Further investigating the cell death mechanisms, we inhibited both the perforin-based cytotoxic pathway using the Ca^{2+} -chelating agent EGTA (Figure 4G) as well as granzyme-b using Z-AAD-FMK inhibitor (Figure 4H). Blocking perforin polymerization and release marked a significant reduction in Annexin V positivity in MSCs exposed to activated CAR-T. Differently, granzyme b inhibition by Z-AAD-FMK showed a decrease annexin V positivity in MSC when co-cultured with activated CAR-T cells, but not when cultured with resting CAR-T.

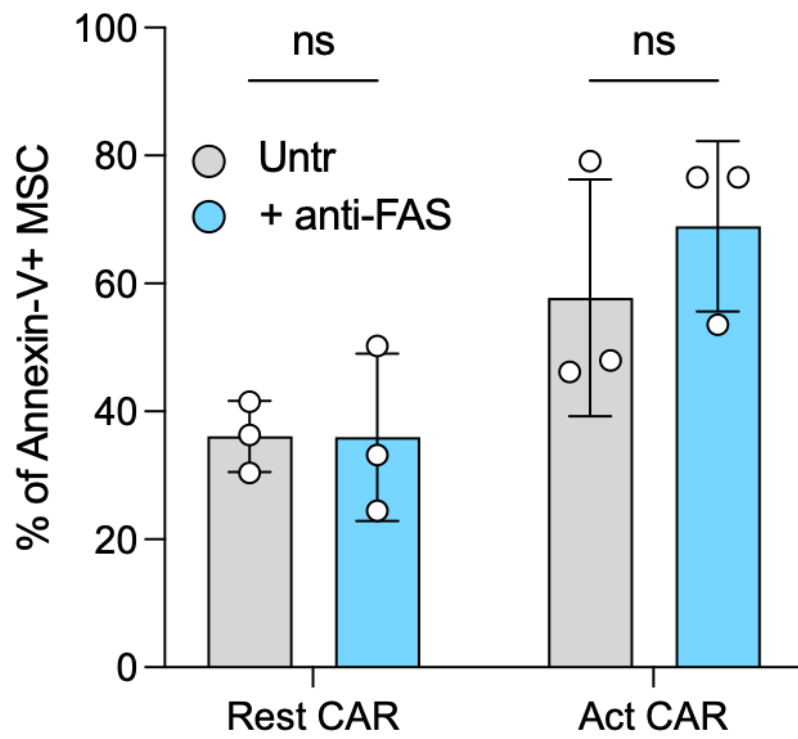
Finally, to better define the pathway of cell death activated in MSCs by activated CAR-T, we employed necrostatin-1, a RIPK3-targeted inhibitor of necroptosis, chloroquine and bafilomycin to exclude autophagy and Z-VAD-FMK, a pan caspase inhibitor. As we can see in figure 4, Receptor-interacting serine/threonine-protein kinase (RIPK) inhibition as well as autophagy blockade did not lead to any changes in cell death (Figure 4I, J, K respectively). On the other hand, caspase inhibition was successful in significantly lower the percentage of dying MSCs in co-culture with both resting and activated CAR-T (Figure 4L).

Figure 4: Activated CAR-T cell mediated MSC apoptosis is contact-dependent and requires caspase activation.

