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Bidirectional interference of mesenchymal stromal cells and anti-leukemic effector cells within the bone marrow microenvironment

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Abstract

Acute myeloid leukemia (AML) is a form of hematological cancer derived from the abnormal expansion of myeloid precursor cells in the bone marrow. It is maintained by a small subpopulation of quiescent and clonal leukemic stem cells (LSCs) that persist after conventional chemotherapy and irradiation, leading to relapse. Current survival rates at first diagnosis are dismal, highlighting the need for novel therapeutic options. Cytotoxic T lymphocyte (CTL)-derived therapies provide a promising alternative avenue for treatment, as LSCs ubiquitously express a range of hematopoietic-specific antigens that could be targeted. However, the effectiveness of an immune response against AML is likely diminished by several constituent cells of the leukemic microenvironment, and this is reflected in the generally disappointing outcomes observed in early clinical trials. Understanding and alleviating the "immunological sanctuary" provided by the leukemic niche is thus central in permitting effective immunity-based therapy. One key regulator of CTL suppression in the bone marrow are mesenchymal stromal cells (MSCs). MSCs are a heterogeneous population of multipotent cells that regulate hematopoiesis and can differentiate into several functional and structural progenitors. Under inflammatory conditions, MSCs undergo a drastic shift in phenotype, leading to the expression of a range of anti-inflammatory and immunosuppressive factors. Previous in vitro and in vivo work has demonstrated that MSCs inhibit the expansion of stimulated CTLs through contact-dependent and independent mechanisms. Less clear is the potential inhibitory role of MSCs concerning the cytotoxic activity of CTLs against their target in the context of AML, which was investigated in this thesis.

In prolonged *in vitro* co-culture assays, a contact-independent modulation of the inflammatory and proliferative capacities of AML-redirected universal chimeric antigen receptor (UniCAR) T cells by healthy donor and patient-derived MSCs was described, inhibiting T cell expansion, $CD4^+$ enrichment, as well as IFNγ and IL-2 release. In addition, MSCs interfered with the inflammatory potential of leukemia-associated WT1- and ROR1-targeting CTL clones, inhibiting the release of IFNγ, TNFα, IL-2 and IL-4. T cells abrogated in this manner were also shown to retain their cytolytic activity. Induction of the $CD28^-CD57^+$ senescent T cell phenotype by MSCs through paracrine mechanisms was demonstrated, a phenomenon that was independent of memory stage, as well as the UniCAR T cell-mediated apoptosis of MSCs. Finally, the expansive changes of MSCs under inflammatory stimulation in the specific context of anti-leukemic UniCAR T cell cytotoxicity were described via transcriptomic and protein analysis, identifying the expression of the immune checkpoint molecules VISTA and HVEM, as-of-yet uncharacterized in bone marrow MSCs. Through whole transcriptome analysis, genetic signatures and molecular programs were identified that were up- or downregulated under inflammation, broadly characterizing MSCs as shifting from multipotent modulators of the bone marrow extracellular matrix mediated by Wnt, Hedgehog, Notch and BMP signaling pathways, to regulators of immunity via the Jak-STAT, MAPK and PI3K-Akt-mTOR pathways. In summary, MSCs were shown to be potent mediators of anti-leukemic immunity, and targeting their modes of action would likely be highly beneficial in a combinatorial approach with AML immunotherapy.

Zusammenfassung

Die Akute Myeloische Leukämie (AML) ist eine hämatologische Krebserkrankung, die durch eine abnormale Expansion myeloischer Vorläuferzellen im Knochenmark ausgeht. Sie wird durch eine kleine Subpopulation ruhender und klonaler leukämischer Stammzellen (LSCs) aufrechterhalten, die nach konventioneller Chemotherapie und Bestrahlung überleben und zu einem Rückfall führen können. Die derzeitigen Überlebensraten bei der Erstdiagnose sind sehr niedrig, was die Notwendigkeit neuer therapeutischer Optionen hervorhebt. Von zytotoxischen T-Lymphozyten (CTL) abgeleitete Therapien bieten einen vielversprechenden alternativen Behandlungsweg, da LSCs eine Reihe von hämatopoetischen spezifischen Antigenen exprimieren, welche als Angriffsziel der Therapien dienen könnten. Die Wirksamkeit einer Immunantwort gegen AML wird jedoch wahrscheinlich durch mehrere Zelltypen der leukämischen Mikroumgebung unterdrückt. Dieser Fakt spiegelt sich in den allgemein enttäuschenden Ergebnissen wieder, die in frühen klinischen Studien erzielt wurden. Von zentraler Bedeutung für die Effizienz immun-basierter Therapien ist es, die „immunologische Schutzzone“ der leukämischen Nische zu verstehen und zu verringern. Ein Schlüsselregulator der CTL-Suppression im Knochenmark sind mesenchymale Stromazellen (MSCs). MSCs sind eine heterogene Gruppe multipotenter Zellen, die die Hämatopoese regulieren und sich in verschiedene funktionelle und strukturelle Vorläuferzellen differenzieren können. Unter inflammatorischen Bedingungen erfahren MSCs eine drastische Veränderung ihres Phänotyps, was zur Expression einer Reihe von entzündungshemmenden und immunsuppressiven Faktoren führt. Frühere *in vitro*- und *in vivo* Experimente haben gezeigt, dass MSCs die Expansion von stimulierten CTLs durch kontakt-abhängige und -unabhängige Mechanismen hemmen können. Weniger klar ist die potentielle inhibitorische Rolle von MSCs bezüglich der zytotoxischen Aktivität von CTLs in Bezug auf die AML.

In dieser Arbeit haben wir versucht, uns diesem Punkt zu widmen. In verschiedenen *in vitro* Kokultur-Assays wurde die kontakt-unabhängige Modulation der entzündlichen und proliferativen Kapazitäten von universellen AML-gerichteten chimären Antigenrezeptor (UniCAR) T-Zellen durch MSCs von gesunden Spendern und AML Patienten nachgewiesen. Diese Modulation führt zu einer Hemmung der T-Zell-Expansion, einer CD4+-Anreicherung, sowie zur reduzierten Freisetzung von IFNγ- und IL-2. Darüber hinaus wurde festgestellt, dass MSCs dem entzündlichen Potential der Leukämie- assoziierten WT1- und ROR1-gerichteten CTL-Klonen entgegen wirkten und die Freisetzung von IFNγ, TNFα, IL-2 und IL-4 hemmten. Interessanterweise konnten jedoch T-Zellen, die auf diese Weise gehemmt wurden, ihre zytolytische Aktivität beibehalten. Die Induktion des seneszenten CD28-CD57+-T-Zell-Phänotyps durch parakrine Mechanismen von MSCs konnte unabhängig vom Gedächtnisstadium der T-Zellen, sowie die von der UniCAR-T-Zell-vermittelte Apoptose der MSCs, beobachtet werden. Die umfangreichen Veränderungen von MSCs unter Entzündungsstimulation im spezifischen Kontext der antileukämischen UniCAR-T-Zell-Zytotoxizität wurden abschließend in Transkriptom- und Proteinanalysen beschrieben. Des Weiteren wurde die Expression der Immun-Checkpoint-Moleküle VISTA und HVEM identifiziert, welche bisher im Knochenmark MSCs noch nicht beschrieben wurde. Durch die Analyse des gesamten Transkriptoms wurden zudem genetische Signaturen und molekulare Programme identifiziert, die unter inflammatorischen

Bedingungen reguliert wurden. Diese Untersuchungen erbrachten den Nachweis, dass MSCs ihren Phänotyp von multipotenten Modulatoren der extrazellulären Matrix des Knochenmarks, vermittelt durch Wnt-, Hedgehog-, Notch- und BMP-Signalwege, zu Regulatoren der Immunität über die Signalwege Jak-STAT, MAPK und PI3K-Akt-mTOR verlagern können.