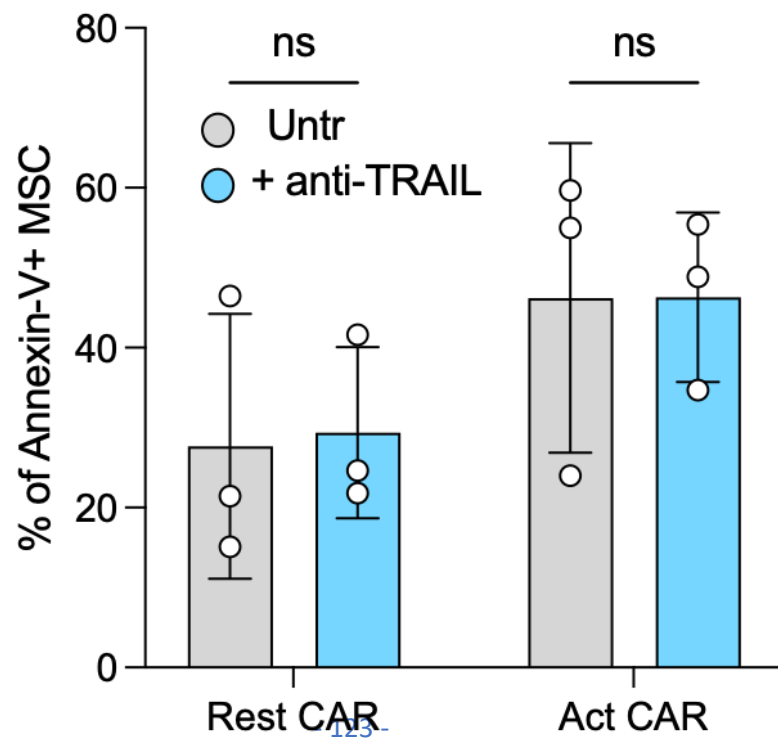




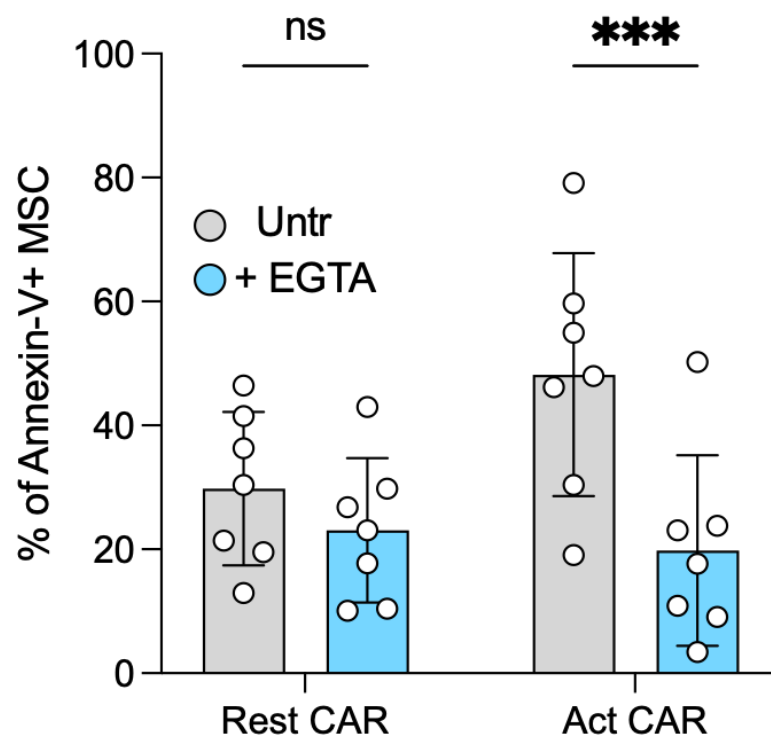
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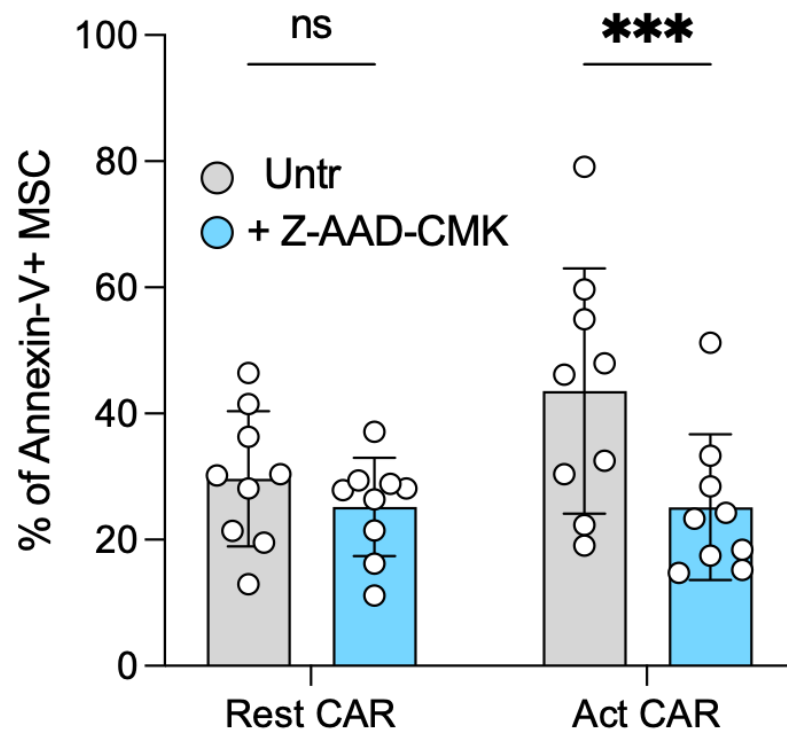
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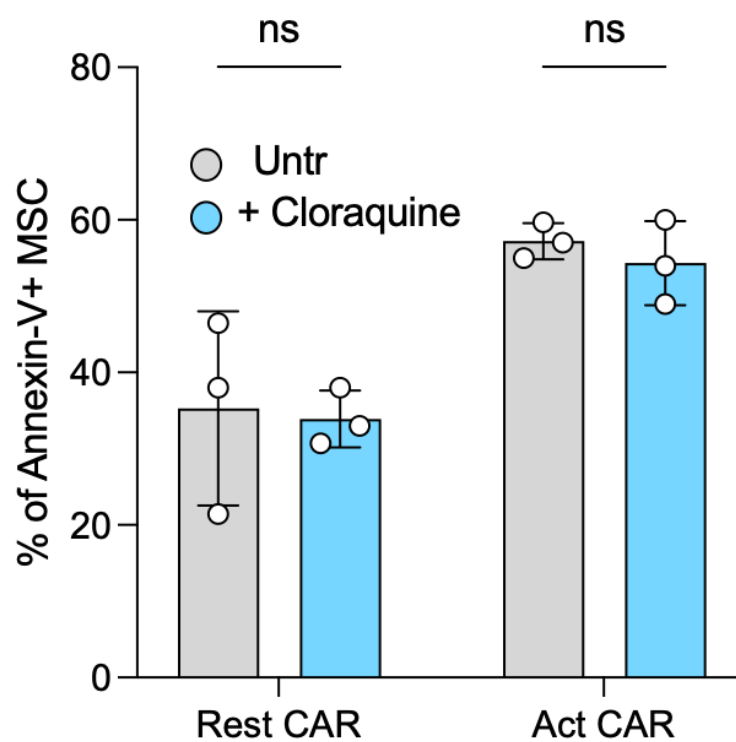
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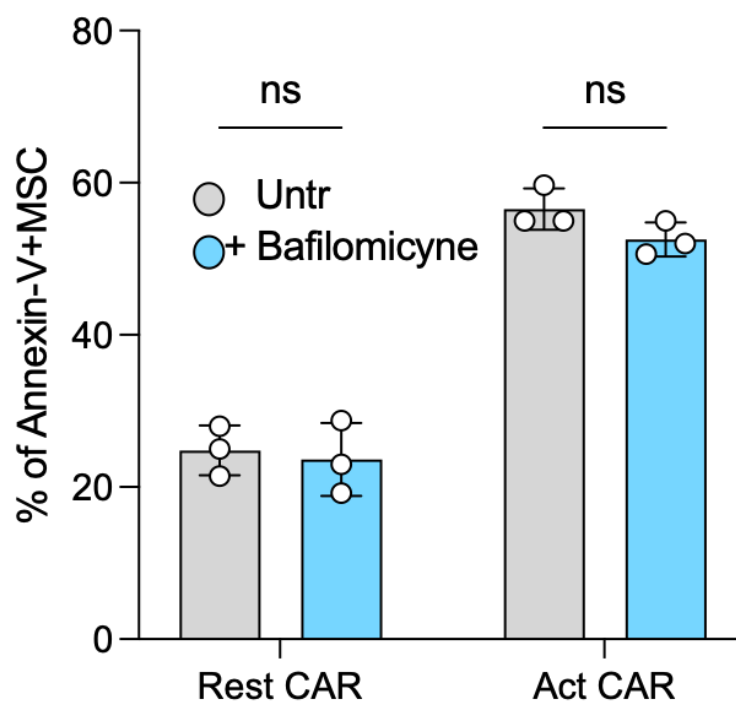
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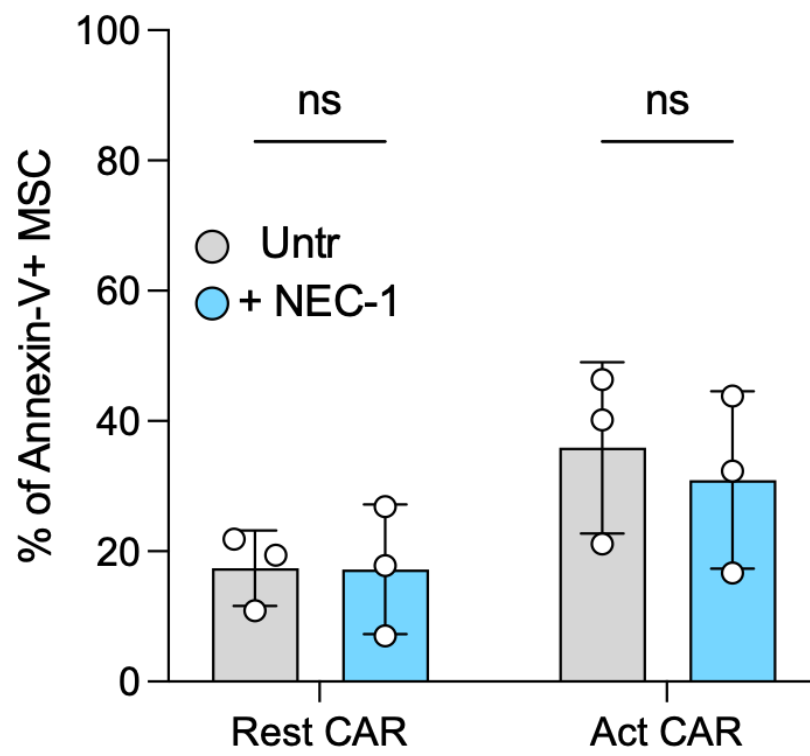
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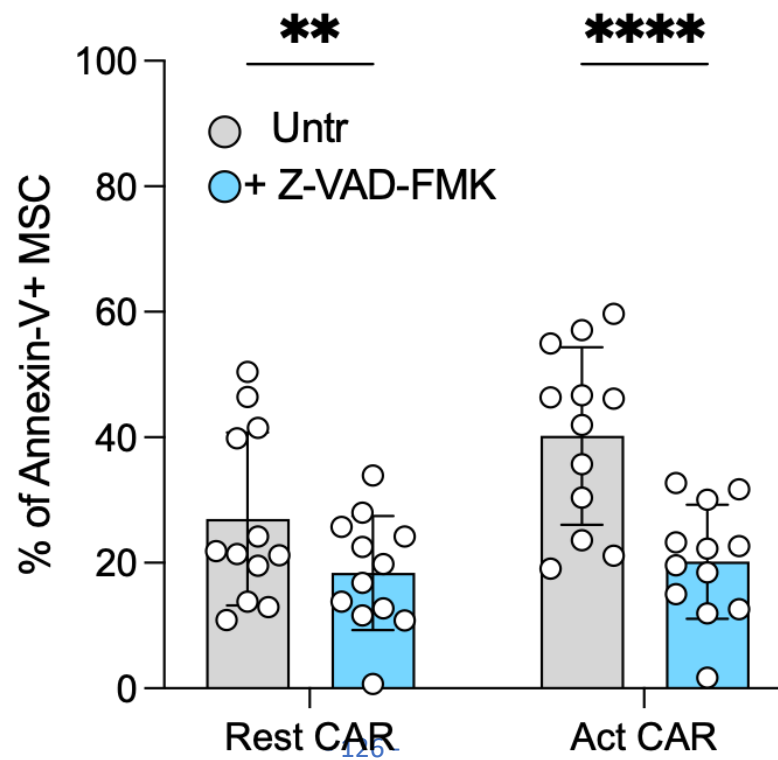
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Activated CAR-T cell mediated MSC apoptosis is contact-dependent and requires caspase activation.

Killing activity of activated and resting CAR-T against MSCs was tested in the presence of several inhibitors. (A) Direct cell contact was blocked with either use of Transwell or by culturing MSCs with CAR-T conditioned medium. Results are expressed as mean $\pm$ SD from 4 independent experiments. * $p < 0.05$, ** $p < 0.005$, and **** $p < 0.0001$ by two-way ANOVA. (B) Blocking antibodies anti MHC-I (100ug/ml) or MHC-II (50ug/ml) were incubated with the MSC monolayer 2h before co-culture and maintained throughout the assay. Results are expressed as mean $\pm$ SD from 5 independent experiments. (C) Blocking antibodies anti CD33 (100ug/ml) were incubated with the MSC monolayer 2h before co-culture and maintained throughout the assay. Results are expressed as mean $\pm$ SD from 3 independent experiments. (D) Blocking antibodies anti NKG2D (100ug/ml) were incubated with CAR-T 2h before co-culture and maintained throughout the assay. Results are expressed as mean $\pm$ SD from 3 independent experiments. (E) Blocking antibodies anti FAS (100ug/ml) were incubated with MSC monolayers 2h before co-culture and maintained throughout the assay. Results are expressed as mean $\pm$ SD from 3 independent experiments. (F) Blocking antibodies anti TRAIL (75ug/ml) were incubated with CAR-T 2h before co-culture and maintained throughout the assay. Results are expressed as mean $\pm$ SD from 3 independent experiments. (G) Perforin inhibitor EGTA (4mM) was incubated with MSC monolayers for 3h before co-culture and maintained throughout the assay. Results are expressed as mean $\pm$ SD from 7 independent experiments. *** $p < 0.001$ by two-way ANOVA. (H) Granzyme b inhibitor Z-AAD-CMK (100uM) was incubated with MSC monolayers for 3h before co-culture and maintained throughout the assay.

Results are expressed as mean $\pm$ SD from 9 independent experiments. *** $p < 0.001$ by two-way ANOVA. (E) Necrosis signalling pathway was inhibited by Necrostatin-1 (50uM), a RIPK3 inhibitor, incubated with MSCs 2h before killing assay. Results are expressed as mean $\pm$ SD from 3 independent experiments. (I-J) Autophagy signalling pathway was inhibited by Chloroquine (100uM) and bafilomycin (50uM), incubated with MSCs 2h before killing assay. Results are expressed as mean $\pm$ SD from 3 independent experiments. (L) Pan-caspase inhibitor Z-VAD-FMK (50uM) was incubated with MSCs 2h before killing assay. Results are expressed as mean $\pm$ SD from 12 independent experiments. ** $p < 0.005$ and **** $p < 0.0001$ by two-way ANOVA.

IV.4 Functional impact of apoptotic MSCs on CAR-T activity

Once established the ability of CAR-T cells to induce caspase-dependent apoptosis in MSCs and as we have demonstrated that MSC underwent apoptosis after contact with activated cytotoxic cells acquire immunosuppressive capabilities, their effect on CAR-T function was investigated. Apoptotic MSCs were generated in vitro using a surrogate model: aFAS activatory antibody was exploded as described by Galleu at al. In brief cells were stimulated for 15 minutes and, after wash out of the stimulus, MSCs were incubated overnight to allow the apoptotic cascade to complete. Resulting cells and conditioned media were frozen as described in methods for future use.