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Abbreviations

1-MT	1-methyltryptophan
^{3}H	^{3}Hydrogen
4-1BB	4-1BB ligand receptor
4-1BBL	4-1BB ligand
^{51}Cr	^{51}Chromium
7-AAD	7-Aminoactinomycin D
A2AR	Adenosine receptor 2A
A2BR	Adenosine receptor 2B
Akt	Serine/threonine protein kinase B
AML	Acute myeloid leukemia
ATP	Adenosine tri-phosphate
B7H3	B7 homolog 3
BiTe	Bispecific T-cell engager
BMP	Bone morphogenetic proteins
BTLA	B- and T-lymphocyte attenuator
CAR	Chimeric antigen receptor
CCR7	CC-chemokine receptor 7
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLL	Chronic lymphocytic leukemia
CLL-1	C-type lectin-like molecule-1
CM	Central memory
COX-2	Cyclooxygenase 2
CRS	Cytokine-release syndrome
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
Cxcl	C-X-C motif chemokine ligand
DAPI	4′,6-diamidino-2-phenylindole
DART	Dual affinity retargeting antibodies
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
E:T	Effector-to-target ratio
ECM	Extra-cellular matrix
EGSEA	Ensemble of gene set enrichment analyses
ELISA	Enzyme-linked immunosorbent assay
EM	Effector memory
FBS	Fetal bovine serum
FLT-3	Fms-like tyrosine kinase 3
FSC	Forward scatter
Gal	Galectin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GFP	Green fluorescent protein
GITRL	Glucocorticoid-induced tumour necrosis factor receptor-related protein ligand
GSEA	Gene set enrichment analysis
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
Gy	Gray
h	Hours
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HMOX1	Heme oxygenase 1 gene
HO	Heme oxygenase
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HVEM	Herpes virus entry mediator A
ICAM-1	Intercellular adhesion molecule 1
ICOSL	Inducible T cell costimulator ligand
IDH	Isocitrate dehydrogenase (NADP(+))
IDO1	Indoleamine 2,3-dioxygenase 1
IFN	Interferon
Il	Interleukin
Jak	Janus kinase
KLRG-1	Killer cell lectin-like receptor G1
La/SS-B	Lupus La protein / Sjögren syndrome type B antigen
LeY	Lewis Y antigen
LFA-3	Lymphocyte function-associated antigen 3
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cell
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mL	Millilitre
MLR	Mixed-lymphocyte reaction
MRD	Minimal residual disease
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stromal cell
mTOR	Mammalian target of rapamycin
Myc	Myelocytomatosis
N	Naïve
NK	Natural killer
nM	Nanomolar
NO	Nitric oxide
NOS	Nitric oxide synthase
PBMC	Peripheral blood mononuclear cell
PCA	Principle component analysis

PD-1	Programmed cell death 1
PDCD1	Programmed cell death 1 gene
PDCD1LG2	Programmed cell death 1 ligand 2
PD-L	Programmed death-ligand
PE	Phycoerythrin
pg	Picogram
PGE2	Prostaglandin E2
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PTGS2	Prostaglandin-endoperoxide synthase 2
qPCR	Quantitative polymerase chain reaction
R/R	Relapsed or refractory
Ras	Rat sarcoma virus
ROR1	Tyrosine-protein kinase transmembrane receptor 1
RPMI	Roswell Park Memorial Institute 1640
SASP	senescence-associated secretory phenotype
scFv	Single-chain variable fragments
SCM	Stem cell memory
SD	Standard deviation
SF3B1	Splicing Factor 3b Subunit 1
sFasL	Secreted Fas cell surface death receptor ligand
SRSF2	Serine and arginine rich splicing factor 2
SSC	Side scatter
STAT	Signal transducers and activators of transcription
TCR	T cell receptor
TE	Terminal effector
TGFB1	Transforming growth factor beta 1
TIL	Tumour-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TM	Target module
TNF	Tumour necrosis factor
TNFAIP6	Tumor necrosis factor alpha-inducible protein 6
TP53	Tumour protein P53
Treg	Regulatory T cell
TSG-6	Tumor necrosis factor-stimulated gene 6 protein
UniCAR	Universal chimeric antigen receptor
VCAM-1	Vascular cell adhesion protein 1
VISTA	V-type immunoglobulin domain-containing suppressor of T-cell activation
Wnt	Wingless-related integration site
WT1	Wilm's tumour protein 1

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1 Introduction

1.1 Acute Myeloid Leukemia

1.1.1 Mutational burden and prognosis

Acute myeloid leukemia (AML) is a form of hematological cancer derived from the abnormal expansion of myeloid precursor cells, resulting in invasion of the bone marrow and ultimately failure of normal hematopoiesis. The World Health Organization classification of AML comprises several recurring cytogenetic and molecular aberrations that reflect the pathophysiology of leukemogenesis and, more importantly, determined prognosis of individual patients in addition to their clinical comorbidities (Döhner et al., 2015). An analysis by the Cancer Genome Atlas of 200 AML samples identified 23 commonly mutated genes that could be grouped into several functional categories, such as constitutive activation of proliferative and cell survival pathways (FLT3), deactivation of tumour suppressors (TP53), deregulation of DNA methylation (IDH1, IDH2) and splicing (SRSF2, SF3BP1), etc. The mutational burden of AML is significantly lower in comparison to most solid tumours, though patients typically present several subclones upon initial diagnosis and at least 2 driver mutations. AML likely develops from hematopoietic stem cells (HSCs) or early myeloid progenitors following the "two-hit" theory of driver mutation acquisition in cancer development. Shlush *et al*. identified in patient samples HSCs harboring mutated DNMT3A, but without the additional NPM1 mutation found in the AML blasts. These mutated HSCs could undergo multilineage differentiation, clonally expand over their normal counterparts and survive chemotherapy (Shlush et al., 2014). Mutations of other regulators of gene expression TET2 and ASXL1 have also been identified in early leukemia development. Mutational analysis and prognostic correlation has permitted the stratification of patients into three risk categories (favourable, intermediate and adverse), with additional factors determining prognosis including age and existing comorbidities, and eventually response to therapy via monitoring of minimal residual disease (MRD) and blast count (Döhner et al., 2017).

1.1.2 Current therapy

Standard treatment regimens for AML have essentially remained unchanged in several decades (Döhner et al., 2017). Typical induction treatment is intensive chemotherapy consisting of a combination of cytarabine and anthracyclines, achieving complete remission at a rate of around 80% in the most favourable circumstances, though this decreases considerably with age and unfavourable mutational profiles. This is followed by more intensive consolidation chemotherapy, with additional allogeneic hematopoietic stem cell transplantation (HSCT) for younger patients with intermediate or adverse genetic risk or primary refractory disease. HSCT is the process of infusing hematopoietic stem and progenitor cells to reconstitute the ablated host hematopoietic system and reinstate durable anti-leukemic immunity. It is currently the most effective post-remission therapeutic option in treating high-risk AML (Stelljes et al., 2014), though it introduces the potential for life-threatening complications, mainly associated graft-versus-host disease (GvHD) (Bornhäuser, 2021). Unfortunately, relapse still occurs in roughly 40% to 70% of

cases (Ehninger et al., 2014), with options for salvage therapy consisting of more chemotherapy and possibly HSCT should remission (or at least major reduction in tumour burden) be achieved. At this stage, survival becomes particularly dismal, especially in patients that have already received HSCT; at best, three year survival can be expected in 38% of patients that have been in successful remission for 3 years, decreasing to only 4% should post-HSCT relapse occur within 6 months (Döhner et al., 2017). Current AML management guidelines thus recommend at this stage an experimental approach and enrollment into ongoing clinical trials.

Aside from treatment-related mortality rates decreasing thanks to improvements in supportive care, the most noteworthy development in AML management in the past several years has been targeted therapies for some of the most frequently mutated genes. Currently, regulatory approval by the U.S. Food and Drug Administration has been given to the FLT-3 inhibitors midostaurin and gilteritinib, and the IDH1 and IDH2 inhibitors ivosidenib and enasidenib (Döhner et al., 2017). Initial investigation of their use as single agents did provide measurable anti-leukemic effects, though their impact on overall survival was disappointing. However, it seems that they are most beneficial as part of a combinatorial approach with their inclusion into standard induction and consolidation regimens, and have been found to even possess some benefit in treating cases of relapsed or refractory (R/R) AML (Thol and Ganser, 2020).

1.1.3 Relapse and the leukemic stem cell theory

As it stands, the 5-year survival rate for newly diagnosed AML patients is less than 30% (SEER, 2019), mainly owing to a high incidence of relapse (though the rate of refractory AML is also considerable). It has thus become critical to investigate the cause(s) of relapse. Early colony formation, cell sorting and xenoengraftment assays (Lapidot et al., 1994; Dick, 2008), and later studies in mutation acquisition (Parkin et al., 2013), described the likely point of origin of AML as a small subpopulation of $CD34^+CD38^-$ "leukemic stem cells" (LSCs). LSCs had been named as such for displaying many similarities to normal HSCs, such as marker expression, pluripotency, quiescence and indefinite self-renewal, as well as being relatively rare. Ishikawa *et al*. demonstrated the repopulating potential of LSCs in murine xenografts, in which the $CD34^+CD38^-$ cell fraction of AML was able to engraft itself within the bone marrow over multiple rounds of transplantation, expanding and differentiating into additional $CD34^+CD38^+$ and $CD34^-$ populations. These cells were also found to be resistant to cytarabine treatment, and further investigation revealed that they largely remained dormant in the G0 phase of the cell cycle (Ishikawa et al., 2007). In the clinical setting, van Rhenen *et al*. showed an inverse correlation between the frequency of $CD34^+CD38^-$ AML cells on initial diagnosis and chemotherapy response, duration of remission and overall survival (Van Rhenen et al., 2005). The body of data from the last two decades establishes a very compelling hierarchal model for AML, with LSCs at the foundation of pre- and post-remission disease. However, recent evidence has emerged that adds a certain degree of nuance to this theory. In clonal evolution studies, Shlush *et al*. showed that the source of relapse may not only be due to rare primitive clones, but could also originate from phenotypically differentiated AML cells (Shlush et al., 2017). Furthermore, Boyd *et al*. demonstrated that contrary to the established paradigm, LSCs could actually be susceptible to

chemotherapy on repeated applications, transitioning into the cell cycle after the initial cytoreduction. They suggest that relapse is not caused by therapy selection of resistant blasts, but rather by an adaptive regenerative state characterized by a transcriptomic signature that is distinct from the LSC phenotype (Boyd et al., 2018). This has since been corroborated by Duy *et al*. in single-cell transcriptomic studies, wherein they describe leukemic cells acquiring a transient quiescent state that is independent of stemness on exposure to chemotherapy (Duy et al., 2021).