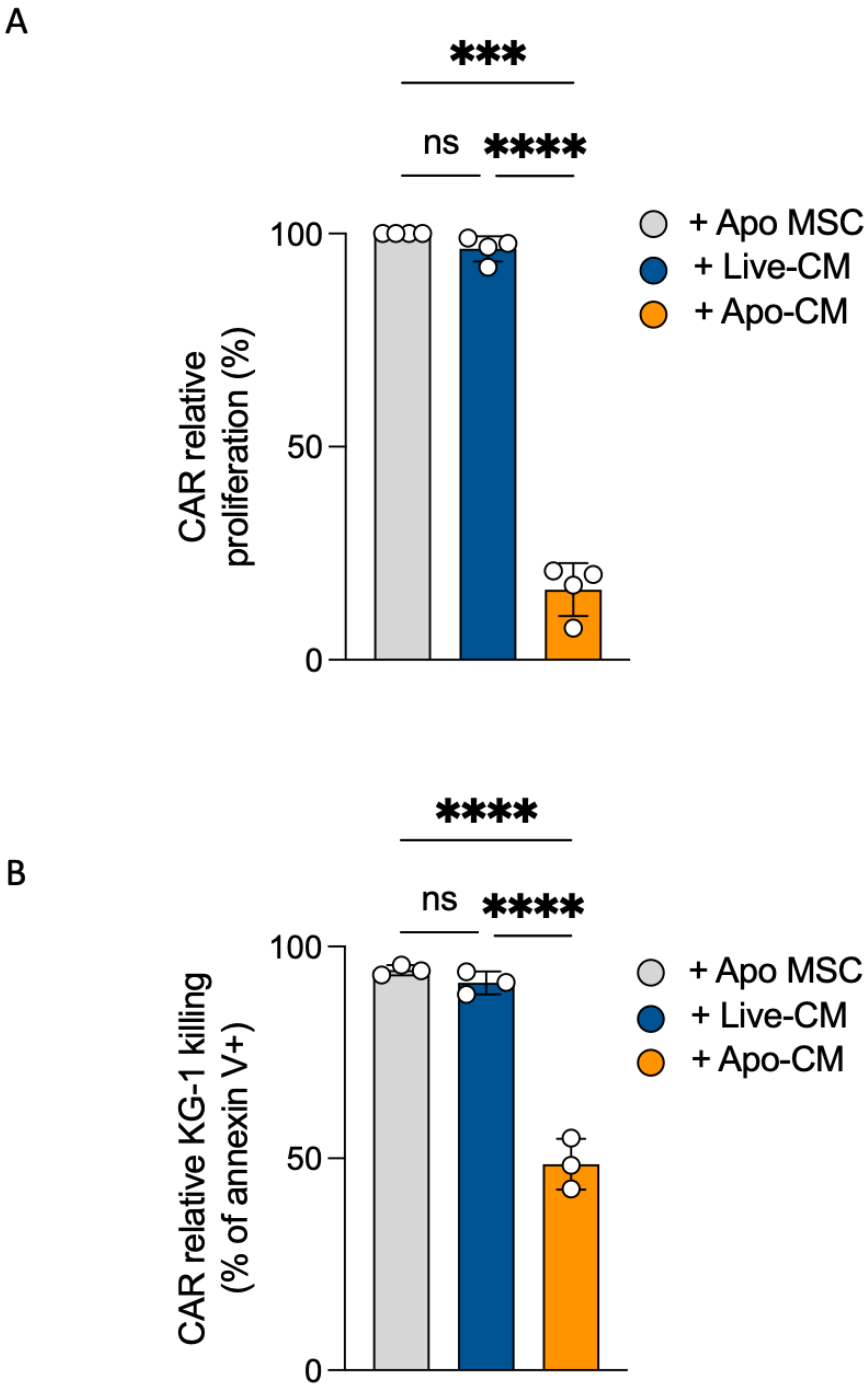
Co-cultured with CAR-T for 72h at a ratio of 5 MSC:1 CAR and their proliferation was assessed. As shown in figure 5A, apoptotic MSCs had no effect on CAR-T cell proliferation. Moreover, the co-culture of CD33.CAR-T and CD33⁺ KG-1 AML cell target with apoptotic MSCs also resulted in no difference in terms of cytotoxicity (figure 5B).

We then assessed whether soluble factors released by MSCs during the process of cell death could potentially impact CAR-T later functions. As depicted in figure 5A, the addition of 25% of apoptotic MSC conditioned media is able to reduce CAR-T cell proliferation in response to plate bound CD33 by over 70% differently from supernatant of live MSCs. Moreover, after 72 hours stimulation, culture supernatant was harvested for cytokine profiling, as well as CAR-T rechallenged in killing assay against KG-1 AML cells at effector to target ratio of 1:10 for 24h.

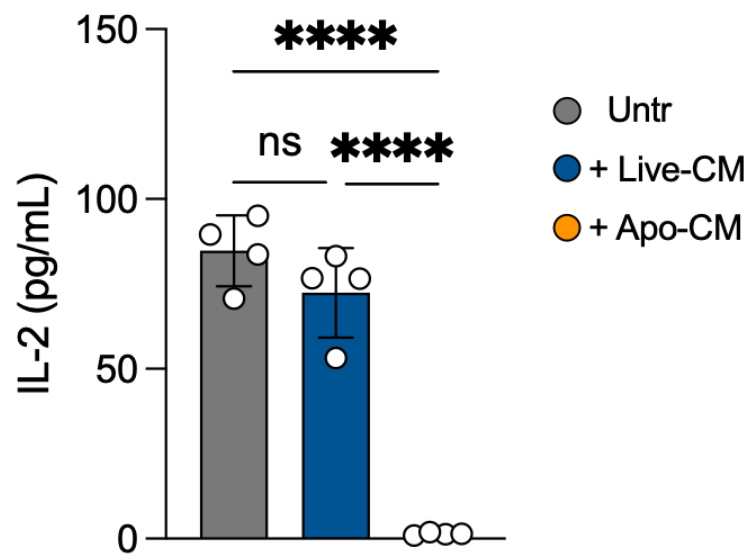
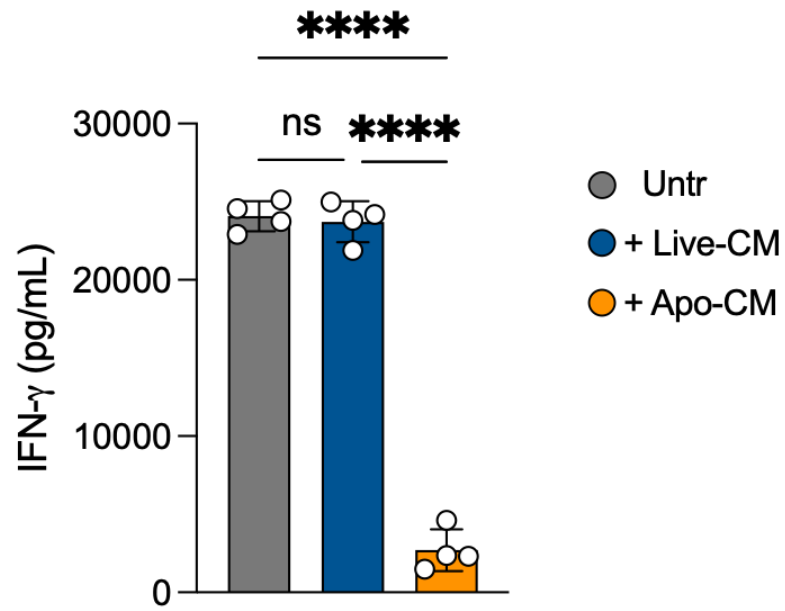
In regards of cytotoxicity against KG-1, CAR-T exposed to apoptotic MSC conditioned media displayed a reduction of the cytotoxic capacity by over 40%, as shown in figure 5B.

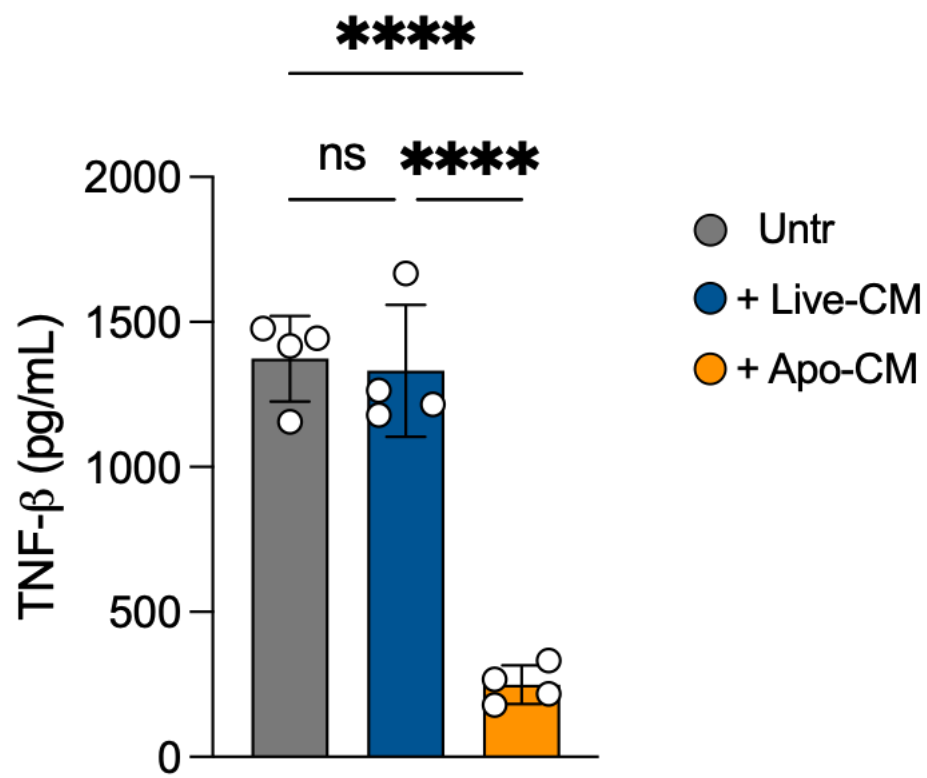
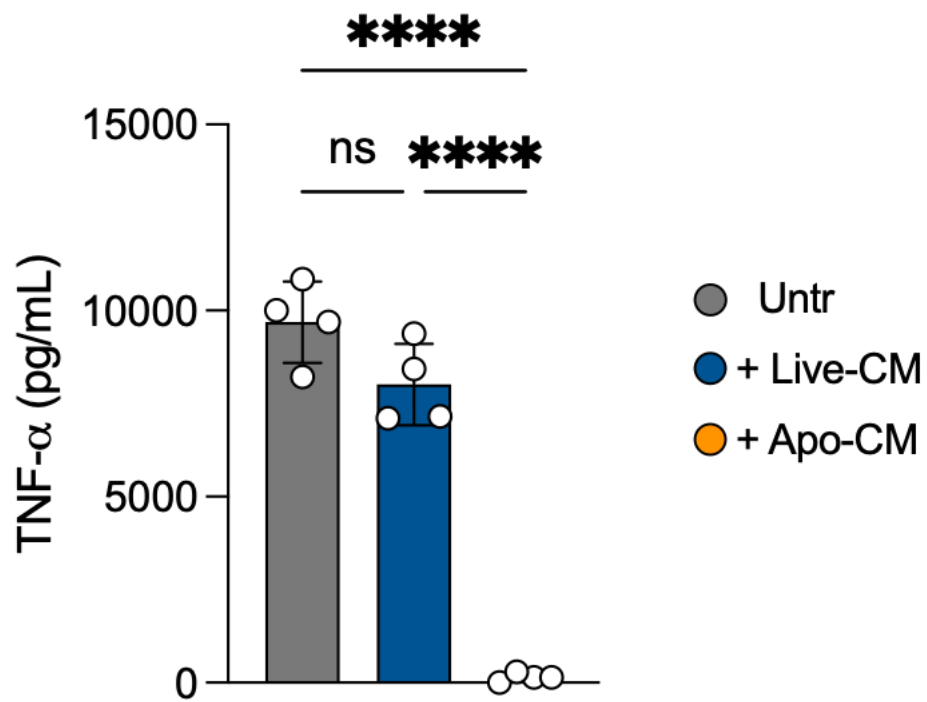
Cytokine profiling revealed that the apoptotic MSC condition media strongly reduced the level of key effector cytokines such as IFN- γ , IL-2, TNF- α and TNF- β (figure 5 C). CAR-T cells viability was not impacted by experimental condition as shown in figure 5D.

Figure 5: Apoptotic MSC conditioned media but not apoptotic cells for itself inhibit CAR-T cells functions.

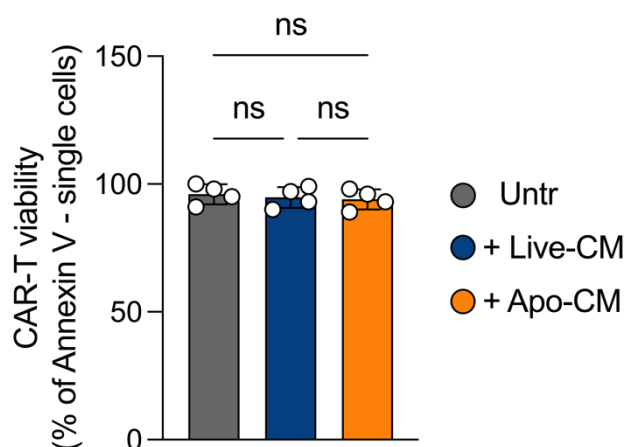


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Apoptotic MSC conditioned media but not apoptotic cells for itself inhibit CAR-T cells functions.

(A) Proliferation of CAR-T upon antigen stimulation in presence of apoptotic MSCs (5:1 MSC: CAR) or apoptotic or live MSC conditioned media (25% of total culture media). Cultures were performed for 72h, and CAR-T proliferation was estimated by CellTrace Violet dilution. Data are represented as relative to control (CAR-T alone) and expressed as mean $\pm$ SD from 4 independent experiments. *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA. (B) Cytotoxicity of CAR-T against KG-1 at E:T ratio of 1:10 in presence of apoptotic MSCs or apoptotic or live MSC conditioned media. Killing of KG-1 cells was quantified via annexin V/7-AAD staining and expressed as relative to control CAR-T. Data are represented as mean $\pm$ SD from 3 independent experiments. **** $p < 0.0001$ by one-way ANOVA. (C) Proliferation assay supernatant was used for cytokine profiling by Luminex assay. Data are represented as mean $\pm$ SD from 4 independent experiments. **** $p < 0.0001$ by one-way ANOVA. (D) Viability of CAR-T in co culture experiments. Data are represented as mean $\pm$ SD from 4 independent experiments.

IV.5 CAR-T inhibition by apoptotic MSCs milieu is dependent on the NF- κ B/COX-2 pathway.

In the previous chapter the activation of NF- κ B and the subsequent increase in COX2 expression and PGE2 production were identified as key players in the immunoregulation mediated by MSCs exposed to activated CAR-T cells. We want to verify if those factors were playing a crucial role also on immunosuppression mediated by MSCs undergoing apoptosis after contact with activated CAR-T.

To this aim we repeated the proliferation assay, using apoptotic MSC conditioned media and an anti-PGE2 antibody, or using conditioned media from apoptotic Rel-A^{KD} MSCs, after testing that REL-A deactivation did not interfere with CAR-T mediated killing.

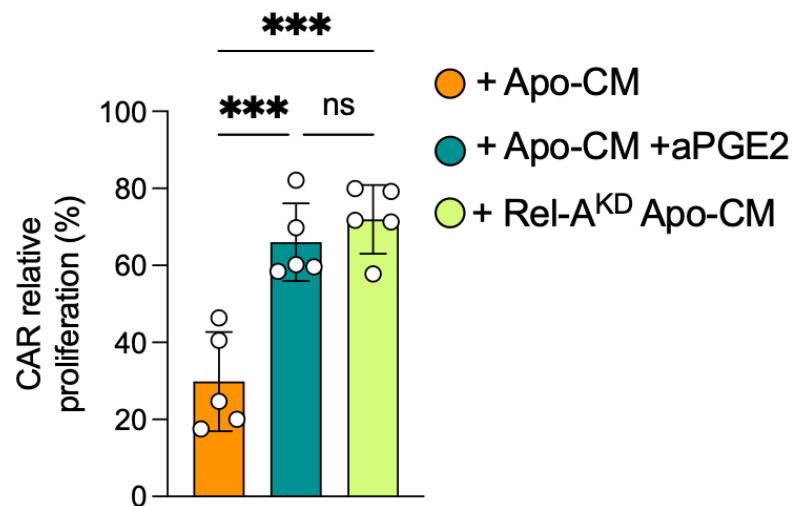
As shown in figure 6A both the block of PGE-2 in apoptotic MSC conditioned media and the suppression of NF- κ B signaling in MSCs underwent apoptosis rescued CAR-T cell proliferation by over 2-fold increase.

The cytotoxicity of CAR-T against KG1 cells after rechallenge was replaced by NF- κ B down-modulation and PGE2 block. As shown in figure 6B both of those conditions restored the cytotoxic activity of CAR-T against malignant cells, with an increase of over 45% compared to the CAR-T exposed to the conditioned media of apoptotic MSCs.

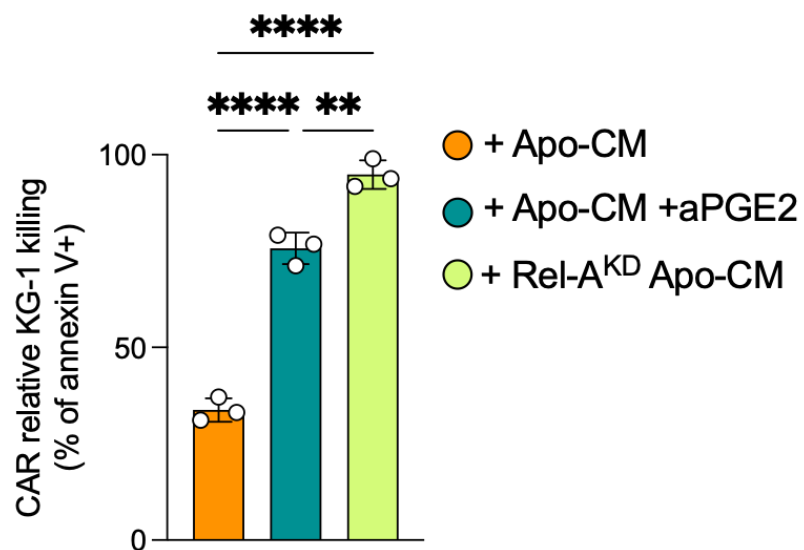
Similarly, key cytokine levels were significantly restored compared to apoptotic MSC conditioned media (figure 6C).

Figure 6: MSC immunosuppression of CAR-T required NF- κ B and was affected via the COX2 pathway.