Regardless, the nature of AML recurrence remains a population of leukemic cells that persist after current therapeutic interventions, and eliminating these cells is critical in order to improve AML survival. This may be achieved by establishing a durable immune response, and immunotherapies are an exciting recent development in cancer management. In the next section, we will be discussing their application in targeting AML.

1.2 Immunotherapy

1.2.1 Immune engagement in standard AML therapy

Standard management of AML already incorporates treatments that elicit an anti-leukemic immune response. Chemotherapy is typically thought to induce tumour cell apoptosis through disruption of DNA replication (Vincelette and Yun, 2014), a form of cell clearance that purposefully avoids triggering inflammation and immunity. Despite being introduced in the late 1960s, the mechanistic functions of anthracyclines were only uncovered decades later in their ability to induce a form of controlled, immunogenic cell death (Galluzzi et al., 2007; Apetoh et al., 2008). Fucikova *et al*. demonstrated that treatment of acute lymphocytic leukemia cells with the anthracyclines doxorubicin and idarubicin, in comparison to other chemotherapies and ionizing irradiation, induced this form of immunological apoptosis, marking them for engulfment by dendritic cells, which could then activate and stimulate T lymphocytes (Fucikova et al., 2011). This was later confirmed in *in vivo* breast cancer models, with treatment efficacy dependent on the presence of cytotoxic T cells, and further enhanced when used in conjunction with immune checkpoint blockers (ICBs, discussed further down) (D'Amico et al., 2019). Doxorubicin induction therapy also provided the most benefit to PD-1 blockade in triple negative breast cancer patients (Voorwerk et al., 2019).

Innate and adaptive immune effector cells have also been identified as the mediators of the anti-leukemic activity of allogeneic HSCT (Wu and Ritz, 2006; Kolb, 2008). Graft-versus-leukemia (GvL) refers to the control of disease by the donor immune system, and T lymphocytes are generally considered the primary mediators of both GvL and GvHD (though this may partly be a reflection of a larger body of evidence spanning many decades, in comparison to other effector cells). GvHD occurs when inflammation due to the preparative myeloablative regimen induces the activation of helper and cytotoxic allo-responsive donor T cells, leading to damage of host tissues such as the gut and skin. This induces further inflammation, reinforcing the allogeneic immune reaction and cascading into what is referred to as a "cytokine storm" (Jacobsohn and Vogelsang, 2007). T-cell depletion reduces the severity of GvHD, but also increases the rate of relapse. In fact, several large scale studies in the 1990s have demonstrated that there is a strong positive correlation

between the presence of GvHD and response to treatment (Horowitz et al., 1990; Ringdén et al., 1996). Likewise, there are reported cases in which cessation of GvHD prophylaxis in R/R hematological disease led to remission (Collins et al., 1992; Libura et al., 1999). In an early clinical study in 1979, Weiden *et al*. demonstrated that that allogeneic bone marrow transplantation decreased the risk of relapse in comparison to syngeneic transplants between identical twins, though survival rates were ultimately similar due to GvHD morbidity in the allogeneic recipients (Weiden et al., 1979). Furthermore, *in vivo* studies have demonstrated that depletion of either the $CD4^+$ or $CD8^+$ T cells led to a decreased GvL effect, with optimal response necessitating both subtypes (Truitt and Atasoylu, 1991). In concert with the characterization of T lymphocytes as important mediators of GvL, numerous AML-associated antigens and their specific HLA class I and/or II-restricted epitopes have been identified (Anguille et al., 2012), such as the transcription factor encoded by Wilm's tumour gene 1 (WT1) (Yang et al., 2007). Overexpressed in the majority of AML cases and having been found to be able to elicit cellular and humoral immune responses (Sugiyama, 2010), there has been a concerted effort to target WT1 with cancer vaccines, particularly as post-HSCT adjuvant therapy (Hashii et al., 2012; Maeda et al., 2013).

In addition to T lymphocytes, B cells may also contribute to the GvL response of HSCT, both as mediators of inflammation as well as through their humoral activity. Indeed, tumour epitope-associated antibodies have been detected in the serum of patients suffering from several hematological malignancies in the months following HSCT, correlating with successful response to therapy, though conclusions differ regarding the potential association with the onset of GvHD (Wu et al., 2000; Bellucci et al., 2004; Miklos et al., 2005). Finally, donor natural killer (NK) cells can also be detected in circulation after HSCT. In fact, NK cells are the earliest lymphocytes to reconstitute after transplantation (Wu and Ritz, 2006). Allogeneic NK cells could be cytotoxic to host leukemic cells due to incompatible HLA-killer inhibitory receptor axes, and several studies have shown correlation with positive outcome (Giebel et al., 2003; Aversa et al., 2005; Ruggeri et al., 2005). Because NK cells are able to activate their cytotoxic machinery without prior exposure to an antigen, much interest has been garnered in their use in adoptive therapies, including in the context of leukemia (Isidori et al., 2021; Myers and Miller, 2021).

1.2.2 Cell surface targets for immunotherapy

In a distinction from the broader engagement of the innate and adaptive immune response produced by HSCT, *bona fide* immunotherapies are designed to recognize and bind to specific cell surface markers on immune and tumour cells. They come in a variety of flavours to prompt the engagement of T cells, NK cells or monocytes (Isidori et al., 2021). Here, we will focus on immunotherapies that are specifically designed to activate the T cell response.

It has been demonstrated that in cases of R/R AML, tumour epitope-recognizing T lymphocytes present dysfunctional proliferative, inflammatory and cytotoxic capabilities, and display markers of senescence (Beatty et al., 2009; Knaus et al., 2018) and exhaustion (Noviello et al., 2019; Rutella et al., 2021). While the mechanisms and contribution of senescent T cells to AML progression are still in the early stages of research, an important driver of immune escape is the engagement by AML blasts and other cells of the leukemic microenvironment of inhibitory

receptors on T cells associated with exhaustion, the so-called immune checkpoints (Toffalori et al., 2019). After promising results in animal studies (Zhang et al., 2009), PD-1, CTLA-4 and TIM-3 are some of the markers currently under investigation for ICB therapy in AML (Taghiloo and Asgarian-Omran, 2021). ICBs are small molecules that are designed to prevent ligand-immune checkpoint interactions. While none have so far achieved unprecedented success compared to the standard of care, early observations have noted successful reduction in blast count, with complete remission being achieved in approximately 35% of R/R AML cases with PD-1 blockade (Davids et al., 2016; Daver et al., 2019; Zeidner et al., 2019), 50% for CTLA-4 blockade (Garcia et al., 2020) and 23% for TIM-3 blockade (Borate et al., 2019), typically in combination with chemotherapy. These results are still preliminary, and it will be some time yet before the completion of any phase III clinical trials to properly study efficacy.

CD33 and CD123 are the primary targets in current T cell redirection-based immunotherapy development and clinical investigation due to their ubiquitous expression on AML cells. In an expansive flow cytometry characterization of 319 newly diagnosed AML patients across various age groups and mutational categorizations, Ehninger *et al*. demonstrated that CD33 and/or CD123 expression could be found on the cell surface of all examined AML samples. When specifically looking at $CD34^+$ blasts (i.e. the LSC compartment), over 70% were double-positive, with only 4% of cases being negative for both markers. However, they likewise noted that both CD33 and CD123 were also detectable on normal myeloid progenitor cells, though at significantly lower levels (Ehninger et al., 2014). This was corroborated in a similar study of 357 AML samples, in which Haubner *et al*. also identified the widespread expression of CD33 and CD123, as well CLL-1, CD7, CD244 and TIM-3, on both bulk AML cells and LSCs at initial diagnosis and on relapse. However, they could also observe the expression of CD33 and CD123 in normal hematopoietic populations. In particular, they noted that CD33 was expressed on the cell surface of granulocytes and monocytes at comparable levels to AML, and that CD123 was positive on hematopoietic stem cells and monocytes, though at slightly lower levels compared to AML (Haubner et al., 2019). These and similar findings (Gill et al., 2014) suggest that while CD33 and CD123 are attractive targets for immunotherapy, careful management of off-target effects will likely be necessary. Indeed, the challenge in identifying an "ideal" AML cell surface target contributed towards delays in translating pre-clinical immunotherapies to the clinical setting in comparison to other cancer types. Haubner *et al*. went on to suggest that to minimize targeting HSCs, more specificity could be achieved with a dual marker approach (CD33/TIM-3 and CLL-1/TIM-3), though monocytes would still be affected.