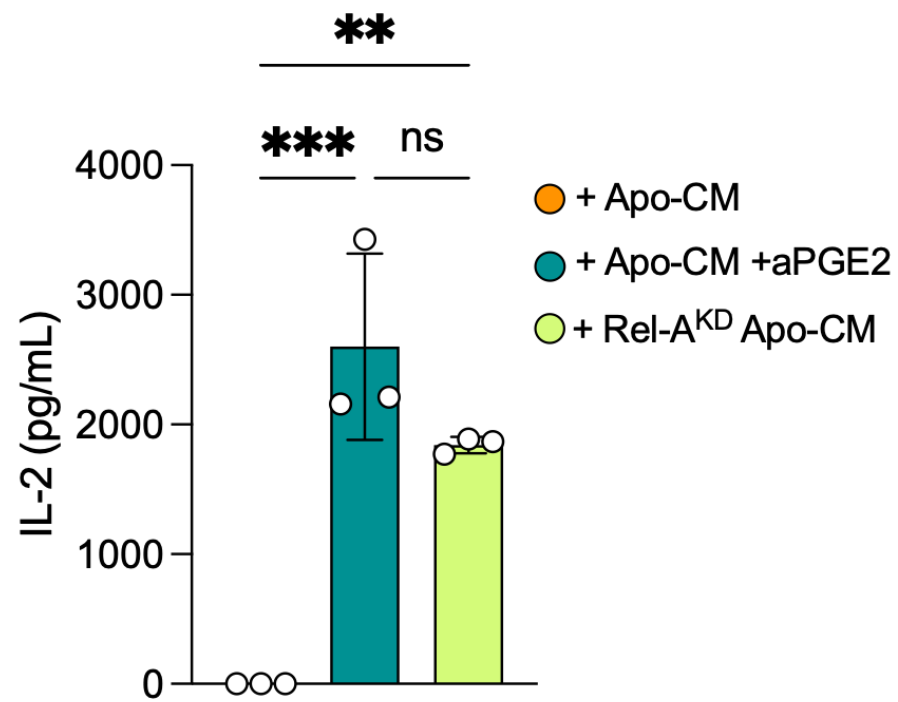
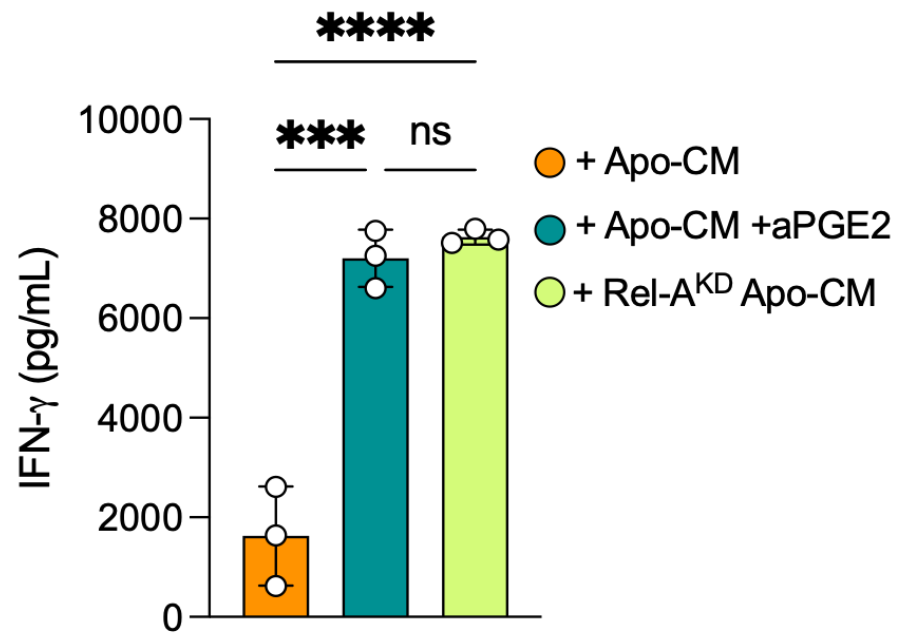
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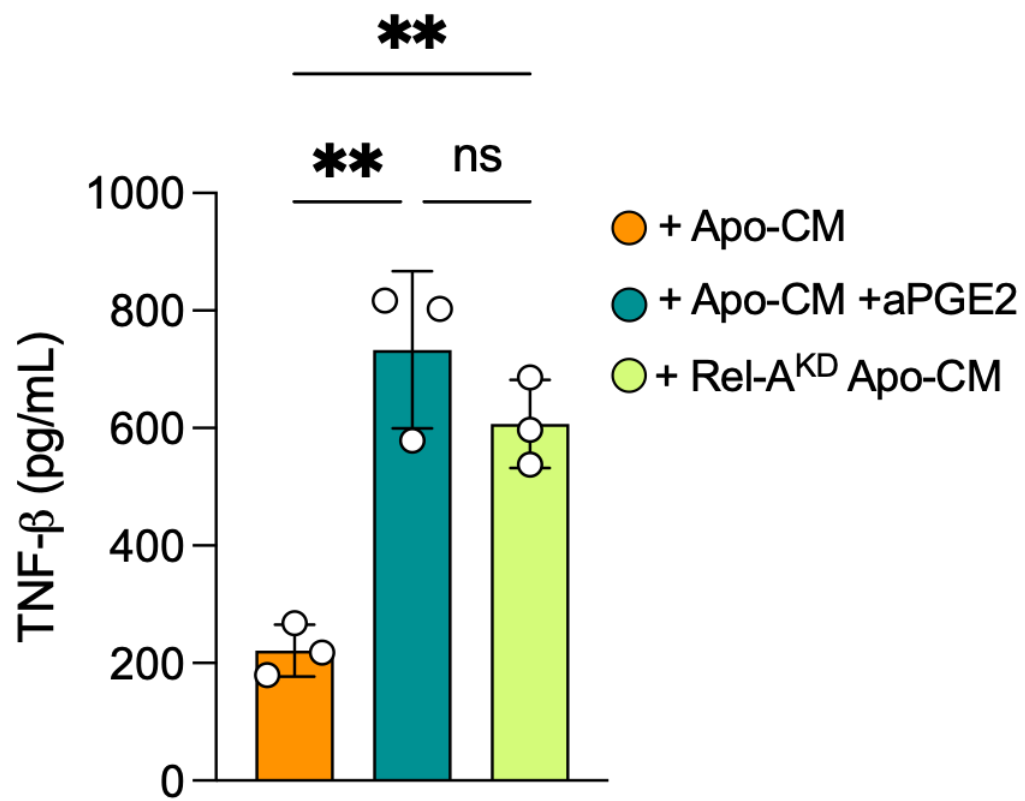
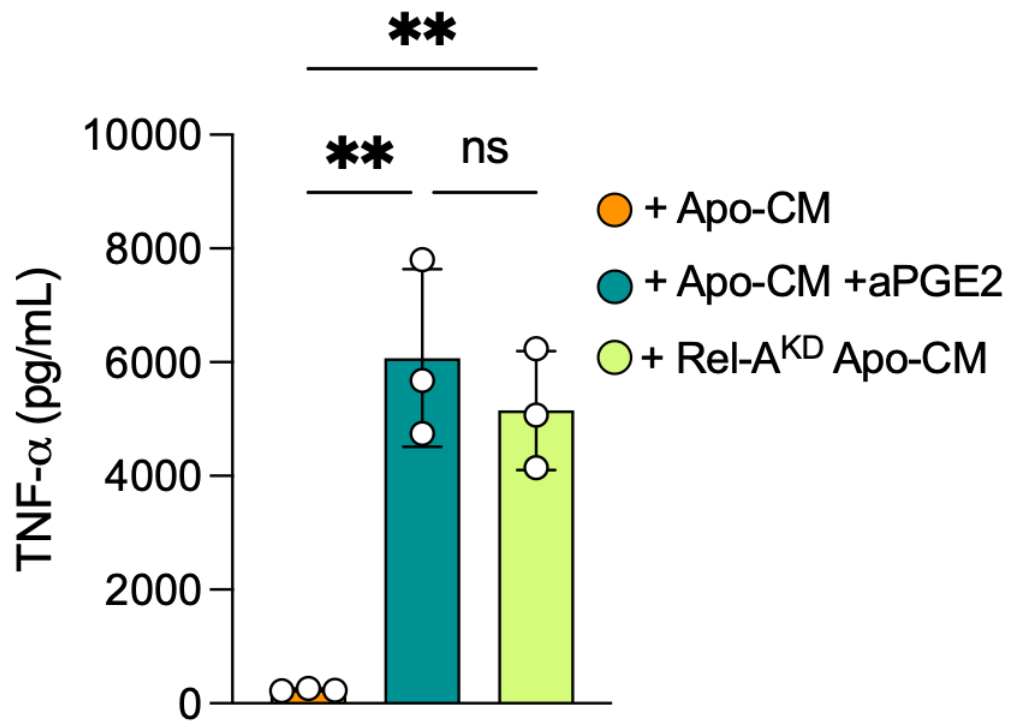


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MSC immunosuppression of CAR-T required NF- κ B and was affected via the COX2 pathway.

(A) Proliferation of CAR-T cells after antigen stimulation was evaluated in presence of media conditioned by apoptotic MSCs wild type or Rel-A^{KD}, with or without anti-PGE-2 antibody. Results are expressed as relative to control and represented as mean $\pm$ SD from 5 independent experiments. *** $p < 0.001$ by one-way ANOVA. (B) Cytotoxic activity of CAR-T against KG-1 cells was evaluated in the same conditions as above. Results are expressed as relative to control and represented as mean $\pm$ SD from 3 independent experiments. ** $p < 0.005$ and **** $p < 0.0001$ by one-way ANOVA. (C) Level of key cytokines (IFN- γ , IL-2, TNF- α and TNF- β) was estimated via Luminex assay in supernatant of proliferating CAR-T. Results are expressed as mean $\pm$ SDT from 3 independent experiments. ** $p < 0.005$, *** $p < 0.001$, and **** $p < 0.0001$ by one-way ANOVA.