1.2.3 Bispecific antibodies

As their name implies, bispecific antibodies possess two recognition domains: one for the tumour marker and the other for the immune effector. Functioning as a bridge, they effectively retarget T or NK effector cells for a more efficient and sustained tumour-specific response (Isidori et al., 2021). Bispecific antibodies can be subdivided into several categories based on their structure: bispecific T-cell engagers (BiTes), tandem diabodies and dual affinity retargeting antibodies (DARTs). BiTes are formed by two connected single-chain variable fragments (scFv)

derived from the antigen-recognition VH/VL antibody domains. One scFv recognizes the leukemia-associated antigen (such as CD33 or CD123), while the other recognizes the T cell co-receptor CD3. This effectively forces the creation of the cytotoxic synapse, leading to activation, proliferation, target lysis and inflammation. Tandem diabodies are essentially 2 connected BiTes for a given target. Their higher molecular weight allows them to exceed the renal clearance threshold and increase their half-life after being administered. DARTs are two polypeptide chains covalently linked by a disulfide bridge, with the VH and VL domains for each antigen specificity split between the two chains.

So far, there have been several early updates from ongoing phase I/II trials for CD3/CD33 BiTes and CD3/CD123 DARTs in the treatment R/R AML (Subklewe et al., 2019; Aldoss et al., 2020; Ravandi et al., 2020b; Ravandi et al., 2020a). In terms of efficacy, blast reduction could be observed in many patients, though complete remission rates ranged from 3% to 19% for the BiTes and 14% to 30% for the DARTs. Most responders would then go on to receive HSCT.

1.2.4 CAR and UniCAR T cells

Chimeric antigen receptors (CAR) are engineered cell surface molecules that link an extracellular antigen-recognition domain to intracellular signaling machinery, effectively retargeting and activating the modified T cell. The basic CAR design is under continuous development, with the latest 3rd generation possessing multiple intracellular co-stimulatory domains (such as CD28 and 4-1BB) fused to the CD3 ζ chain-signaling domain (Acharya and Walter, 2020).

Regarding the administration of CAR T cells for AML therapy, preliminary observations from the first clinical trials have only recently been emerging and it will be some time before efficacy can truly be evaluated. In the first reported clinical trial for adoptive CAR T cell therapy in AML, Ritchie *et al*. treated 4 cases of R/R AML with Lewis Y antigen (LeY) -specific CAR T cells. They noted some response to the treatment, including blast reduction in a patient previously resistant to chemotherapy, though ultimately all patients would relapse (Ritchie et al., 2013). These outcomes stand in contrast to the pre-clinical data, in which LeY CAR T cells significantly reduced tumour burden and increased survival in animal models (Westwood et al., 2005; Peinert et al., 2010).

In vivo experiments with CD33 (Kenderian et al., 2015; Li et al., 2018) and CD123 (Mardiros et al., 2013; Gill et al., 2014; Baroni et al., 2020) CAR T cells likewise looked extremely promising, with treatment leading to persistent immunosurveillance and increased survival in murine models. The results of several phase I trials have now been published, though they generally reflect the outcomes observed with the LeY CAR T cell studies. Wang *et al*. treated a single patient with refractory AML with CD33-targeting CAR T cells, observing decrease in blast count in the short term before disease progression and death (Wang et al., 2015). Budde *et al*. treated 6 post-HSCT refractory AML patients with CD123 CAR T cells and noted more promising outcomes: 3 patients had a reduction in blast count and 2 achieved complete remission permitting follow-up HSCT, one of which maintained remission for over 6 months of follow-up. The last

patient did not receive any benefit from the adoptive therapy however (Budde et al., 2017). The trial is currently on-going (NCT02159495) with an enrollment of 31 patients and a study completion date set for December 2022. As mentioned above, a primary concern with CD123 is off-target hematological toxicity, and indeed pre-clinical data has shown that CD123 CAR T cells induced lower hematopoietic potential and *de novo* reconstitution in xenograft models (Gill et al., 2014; Baroni et al., 2020). Fortunately, Budde *et al*. noted that none of the patients had any observable myeloablative effects. This was corroborated in another trial by Cummings *et al*. composed of 7 R/R AML treated with CD123 CAR T cells, though in this instance they could not detect any reduction of blast count (Cummins et al., 2017). In addition to CD33 and CD123, other targets being considered are CD44v6, FLT-3 and CLL-1 (Isidori et al., 2021).

A common side effect in the above-mentioned clinical trials was cytokine-release syndrome (CRS), which is defined as an acute and widespread cytokine-mediated inflammatory response to treatment, leading to symptoms of fever, hypotension and excessive bleeding. It has been reported to occur in up to 91% of patients receiving CD19 CAR T cell treatment, with particularly severe incidence in 8% to 43% of cases (Hay et al., 2017). CRS has also been observed in the administration of BiTes (Barrett et al., 2014), though it has been associated with both toxicity and treatment effectiveness. This draws obvious parallels with HSCT and induction of GvHD, but whether CAR T cell-mediated CRS is more or less debilitating than GvHD still needs be determined. In any case, this highlights the need for continued refinement of CAR T cells with the implementation of safety mechanisms, such as incorporation of suicide switches or transient CAR expression (Koedam et al., 2022). Another such effort is the modular universal CAR (UniCAR) (Cartellieri et al., 2016) (**Figure** 1). UniCARs follow the 2nd generation CAR design, with intracellular CD3 ζ chain and CD28 signaling domains. For the extracellular antigen recognition element, the UniCAR replaces typical tumour antigen recognition with specificity for an epitope of the nuclear La/SS-B peptide. Tumour recognition is instead dictated by the second component of the system, the target module (TM), which combines an antigen recognition scFv (i.e. for CD33 or CD123) with the La/SS-B epitope. Akin to a bispecific antibody, the TM effectively functions as a bridge between the target cell and the UniCAR-expressing T cell. In the clinical setting, this modular TM-dependent UniCAR system provides two distinct advantages over the classic CAR approach. Firstly, the low half-life of the TM after infusion (Loff et al., 2020) allows for more precise management of UniCAR T cell activity, such as sudden retraction in instances of overt toxicity. Secondly, circulating UniCAR T cell redirection may be achieved with the sequential or even concurrent use of TMs for different targets. The UniCAR system has been shown to efficiently kill AML blasts *in vitro* with CD33 and CD123-redirecting TMs, and withholding the TM led to minimal activity (Cartellieri et al., 2016; Loff et al., 2020). Likewise, CD123-redirected UniCAR T cells effectively reduced the tumour burden and improved survival in murine models (Loff et al., 2020). The first preliminary observations of the clinical efficacy of UniCAR T cells with the anti-CD123 TM have recently been reported, of which all three R/R AML patients responded to treatment and two achieved complete remission (Wermke et al., 2021).

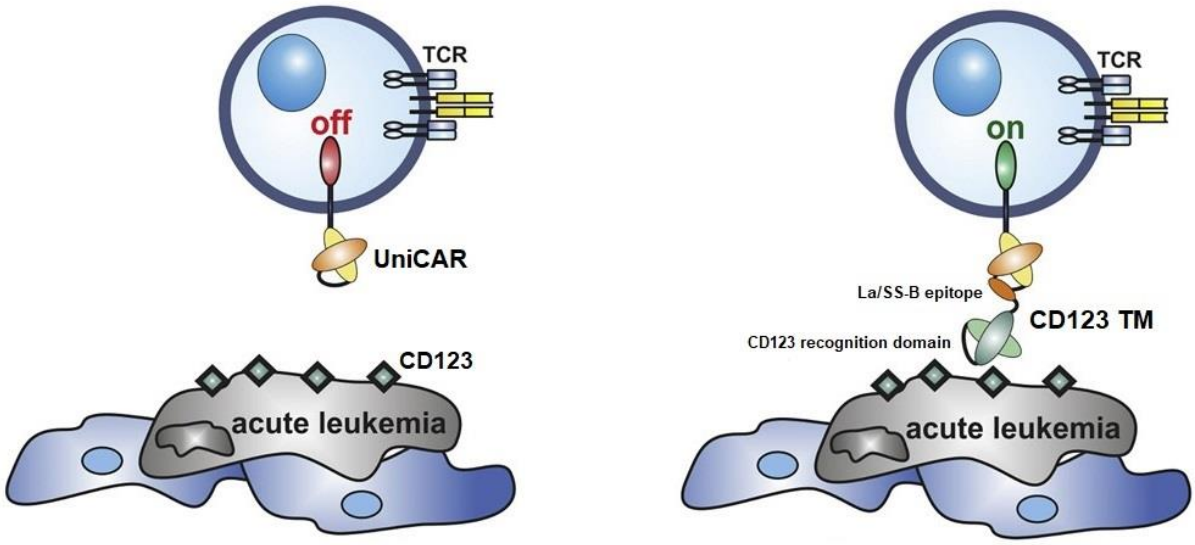


Figure 1. The modular UniCAR T cell system.
T cells modified to express the universal chimeric antigen receptor (UniCAR) remain inactive until redirected by the target module (TM). The TM is composed of a tumour marker recognition domain (for CD123 in this example) attached to an epitope of the nuclear protein La/SS-B. The UniCAR is composed of an extracellular La/SS-B recognition domain linked to intracellular activation machinery. Modified from (Loff et al., 2020).