IV.6 Discussion

In this chapter we proved how MSCs can undergo caspase-dependent apoptosis when co-cultures with both resting and especially activated CAR-T. This result has been firmly confirmed using 3 different experimental methods (figure 3B-D). This is in accordance with previously published data by Dazzi lab, differently from the PBMC context it must be highlighted that also resting CAR-T are able to induce basal level of apoptosis in MSCs, this can be explained by the manufacturing process of CAR-T cells, in fact the original PBMCs are activated and expanded in vitro in an IL-2 enriched environment which have been described to maintain T-cell activation (Cheung et al., 2019; Galleu et al., 2017; Giacomini et al., 2023). In regards of resting CAR-T killing, we speculate that resting CAR-T have a higher level of basal activation retained by the CAR manufacturing process in which they have been activated by CD3/28 stimuli and kept in IL-2 media for prolonged time.

Once established the ability of CAR-T to induce cell death in MSCs we further investigated mechanisms by which the signal is delivered. It appears clear from figure 4A that the process requires cell to cell contact and it is not triggered by MSC expression of CD33 antigen, nor by allorecognition (figure 4B). To date it still remains unclear what activated lymphocytes, both CAR-T and activated PBMCs, recognize on MSC surface.

Mechanistically it was observed that activated CAR-T mediated MSC death is driven by the release of perforin and granzyme b, as shown by using respectively the Ca^{2+} -chelating agent EGTA and the Z-AAD-CMK inhibitor (figure 4C-D). This enforces our believe that CAR-T cells recognize a specific molecule on MSC surface which form an immune synapse, as it was proven that both

perforin and granzyme are unable to induce cell death unless enclosed in the synaptic space (Prager & Watzl, 2019). MSCs cell death strictly caspase dependent, as shown in figure 4F, as caspase activation is hallmark of different programmed cell death pathways such as apoptosis and necroptosis (Galluzzi et al., 2018a). To exclude necroptosis, RIPK3 inhibitor was used (Figure 4E), as no significant changes were observed, necroptosis was excluded, condolences MSCs undergoing apoptosis.

It is also important to emphasize that even if several inhibitors were employed, a basal level of cell death was still observed. This suggests that further mechanisms may contribute to the contact-dependent caspase dependent CAR-T cytotoxicity .

We then focused on exploring the effect of apoptotic MSCs on CAR-T cells. As testified by figure 5A-B apoptotic nor live MSCs themselves did not have any effect in inhibiting CAR-T proliferation or cytotoxicity against target cells. Instead, when CAR-T cells were exposed to apoptotic MSCs conditioned media, the milieu of molecules released by dying cells strongly affected CAR-T proliferation (figure 5A), cytotoxicity (figure 5B) and cytokine release (figure 5C).

To find key molecules involved in the immunosuppression mediated by apoptotic MSCs we explored, as in the previous chapter, the NF- κ B-COX2-PGE2 pathways. Notably, a strong reduction of proliferation suppression was observed using both conditioned media from apoptotic MSC Rel-A^{KD}, as well as blocking PGE2 in the media conditioned by apoptotic wild-type MSCs (figure 6A). Those treatment not only restore CAR-T cell proliferation but also increased the release of cytokines (figure 5C) and the cytotoxic activity against KG1 cells (figure B6) inhibited by apoptotic MSC conditioned media alone.

Although not explored in this work, it is plausible to speculate that further mechanisms of immunosuppression could be ongoing in the leukemic microenvironment in vivo. In particular, as shown by previous works of this group (Galleu et al., 2017; Giacomini et al., 2023), apoptotic bodies generated from dying MSCs are efferocytosed by tissue resident macrophage and chemoattracted monocytes. This phenomenon was shown to trigger an M2 polarization determining an upregulation of COX2 in the myeloid cells leading to production of PGE2.

Despite not being investigated in this work we can speculate that, as MSCs are present in different tissue of the body, the above-described effect, which is not antigen restricted, could be expanded to other context where CAR-T demonstrate lack of activity such as solid tumors.

Chapter V – Results:

In-vivo validation

V.1 introduction

As discussed in previous chapters, two different mechanisms by which MSCs regulate CAR-T function were identified. Both, contact and cytokine dependent, relies on NF- κ B activation in MSCs and the consequent upregulation of COX2 and PGE2 release in the microenvironment.

We tested the impact of ApoMSC on CAR-T antileukemic activity in vivo in a xenograft model of AML (Biondi et al., 2023). CD33-positive KG-1 cells were injected in NSG mice and 2 weeks later, CD33-specific CAR-T were administered with or without a simultaneous dose of ApoMSC. Peripheral blood samples were collected 21, 28, 35 and 84 days after, and the percentage of human T-cells (hCD45+hCD3+) and human AML cells (hCD45+hCD33+) was evaluated by flow cytometry.

Finally, survival was measured to assess in vivo activity of apoptotic MSCs in suppressing CAR-T function.

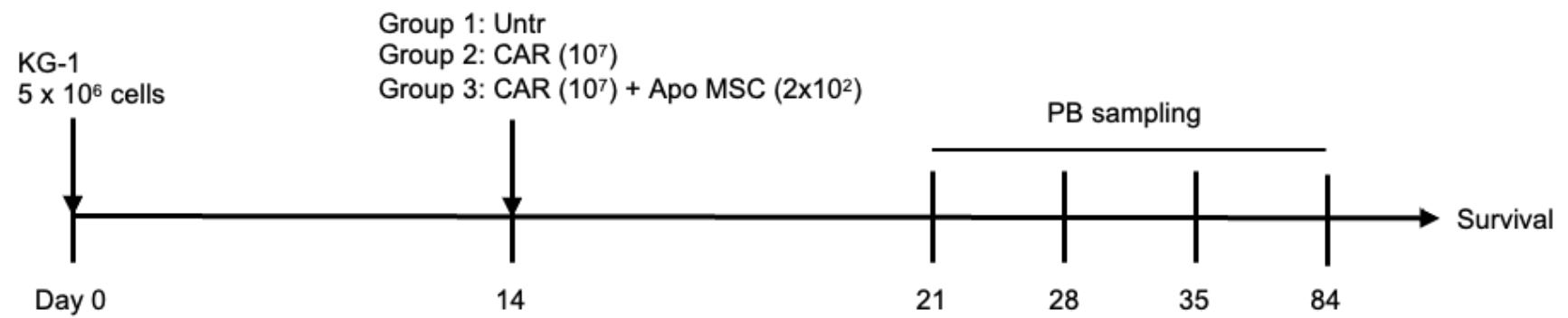
V.2 Apoptotic MSC administration impairs CAR-T cell persistence and inhibit their antileukemic activity in-vivo.

To evaluate the immunosuppressive effect of apoptotic MSC on CAR-T antitumor activity in vivo, we established a leukemia xenograft model by injecting CD33⁺ KG-1 AML cells into NSG mice (Biondi et al., 2023). Fourteen days later, mice were treated with CD33.CAR-T cells alone or together with ApoMSCs (Figure 7A). Peripheral blood was collected from the tail vein of mice at day 21, 28, 35, and 84 after KG-1 infusion to monitor the frequency of hCD33⁺ CAR-T and of residual hCD33⁺ AML cells. Animals treated with CD33.CAR-T displayed a reduction of the frequency of hCD33⁺ cells in the PB starting at day 21 after administration and the disease remain controlled until day 84. In the presence of ApoMSCs, CAR-T failed to control AML and the disease progress during time (Figure 7B). Percentage of hCD33⁺ CAR-T cells detected in the PB was significantly higher in mice treated with CD33.CAR-T alone compared to those receiving Apo-MSC until day 35. At day 84, 3 out 5 mice of the CD33.CAR-T alone group still present hCD33⁺ cells detectable in the PB (Figure 7B).