It must be stressed once again that it is impossible to properly assess at this stage the clinical benefit of adoptive CAR T cells and other immunotherapies in AML, as clinical trials are still in the very early stages wherein the primary focus is determining dosage and toxicity in a limited number of patients. With this in consideration, in broad terms, initial clinical trials with immunotherapies have thus far produced less-than-ideal results. It should be noted however that these early observations are more or less on par with the current standard of care. As a point of comparison, complete remission with HSCT is achieved in 42% of patients with refractory AML, leading to long-term survival in only 10% to 20% of cases (Döhner et al., 2017). In addition, having any response at all against post-HSCT relapsed AML should not be disregarded, as current survival rates in these instances are particularly bleak. The potency of immunotherapies so far parallels initial investigations with mutation-specific targeted therapies such as FLT-3 inhibitors, which would later find a role as part of a combinatorial approach to treatment. Though this would need to be investigated, it is possible that immunotherapies would provide the most benefit if used as a bridge between induction and consolidation therapies (Budde et al., 2017; Buechner et al., 2022), however whether treatment with multiple costly cell therapeutics would be realistically feasible is another question. It is thus paramount to explore the mechanisms by which tumours escape cytotoxic T cell activity.

1.3 Immune evasion and the immunosuppressive AML bone marrow niche

1.3.1 Intrinsic mechanisms of immune escape by AML

Escape from immunity and active suppression of the adaptive immune response are necessary steps in cancer development. The intrinsic strategies with which AML blasts escape T cell immunity can broadly be grouped into two categories: concealment from T cell recognition and expression of T cell mediators. T cell recognition normally functions through antigen presentation mediated by major histocompatibility (MHC) molecules on the surface of target cells. AML blasts have been shown to downregulate these molecules, as noted in cases of post-HSCT relapse (Jan et al., 2019). MHC-independent immunotherapies such as bispecific antibodies and CAR T cells effectively circumvent this by directly targeting cell surface markers. However, AML blasts are also able to express immune checkpoint ligands, such as programmed death ligand 1 (PD-L1) (Berthon et al., 2010), galectin-9 (Gonçalves Silva et al., 2016) and CD80/CD86 (Costello et al., 1998), denoting the importance of ICBs for effective immunotherapeutic interventions. Furthermore, AML blasts have been shown capable of secreting microvesicles and soluble factors that inhibit T cell activation and proliferation, including transforming growth factor beta (TGFβ) (Szczepanski et al., 2011) and kynurenine mediated by the enzyme indoleamine 2,3-dioxygenase 1 (IDO-1) (Folgiero et al., 2014).

1.3.2 Role of the leukemic microenvironment

In addition to mechanisms intrinsic to the leukemic blasts themselves, other neighbouring constituents of the bone marrow provide additional immune protection (Shafat et al., 2017; Sendker et al., 2021). Indeed, upon transformation, leukemic blasts engage in reciprocal interactions with local stromal, myeloid and lymphoid cells, deviating support from normal HSCs and ultimately providing protection from chemotherapeutic agents and immunity (**Figure 2**). Immunosuppressive regulatory T cells (Tregs) have been found to be particularly elevated in the peripheral blood and bone marrow of AML patients, and have been associated with poor outcome (Shenghui et al., 2011). Leukemic blasts can polarize effector T cells to acquire the Treg phenotype through the action of IDO-1 (Curti et al., 2007) and TGFβ (Chen et al., 2003). Mussai *et al*. demonstrated that AML blasts secrete high amounts of arginase 2, through which they can promote the immunosuppressive M2 macrophage phenotype (Mussai et al., 2013). M2 macrophages were also found to be enriched in the bone marrow of AML patients (Al-Matary et al., 2016). Similarly, Pyzer *et al*. characterized the AML bone marrow as being elevated for myeloid-derived suppressor cells (MDSCs). They further showed that engulfment of AML-excreted extracellular vesicles by myeloid precursors led to the acquisition of this regulatory phenotype (Pyzer et al., 2017).

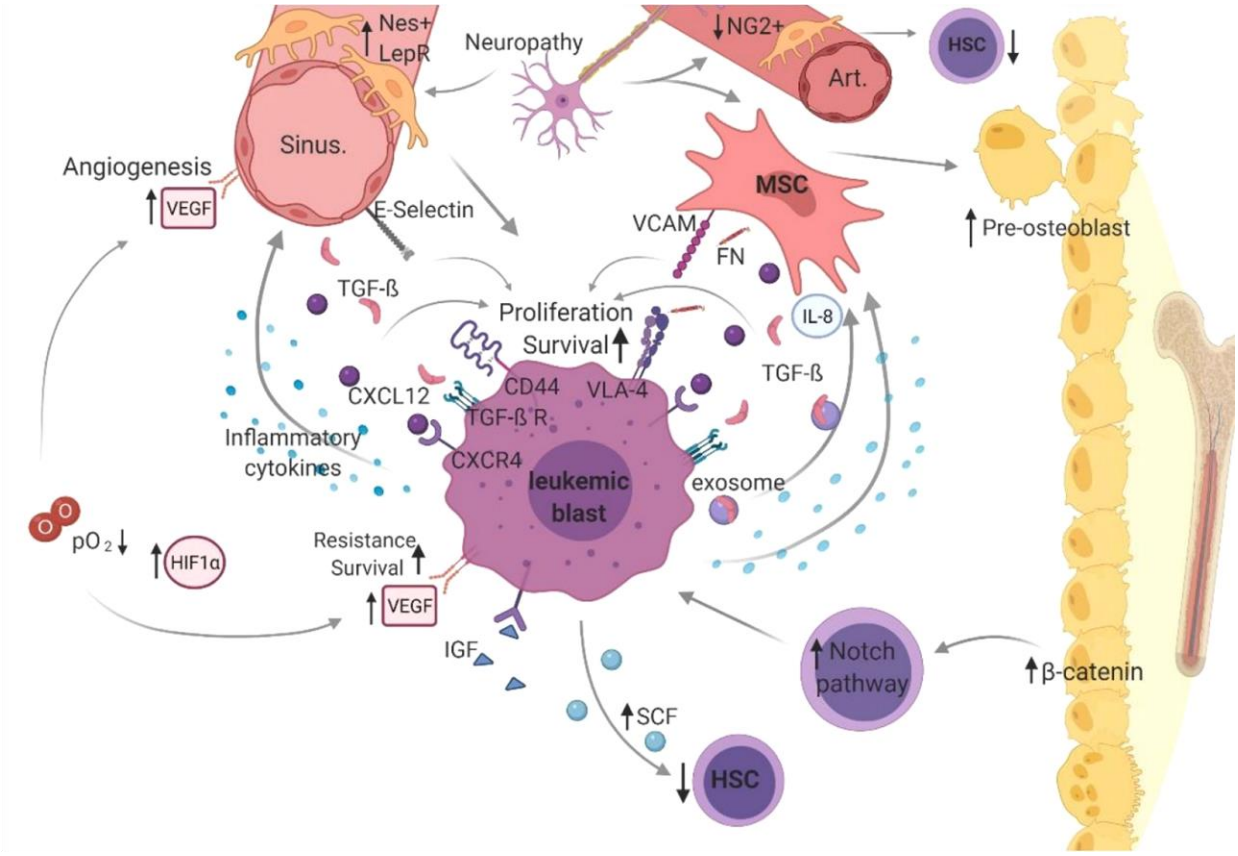


Figure 2. The leukemic bone marrow microenvironment.
A schematic representation of the molecular crosstalk between leukemic blasts and other resident bone marrow cells that mediate treatment resistance, survival and proliferation, while simultaneously decreasing support for normal hematopoietic stem cells (HSCs). Adapted from (Sendker et al., 2021).