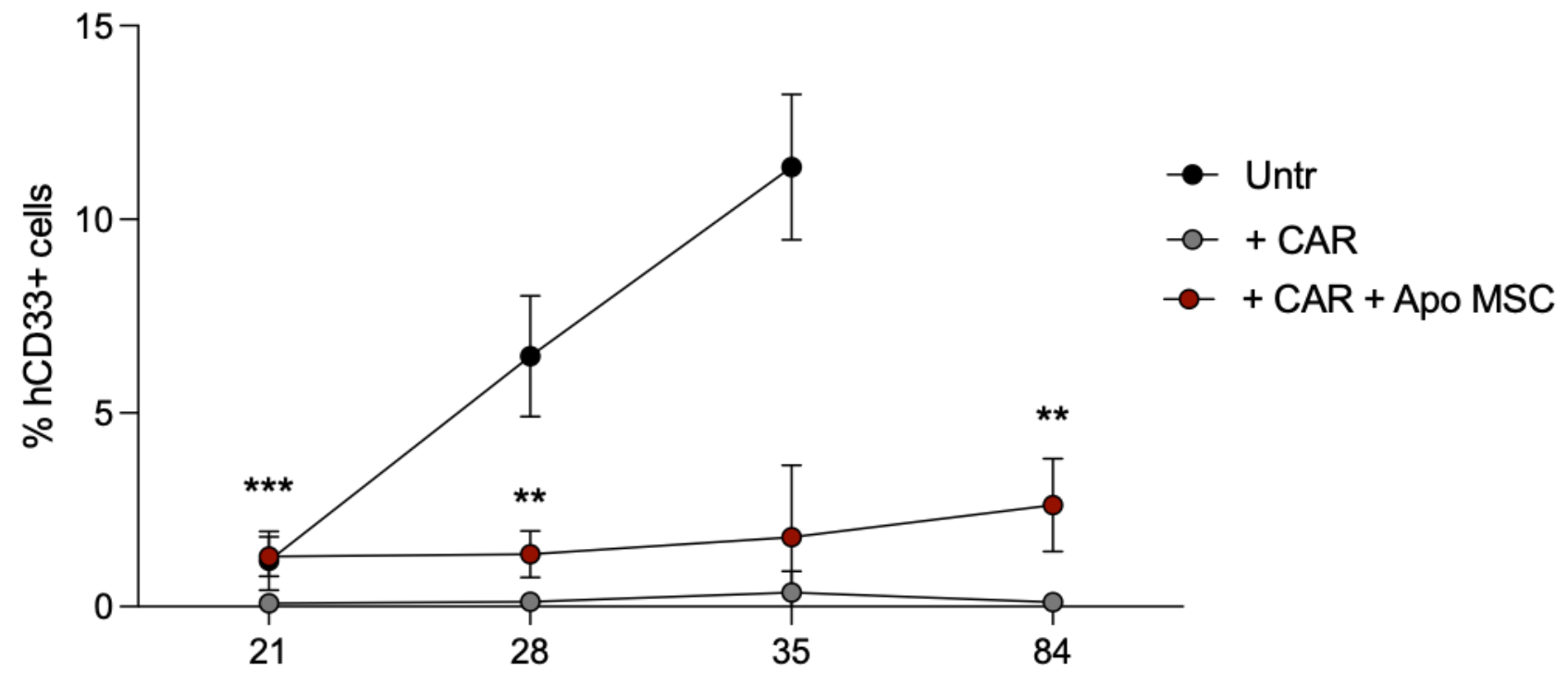
Accordingly, the survival of the KG-1 cell-inoculated mice treated with CD33.CAR-T was significantly prolonged (n=6 mice per group, p=0.0001, Log-rank test), with mean survival time increased from 60.5 in the untreated to >117 days (Figure 7C). On the contrast, the presence of ApoMSCs significantly reduce the mice survival compared to those receiving CAR-T cells only (83.5 days, p=0.001), further confirming their negative impact on disease control mediated by CAR-T.

Figure 7: Apoptotic MSCs inhibit CAR-T antileukemic activity in-vivo.

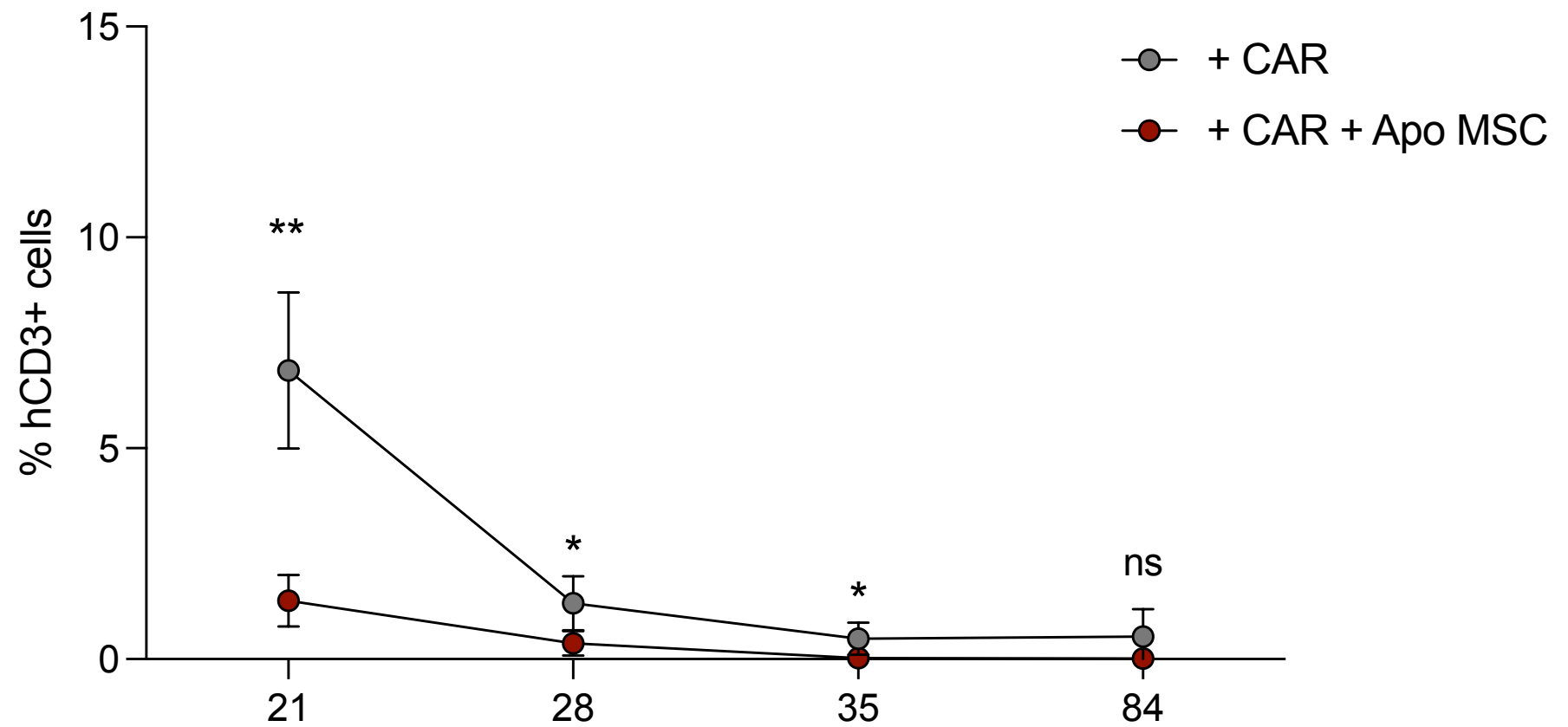
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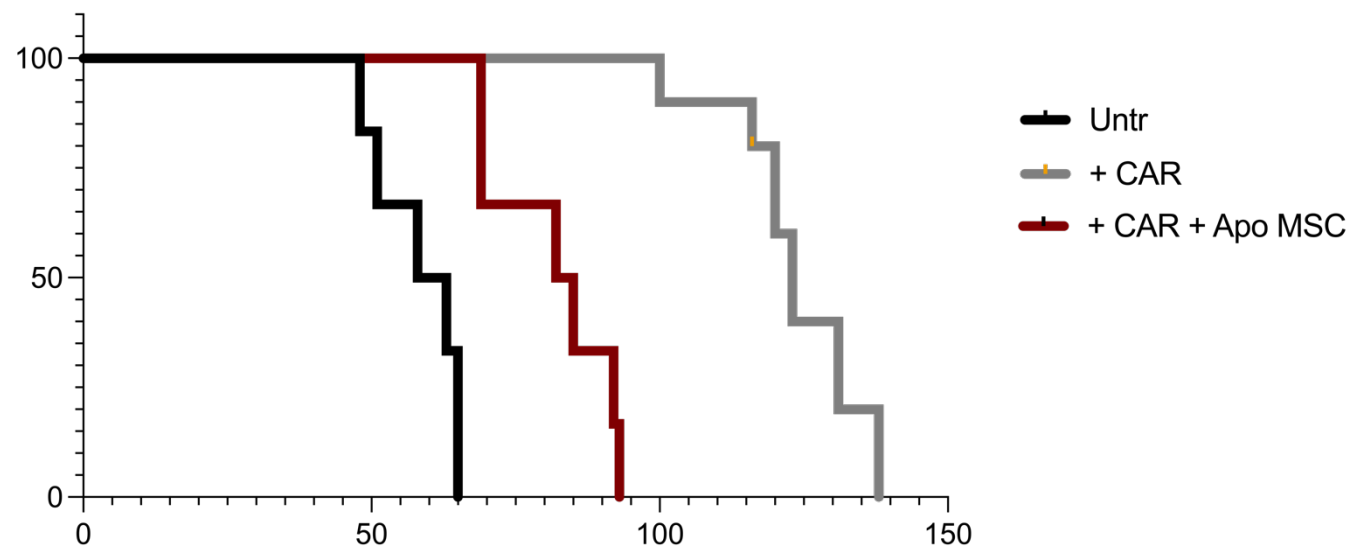
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Apoptotic MSCs inhibit CAR-T antileukemic activity in-vivo.

(A) Experimental design of in vivo experiments. NSG mice were inoculated i.v. with KG-1 AML cells (5×10^6 cells/mouse). 14 days later, CD33.CAR-T cells (10^7 cells/mouse) were administered i.v. alone or in combination with apoptotic MSCs (2×10^6 cells/mouse). FACS analysis on PB was performed at day 21, 28, 35 and 84 to evaluate the disease progression and the CAR-T cells persistence. (B) Percentage of human CD33⁺ AML cells in mice PB. Results are represented as mean $\pm$ SD from 1 experiment with 5 mice each group. ** $p < 0.005$ and *** $p < 0.001$ by t-test. (C) Percentage of human CD3⁺ CAR-T cells in mice PB. Results are represented as mean $\pm$ SD from 1 experiment with 5 mice each group. * $p < 0.05$ and ** $p < 0.005$ by t-test. (D) Kaplan-Meier survival curves of the same mice. Comparisons of survival curves were determined by Log-rank test. Results from 1 experiment with 6 animal per group. **** $p < 0.0001$ by Mantel-Cox test.

V.3 Discussion

In this chapter we aimed to prove an in-vivo effect of the mechanisms identified in-vitro. Due to technical issues, as human MSC engraftment in animal model is still largely unsuccessful (Pievani et al.,2020), only the contact dependent pathway was tested. This was achieved using a surrogate model where in-vitro apoptotic MSCs were generated, as previously described, and co-administered in a single dose with CAR-T cells in an AML xenograft model.