1.3.3 Mesenchymal stromal cells

In addition to the recruitment of regulatory hematopoietic cells to the leukemic niche, resident bone marrow stromal cells are also proficient mediators of immunity. A lot of interest was garnered for these multipotent cells in the early 2000s when the first reports were published of their impressive immunomodulatory potential (Bartholomew et al., 2002; Di Nicola et al., 2002). However, it was noted soon after that mounting inconsistencies appearing in the field could be due to a lack of established characterization of the cells. In 2006, the International Society for Cellular Therapy (ISCT) proposed a minimal set of criteria for the definition of what are now called mesenchymal stromal cells (MSCs): plastic-adherent cells that must possess the capacity to differentiate down osteoblastic, adipocytic and chondrocytic lineages. In addition, they must fulfill a defined immunophenotypic classification to distinguish them from hematopoietic cells (Dominici et al., 2006). This definition standardized subsequent research, though "MSC" is still an umbrella term for a heterogeneous group of stem and progenitor cells. Spatial studies of the murine and human bone marrow have identified further subgroups within MSCs, differing in location, transcriptomic profile and phenotype. These include adipogenic Cxcl12-abundent reticular cells and leptin receptor$^+$ MSCs of the sinusoidal niche, and osteogenic reticular cells and nestin$^+$ MSCs of the arteriolar niche (Tikhonova et al., 2019; Baccin et al., 2020; Méndez-Ferrer

et al., 2020). MSCs have also been identified in other tissues, such as adipose tissue and the umbilical cord, conserving many characteristics of bone marrow MSCs, including anti-inflammatory capabilities (Gonzalez-Rey et al., 2009; Deuse et al., 2011).

Di Nicola *et al*. were the first to characterize primary human MSCs of the bone marrow as potent immunoregulators when they noted a drastic reduction in the proliferative capacity of stimulated peripheral blood mononuclear cells (PBMCs) when co-cultured with MSCs (Di Nicola et al., 2002). Furthermore, this effect was at least partially independent of cell-cell contact and could be reversed with neutralization of the cytokines TGFβ, interleukin (IL)-6, IL-11 and hepatocyte growth factor (HGF). Since then, a plethora of other immunosuppressive molecules have been identified. These include other soluble factors such as kynurenine (Meisel et al., 2004), prostaglandin E2 (PGE2) (Aggarwal and Pittenger, 2005), galectin-3 (Sioud et al., 2010), heme oxygenase (HO) (Mougiakakos et al., 2011) and tumor necrosis factor-stimulated gene 6 (TSG-6) (Lee et al., 2009), as well as the immune checkpoint ligands PD-L1, PD-L2 (Augello et al., 2005; Davies et al., 2017), B7 homolog 3 (B7H3) (Chinnadurai et al., 2021), CD155 (Najar et al., 2018) and galectin-9 (Ungerer et al., 2014). In addition to anti-proliferative effects, Krampera *et al*. demonstrated that MSCs were also potent suppressors of inflammation, using intracellular staining to show a marked decrease in interferon gamma (IFNγ)-expressing $CD8^+$ T cells in MSC co-cultures (Krampera et al., 2003). Laranjeira *et al*. would later show that MSCs also decreased the expression of other pro-inflammatory cytokines, including tumour necrosis factor alpha (TNFα) and IL-2 in both $CD4^+$ and $CD8^+$ T cells, and moreover that this was largely independent of lymphocyte maturation stage (Laranjeira et al., 2015). In addition, MSCs were shown to be able to regulate the activity of other adaptive immune cells, efficiently suppressing the proliferation and humoral activity of B-cells, as well as promoting the regulatory phenotypes of dendritic cells and macrophages (Müller et al., 2021) (**Figure 3**). MSCs can also promote and recruit Tregs and MDSCs to the leukemic niche, similar to blasts (Pleyer et al., 2016).

One interesting property of MSCs is that in a resting state, most of the above-mentioned immunoinhibiting molecules are only expressed at low levels, if at all (Ryan et al., 2007; Li et al., 2014; Davies et al., 2017). Instead, MSCs are polarized towards an immunosuppressive phenotype by inflammatory stimuli, in a process often referred to as "licensing". This was first demonstrated with murine MSCs by Ren *et al*., wherein neutralization of IFNγ signaling was largely sufficient to reverse MSC-mediated suppression of T cell proliferation, though effective licensing required other cytokines such as TNFα and IL-1β in combination with IFNγ (Ren et al., 2008). In addition to immunomodulating molecules, MSCs in a licensed state also upregulate the expression of lymphocyte-attracting chemokines such as chemokine C-X-C ligand 9 (Cxcl9) and Cxcl10, among many others (Ren et al., 2008), as well as the cell surface lymphocyte adhesion molecules intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1) and lymphocyte function-associated antigen 3 (LFA-3) (Najar et al., 2010; Ren et al., 2010).

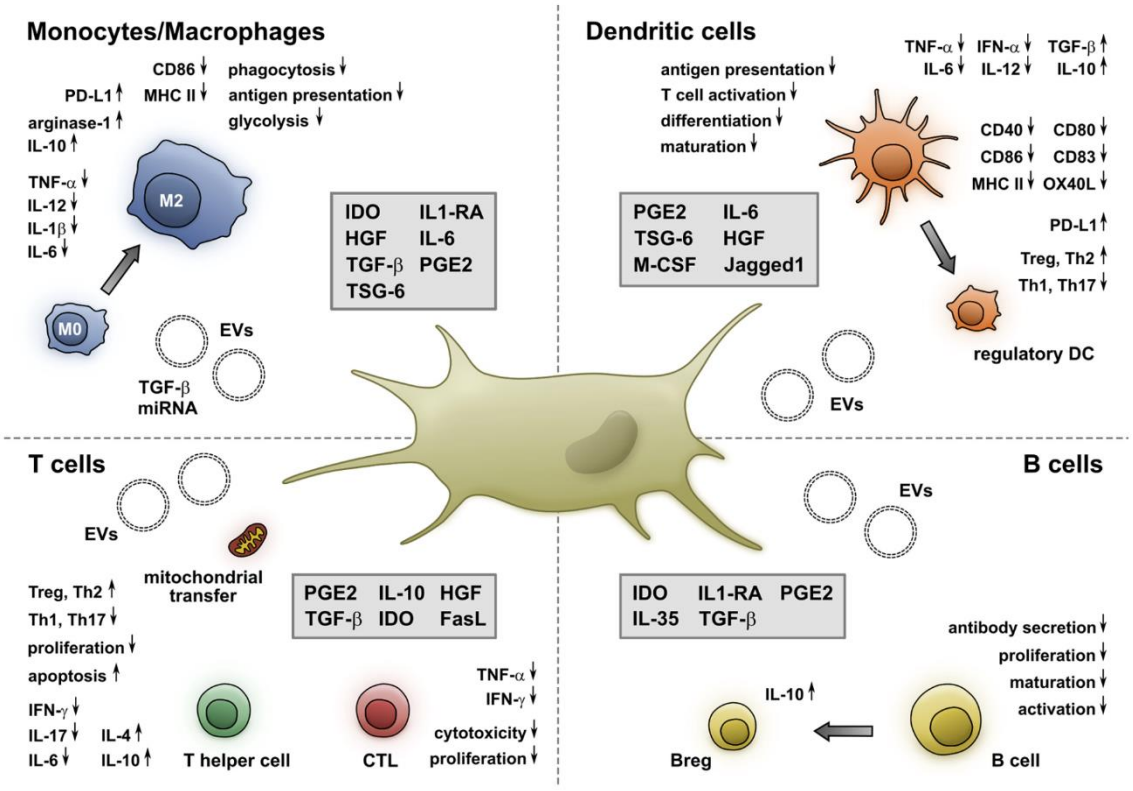


Figure 3. Suppression of adaptive immunity by mesenchymal stromal cells.
Mesenchymal stromal cells express soluble and cell-surface factors that promote regulatory phenotypes and suppress effector functions of monocytes, dendritic cells, T cells and B cells. Adapted from (Müller et al., 2021).

It follows to ask if MSCs can interfere with the action of T cells and immunotherapies in the context of hematological malignancy. At the time of writing, very few reports have come out addressing this topic, and the results are somewhat paradoxical. Ramasamy *et al*. were the first to consider this question in 2008, when they investigated whether MSCs could ablate the cytotoxic potential of T cells specific for the AML antigen WT1, of which they found no effect (Ramasamy et al., 2008). Over a decade later, Zanetti *et al*. published a report on the potential interference of MSCs against immunotherapies, specifically CD19 CAR T cells directed against blasts isolated from pediatric B-cell acute lymphocytic leukemia patients. They demonstrated *in vivo* that MSC infusion did not affect CAR T cell activity in xenografts despite being able to efficiently mediate inflammation in a murine model of acute colitis (Zanetti et al., 2020). Holthof *et al*. have recently investigated the effect of MSCs on different CAR designs in the context of multiple myeloma, and in contrast to previous reports demonstrated that MSCs could in fact decrease cell lysis mediated by several of the constructs (Holthof et al., 2021). To reconcile these contradictory reports, it is apparent that more research in the field is needed. Furthermore, with exception of the work by Ramasamy *et al*., MSC-interference of AML-targeting T cells is woefully lacking, which we wish to address with this thesis.

1.4 Rationale

To summarize, treatment options for AML are currently insufficient, manifested by the high incidence of relapse and death due to rare persistent leukemic subpopulations. The elimination of these resistant cells is highly desirable and target-specific immunotherapy is an attractive approach to this end. However, disappointing preliminary data from ongoing clinical trials suggest that other factors need to be addressed for these therapies to achieve their full potential. The AML microenvironment and MSCs in particular are likely contributors to leukemic escape from immunity. The immunosuppressive capacity of MSCs on general T cell populations has been well established over the last couple of decades, but their ability to suppress anti-leukemic immunotherapies such as CAR T cells is currently understudied. At the outset of this project, we wished to address the following main questions:

1) Can bone marrow-derived MSCs interfere with the proliferative, inflammatory and cytotoxic activities of AML-targeting T cells?

2) If so, through which mechanisms do MSCs mediate this effect and could their inhibition restore anti-leukemic T cell activity, with a particular focus on actionable targets for clinical translation?

2 Materials and Methods

Isolation, expansion and characterization mesenchymal stromal cells

Bone marrow aspirates were procured from healthy (ethical approval no. EK307082018) and acute myeloid leukemia (AML) patient donors (ethical approval no. EK98032010) after obtaining written consent. Mononuclear cells were isolated by density gradient centrifugation using Percoll (Sigma-Aldrich, USA), washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, USA) and resuspended in low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA). Cells were expanded at 37°C/5%CO_2 (passage 0) and non-adherent cells were removed with DPBS after 2 days. Once a confluency of 80% had been reached, cells were detached with 0.25% Trypsin-EDTA solution (Thermo Fisher, USA) and replated up until passage 4.

Mesenchymal stromal cells (MSCs) at passage 1 were characterized by technical expert Katrin Müller based on the criteria set out by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). Briefly, adherent cells were demonstrated by flow cytometry to be positive for the CD73, CD90, CD105, CD44, CD146 and CD166, and negative for the hematopoietic markers CD11b, CD14, CD34 and CD45. Osteogeneic potential was established externally by alkaline phosphatase colorimetric assay (Trivedi et al., 2020).

Isolation and culture of peripheral blood mononuclear cells

Blood was donated by healthy volunteers after obtaining written consent (ethical approval no. EK206082008). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Pancoll (PAN-Biotech, Germany), washed twice with DPBS, and stored at -80°C in Roswell Park Memorial Institute 1640 (RPMI) medium (Gibco, USA) supplemented with 10% FBS and 10% dimethyl sulfoxide (WAK-Chemie Medical, Germany). Aliquots were thawed when needed on the day of the assay.

Generation of $CD8^+$ cytotoxic T lymphocyte clones

High avidity WT1- and ROR1-specific $CD8^+$ cytotoxic T lymphocyte (CTL) clones were generously provided by the laboratory of Marc Schmitz (University Hospital Carl Gustav Carus Dresden). They were generated and expanded as described elsewhere (Tunger et al., 2017). In brief, $CD8^+$ T cells and $CD14^+$ monocytes were isolated from the PBMCs of a healthy HLA-A*02:01 or HLA-B*07:02 donor. The $CD8^+$ T cells underwent stimulation with monocyte-derived dendritic cells pulsed with HLA-A*02:01-restricted $WT1_{126}$ nonamer (RMFPNAPYL) or HLA-B*07:02-restricted $ROR1_{783}$ nonamer (NPRYPNYMF), before being isolated, clonally expanded and finally validated for high purity and avidity.

Generation of UniCAR T cells and target modules

Retargetable universal chimeric antigen receptor (UniCAR) T cells were graciously provided by the laboratory of Anja Feldmann and Michael Bachmann (Helmholtz-Zentrum Dresden-Rossendorf). As previously described (Cartellieri et al., 2016), the lentiviral UniCAR vector was constructed with a La/SS-B epitope recognition domain linked to a CD3ζ/CD28 signaling domain, and fused to an enhanced green fluorescent protein (eGFP) open reading frame. $CD4^+$ and $CD8^+$ T cells were freshly isolated from healthy donor PBMCs and transduced the week preceding their use.

Target modules (TMs) were constructed with a CD33 or CD123-recognition single chain variable fragment (scFv) fused to the La/SS-B epitope followed by a His tag. Lentiviral vectors were transduced into 3T3 cells and selected for stable TM expression. The TM is purified from the supernatant using Ni-NTA affinity chromatography columns, dialyzed and finally assessed for purity and concentration with SDS–polyacrylamide gel electrophoresis.

Mixed lymphocyte reaction – conditioned medium preparation

Mixed lymphocyte reaction – conditioned medium (MLR-CM) was prepared as previously described (Faßlrinner et al., 2012). PBMCs from 2 allogeneic donors were co-cultured in RPMI supplemented with 10% FBS at 1×10^6 cells/mL for each donor. After 6 days, the medium was collected by centrifugation and diluted 1:1 with fresh RPMI supplemented with 20% FBS.

Target cell culture

T2, K562, MV4-11 and MOLM-13 cells were maintained in RPMI supplemented with 10% FBS at 37°C/5%CO_2. AML blasts were isolated from the bone marrow of patients (ethical approval no. EK98032010) and delivered frozen. Frozen AML blasts were thawed in RPMI supplemented with 5% FBS, 20U/mL Heparin and 8U/mL DNAse I (Sigma-Aldrich, USA), and maintained in StemSpan medium (Stemcell Technologies, Canada) supplemented with 2% FBS, 1% penicillin/streptomycin, 1% glutamine, 10ng/mL FLT3-L, 10ng/mL SCF, 10ng/mL TPO and 10ng/mL IL-3 (ImmunoTools, Germany) at 37°C/5%CO_2.

PBMC co-culture

MSCs were plated the day before the assay in RPMI supplemented with 10% FBS. PBMCs were added at a PBMC:MSC ratio of 5:1 or 100:1 and were stimulated with anti-CD3/CD28 Dynabeads (Gibco, USA) for 6 days at 37°C/5%CO_2. For non-contact cultures, MSCs were plated in the basolateral chamber of the Transwell Permeable Support system (Corning, USA), and PBMCs and beads in the apical chamber, separated by 0.4µm membranes. For proliferation assays, MSCs were first irradiated to induce senescence (30 Gy) and ^{3}H-thymidine (Hartmann Analytic, Germany) was added at 0.2 µCi/mL after 5 days, after which cultures were further incubated for

an additional 18 hours. Cells were harvested and lysed onto filtermats with the FilterMate Harvester, which were then dried at 70ºC for 1 hour and overlaid with MeltiLex scintillation plates (PerkinElmer, USA). Proliferation was assessed as a measurement of ^{3}H-thymidine incorporation into DNA as determined with the MicroBeta 2 (PerkinElmer, USA), converting radioactive β particles into counts per minute.

CTL clone co-culture

MSCs were plated the day before the assay in RPMI supplemented with 10% FBS. On the day of the assay, T2 target cells were pulsed with HLA-restricted peptides in RPMI supplemented with 20 µg/mL $WT1_{126}$ nonamer or HIV $Gag\text{-}Pol_{896}$ nonamer (ILKEPVHGL), and K562 with 20µg/mL $ROR1_{783}$ nonamer or HIV $Gag\text{-}Pol_{355}$ nonamer (GPGHKARVL). The cells were then incubated at 37°C/5%CO2 for 3 hours. For cytotoxicity assays, ^{51}Cr (Hartmann Analytic, Germany) was added at 100µCi/mL after 2 hours of incubation. These cells were collected, washed thrice with DPBS and resuspended in RPMI supplemented with 10% FBS. T2 cells with WT1-reactive CTLs, and K562 cells with ROR1-reactive CTLs, were co-cultured with MSCs at an E:T:MSC ratio of 10:1:2 in RPMI supplemented with 10% FBS. Spontaneous ^{51}Cr release cultures were prepared with target cells cultured alone, and maximal ^{51}Cr release cultures were prepared with target cells lysed with the addition 10% Triton X-100 (Serva Electrophoresis, Germany). For ^{51}Cr release determination, cultures were centrifuged after 4 hours and 25µL of supernatant was collected and mixed with 150µL of Ultima Gold scintillation cocktail (PerkinElmer, USA). Cytotoxicity was established as a measurement of ^{51}Cr release assessed with the MicroBeta 2 (PerkinElmer, USA), converting radioactive γ-particles into counts per minute. For cytokine analysis, supernatants were collected after 4 hours or 24 hours of incubation.