To monitor the effect of Apo MSC on disease burden and CAR-T peripheral blood of mice was harvested at day 21, 28, 35, 84 post KG-1 infusion (day 7, 14, 21, 70 after CAR-T infusion respectively). As Figure 7A shows in the group where Apo MSC were administered significantly more AML was detected (except day 35). Those data show a clear difference in the CAR-T ability to control the disease in presence of apoptotic MSCs.

Similarly, CAR-T cell levels were monitored at the same timepoints. Figure 7B clearly shows a significant decrease in CAR-T cells in the animal blood stream for the first 3 timepoints in mice treated with Apo MSC. Even though our experiments did not prove what is the reason of this change in CAR-T cell frequency in peripheral blood, based on in-vitro data we could speculate that is due to an impairment of proliferation.

Finally, mice were carefully monitored, and overall survival is reported in figure 7D. This panel clearly shows that mice treated with Apo MSCs display a shorter survival compared to the CAR-T cell only group.

Those data together prove that in the presence of Apo MSC the effect of CAR-T on disease control is impaired, proving their role in supporting a backfire against therapy.

Chapter VI - Discussion & Conclusion

VI.1 Discussion

This work aimed to find possible explanation on why CAR-T cell therapy still remains unsuccessful out of the CD19⁺ or BCMA⁺ oncological malignancies, such as solid tumors and AML. In our study we decided to employ an AML targeting CAR-T cells model as is the most similar to the scenarios where CAR-T showed successful in controlling patients' disease. Those similarity encompass the organ involved in the disease, the presence of a defined antigen (CD33 in our case) and the mechanism by which both CD19 and CD33 targeting CAR-T cells induce cytotoxicity also in healthy cells as the antigen is not disease specific.

To dissect the possible interactions between CAR-T and bone marrow MSCs, we looked at two different mechanisms to map those interactions and define their effect on the microenvironment. The first one investigated the phenotypic and functional changes of MSC when exposed to CAR-T cells released cytokines, and on the other hands, how those re-educated stromal cells impact CAR-T later functions. The second one instead relays on direct contact between CAR-T and stromal cells which triggers a cascade of events which also leads to an antagonistic effect on CAR-T therapy.

In the first mechanisms we highlighted how CAR-T cell secretion are able to leverage on the intrinsic plasticity of MSCs to re-educate them towards an immunosuppressive phenotype. This is characterized by the upregulation of known immune checkpoint ligands such as CECAM1 and PDL1 as well as other molecules typically involved in MSC mediated immunosuppression like IDO1.

More interestingly our analysis highlighted the upregulation of COX2 and Prostaglandin E2 synthetase, the two main players in PGE2 synthesis. PGE2 is known for its immunosuppressor effect in many different settings and recent studies demonstrated its role in polarizing immune cells to promote a cold microenvironment.

Gene ontology analysis was employed to understand the key regulators of this phenotypic changes in MSC upon exposure to CAR-T released cytokines. NF- κ B signature emerged in this analysis and was later validate by FACS analysis and shRNA silencing of Rel-A which decreased the COX2 upregulation and PGE2 production. Gene ontology analysis also revealed the encroachment of TNF- α and IFN- γ signaling signatures in re-educated MSCs, but the identification of the key mediator responsible for the NF- κ B activation in MSCs have not been investigated in this project.

To investigate the potential of re-educated MSCs in impairing CAR-T cell function it was decided to perform proliferation, cytotoxicity, and cytokine quantification assay. In each case re-educated MSCs demonstrated their active role in the inhibition, role which was abrogated by the use of MSC Rel-A^{KD}.

The second mechanism investigated instead looked at the direct contact interaction between CAR-T and stomal cells. It was observed, with several methods, that upon contact MSCs undergo contact-dependent, caspase-dependent, granzyme and perforin-mediated apoptosis. This process, which we described as bystander, is not allorecognition or antigen dependent. Despite our effort in this work and in other published by our group (Galleu et al., 2017) it is still unclear what is recognized by CAR-T cells (as well as other activated T-cells in other context) on MSC surface to trigger apoptosis. Understanding this could be achieved by high throughput knockout library

screen and could be important to shed light on MSC role in controlling inflammation.

It was proved before how the apoptotic cascade can trigger NF- κ B activation and how the cytokine released in this cascade can be strongly influence the immune response, among which the key role of PGE2 was highlighted (Cheung et al, 2023 in press). Indeed, also in the CAR-T scenario it was proven how apoptotic conditioned media is able to suppress proliferation, reduce cytotoxicity and reduce key cytokine secretion in a NF- κ B and PGE2 dependent fashion.

Finally, our findings were tested in an in-vivo xenograft model of AML. Due to technical reasons, as the lack of a strategy to engraft human MSCs in mice, only the effect of apoptotic MSCs was tested (Pievani et al., 2021). Experiment clearly shows that the co-administration of a single dose of apoptotic MSCs is sufficient to reduce the ability of CAR-T to control the disease and significantly reduce the overall survival of the animals.

All together those findings present 2 different ways in which bone marrow MSC can promote an immunosuppressive microenvironment and shows the molecular key player conserved among both, as in each case either the NF- κ B dependent upregulation of COX2 which leads to CAR-T loss of function in terms of proliferation, cytotoxicity levels and cytokine production.

VI.2 Conclusions

All together our data clearly shows that the immunomodulatory effect of MSCs on CAR-T is related to the activation of NF- κ B in the MSC and the consequent COX2 upregulation and PGE2 release in the microenvironment. Moreover, recently a gene signature of COX2 mediate immunosuppression was identified and correlate with poor prognosis in different solid tumor setting. Upregulated genes published in the signature are shared with the one resulting of our analysis. This enforce our hypothesis on the mechanism by which MSCs contribute to an immunosuppressive microenvironment which results in inadequate CAR-T performace (Bonavita et al., 2020).

It is important to highlight that in our study we focused on direct ways MSC can produce PGE2. Nevertheless, even if not part of this investigation Cheung and colleagues demonstrated how monocytes and macrophages, chemoattracted by MSCs' released cytokines, can efferocytose cell debris and upregulate themselves COX2 working as a 3rd mechanism which enrich the microenvironment of PGE2 (Cheung et al., 2019).

Another important aspect not explored in this work is the rest of the immune repertoire found in the microenvironment. Recent studies have highlighted how PGE2 released by MSCs can reprogram the neutrophil population found in lung metastasis (Gong et al., 2023). This study clearly shows that the pharmacological blockade of PGE2 or its receptors EP2 and EP4 strongly reduced the metastasis and improved the efficacy of adoptive Tcell therapy in this setting (Gong et al., 2023).

Similar results, which underline the importance of PGE2 as an immunosuppressive molecule in the tumor microenvironment are several. An example is the study by Pelly et al., which shows how the administration of PGE2 receptor (EP) EP2 and EP4 antagonist in combination with immune checkpoint inhibitor can boost antitumoral response in different primary tumor samples as well as xenograph melanoma models (Pelly et al., 2021).

Together those finding indicate that the target of COX2-PGE2 axes is an attractive druggable pathway to improve patient outcome, and for this reason is not a surprise as selective COX2 inhibitors, like Celecoxib, have been used in several solid tumor setting to boost the natural immune response (R. E. Harris et al., 2014; B. Liu et al., 2015; Mantovani et al., 2010; Regulski et al., 2016).

In the CAR-T setting only one report was published to date in regards of MSCs and CAR-T interaction. This report highlights the role of Stanniocalcin-1 (STC1) as key modulator of MSCs suppression (R. Zhang et al., 2023). Although in the article a precise mechanism for this phenomenon is not described. It is possible to speculate based on literature STC-1 immunomodulatory effect is directly linked to the mechanism described in this work. It is known that STC-1 in its role as a calcium ion regulator can increase the cytoplasmic concentration of free calcium which in turn can activate proteins such as phospholipase A2 to initiate phospholipid metabolism and the release of free arachidonic acid, which is PGE2 precursor (Rubin & Laychock, 1978; Song et al., 2009).

Finally, all the described mechanisms are independent from CAR – antigen recognition and for this reason is logical to suppose that the concept can be applied to any other context of CAR-T cell therapy especially if their microenvironment is reach in stromal cells like AML and solid tumors. Proof of concept studies are currently being conducted using a CAR construct

recognizing CD19 and with different costimulatory domains, obtaining similar results (data not shown)

To overcome this stromal counterattack to CAR-T cells several strategies could be implemented.

COX2 inhibition seem in this case the low hanging fruit as it based on common molecules such as ibuprofen with minor side effects, but reports proves that long term exposure to this type of therapy can interfere or delay with the Tcell activation processes (Iñiguez et al., 1999; Mortensen et al., 2019). PGE2 instead given the very short half-life of the molecule could be challenging target for clinical setting as the kinetics of its release can be highly variant.

Blocking of PGE2 receptors EP2 and EP4 is now an attractive target also due to the availability on the market of several antagonists. Proof of concept study shows how the administration of those can increase CAR-T efficacy against pancreatic cells *in-vitro* (Akbari et al., 2023). Major issue with this approach is again the half-life of the molecules which is restricted, in fact in *in-vivo* models needs to be administered every 12h (Pelly et al., 2021).

Kept into account the previous information, we propose a more elegant way to address the problem and boost CAR-T cell efficacy in stromal reach environments. An armored CAR based on a polycistronic vector which expresses a CAR construct targeting the desired antigen together with a sequence of shRNA targeting both EP2 and EP4 receptor transcripts. With this solution it would be possible to maximize CAR-T cell therapy efficacy by interfering at the source of the PGE2 immunosuppressive mechanisms.

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