UniCAR T cell co-culture

MSCs were plated the day before the assay in RPMI supplemented in 10% FBS. For MSC apoptosis determination, MSCs were first labelled with CellTrace Violet (Thermo Fisher, USA): MSCs were collected and resuspended in DPBS supplemented with 2.5 µM CellTrace Violet for 10 minutes at 37°C, after which 1 volume of FBS was added. MSCs were then washed and plated in medium. If needed for cytotoxicity determination, target MOLM-13 or AML blasts were labelled as follows: cells were collected, washed with DPBS, resuspended at a maximum concentration of $1x10^{7}$ cells/mL in DPBS supplemented with 5µM fluorescent eFluor 670 dye (Thermo Fisher, USA) and incubated in the dark at 37°C. After 10 minutes, the reaction was terminated with cold RPMI/Complete (RPMI supplemented with 10% FBS, 100 µg/ml penicillin/streptomycin, 1% nonessential amino acids, 2 mM N-acetyl-l-alanyl-l-glutamine, and 1 mM sodium pyruvate (Biochrom, UK)). After 5 minutes of incubation on ice, the cells were washed twice with DPBS and incubated overnight in RPMI/Complete at 37°C/5%CO_2. On the day of the assay, target cells and UniCAR T cells were co-cultured with MSCs at a E:T:MSC ratio of 16:16:3 or 20:20:3 in RPMI/Complete supplemented with 0.5nM TM and incubated in 37°C/5%CO_2. Cultures without TM served as a negative control. For flow cytometry-based assays,

a sample of each culture was collected at the appropriate time point and diluted in DPBS supplemented with 1 µg/mL propidium iodide (PI) (Sigma-Aldrich, USA) and surviving UniCAR T cells (PI^-eGFP^+) and target cells ($PI^-eFluor670^+$) were quantified with the MACSQuant X (Miltenyi Biotec, Germany). Additional supernatant was collected for cytokine analysis. Equal volume of fresh RPMI/Complete with 0.5nM TM was added back to the cultures after each collection. Cultures were restimulated twice with target cells on day 2 and day 4 or 5. For live fluorescent imaging assays, cultures were supplemented with 40ng/mL DAPI (Sigma-Aldrich, USA) and analyzed with the Celigo Image Cytometer (Nexcelom Bioscience, USA). For non-contact cultures, the MSCs were plated in the basolateral chamber of Transwell Permeable Support wells with 0.4µm polycarbonate membranes (Corning, USA), and UniCAR T cells and target cells were plated in the apical chamber.

Cytokine assessment

Cytokines within cell culture supernatant were assessed with commercially available kits following the manufacturer's protocols. Human IFNγ ELISA Set and Human IL-2 ELISA Set (BD, USA) were measured on the Sunrise spectrophotometer and analyzed with the accompanying Magellan software (Tecan, Switzerland). LEGENDplex Human CD8/NK Panel (Biolegend, USA) was measured on the MACSQuant X (Miltenyi Biotec, Germany) and analyzed with Legendplex software (Biolegend, USA).

Cell surface molecule expression and viability assays

Cells were collected and resuspended in DPBS supplemented with 5% FBS and fluorochrome-labelled antibodies (**Table 1**) at concentrations following the manufacturer's guidelines, and incubated on ice for 20 minutes in the dark. Samples were then washed thrice with DPBS and resuspended in DPBS/5% FBS supplemented 40ng/mL DAPI. MSC viability was determined with the commercially available PE Annexin V Apoptosis Detection Kit (BD, USA). Acquisition was performed on the LSR II or the LSRFortessa flow cytometers (BD, USA) and analysis with FlowJo software (BD, USA).

Target module binding assay

Cells were collected, washed and resuspended in DPBS supplemented with 2% FBS and 10ng/µL TM for 1 hour at 4°C. Samples were then washed and incubated in DPBS/2%FBS supplemented with 5µg/mL mouse anti-La/SS-B epitope (Feldmann lab) for 1 hour at 4°C. Cells were washed again and incubated a final time with DPBS/2%FBS supplemented with 2% PE-conjugated goat anti-mouse IgG (Biolegend, USA) for 30 minutes at 4°C. Cells were washed, resuspended in DPBS/2%FBS supplemented with 7-AAD (BD, USA) and assessed by flow cytometry on the LSR II.

RNA isolation

MSCs were lysed with 1mL TRIzol reagent (Thermo Fisher, USA). Lysates were collected, mixed with 200µL of chloroform and incubated for 3 minutes. After centrifugation, the upper aqueous layer was recovered and supplemented with 10µg of glycogen. RNA was precipitated by adding 500µL isopropanol and incubating for 10 minutes. After centrifugation, RNA pellets were washed with 75% ethanol, air dried and resuspended in ultrapure DNAase/RNAase-free water. Concentration and quality control was determined by Nanodrop 2000 (Thermo Fisher, USA).

cDNA library preparation and quantitative polymerase chain reaction

Approximately 200ng of RNA were used for cDNA library preparation with the commercially available RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Validation was done by polymerase chain reaction (PCR) for the housekeeping gene GAPDH with DreamTaq PCR Master Mix (Thermo Scientific, USA) on the MyCycler thermal cycler (Bio-Rad Laboratories, USA). PCR samples underwent agarose gel electrophoresis loaded with Redsafe (iNtRON Biotechnology, South Korea) and visualized by the G:Box gel imaging system (Syngene, India). Quantitative PCR (qPCR) was performed with the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) on the QuantStudio 3 (Applied Biosystems, USA). Primers are listed in **Table 2.**

Whole transcriptomic sequencing and analysis

Quality control, library preparation and whole transcriptomic sequencing was performed at the Deep Sequencing facility at the Center for Molecular and Cellular Bioengineering (Dresden, Germany). The initial analysis was performed by Katrin Sameith, wherein alignment and sequencing quality were assessed using RNA-SeQC (Deluca et al., 2012), and differential gene analysis was generated using DESeq2 (Love et al., 2014). Further analysis was performed by Anupam Sinha. Reads were first subject to the Illumina FASTQ filter and Cutadapt (Martin, 2011) to remove low quality nucleotides and adaptor sequences, respectfully. Remaining reads were aligned to the Human Genome version GRCh38 using STAR Aligner. Aligned reads were counted using HTSeq-0.6.1p1 (Anders et al., 2015). Differential genes were identified with DESeq2_1.8.1 (Anders and Huber, 2010), and the resulting ranked list was evaluated by Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) and Enhanced Gene Set Enrichment Analysis (EGSEA) (Alhamdoosh et al., 2017), which query the Molecular Signatures Database (MSigDB).

Statistical Analyses

Standard deviation (SD) and analysis of variance (ANOVA) were calculated with Graphpad Prism. p values ≤ 0.05 were considered statistically significant and were further stratified as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

Table 1. Flow cytometry antibodies.

Antigen	Fluorophore	Manufacturer
4-1BBL	BV510	BD
B7H3	BV605	BD
CCR7	BV421	BD
CD105	APC	Miltenyi
CD123	BV421	BD
CD123	PE-Cy7	Biolegend
CD155	PE-Cy7	Biolegend
CD28	PE-Cy7	BD
CD28	APC	eBiosciences
CD33	APC	eBioscience
CD4	APC	eBioscience
CD4	V500	BD
CD45	V500	BD
CD45	AF700	BioLegend
CD45RA	FITC	eBioscience
CD45RA	PE-Cy7	eBioscience
CD45RO	PerCP-eFl710	eBioscience
CD57	PE	Biolegend
CD80	PerCP-eF710	eBioscience
CD86	BV421	Biolegend
CD8a	APC-eFl780	eBioscience
CD90	FITC	Miltenyi
CD90	PerCP-Cy5.5	eBioscience
Galectin 9	BV421	Biolegend
GITRL	PE	R&D Systems
HLADR	APC	Miltenyi
HVEM	PE-Cy7	Biolegend
ICAM-1	APC/Fire 750	Biolegend
ICOSL	BV605	BD
LFA-3	BV421	BD
mouse IgG	PE	Biolegend
PD-1	BV421	BioLegend
PD-1	BV510	Biolegend
PD-L1	AF700	BD
PD-L1	APC	eBioscience
PD-L2	BV421	BD
PD-L2	PE	eBioscience
VCAM-1	PE-Cy7	eBioscience
VISTA	PE	R&D Systems

Table 2. Primers.

Gene	Primer Sequence
GAPDH fwd*	GCAGGGGGGAGCCAAAAGGG
GAPDH rev*	TGCCAGCCCCAGCGTCAAAG
GAPDH fwd	GAAGGTGAAGGTCGGAGTC
GAPDH rev	GAAGATGGTGATGGGATTTC
PTGS2 fwd	TGAGCATCTACGGTTTGCTG
PTGS2 rev	TGCTTGTCTGGAACAACTGC
IDO1 fwd	GCCAGCTTCGAGAAAGAGTTG
IDO1 rev	TGACTTGTGGTCTGTGAGATGA
TGFB1 fwd	GTACCTGAACCCGTGTTGCT
TGFB1 rev	GTATCGCCAGGAATTGTTGC
HGF fwd	CCCTGTAGCCTTCTCCTTGA
HGF rev	CGCTGGGAGTACTGTGCAAT
TNFAIP6 fwd	AAGCACGGTCTGGCAAATACAAGC
TNFAIP6 rev	ATCCATCCAGCAGCACAGACATGA
HMOX1 fwd	CTTCTTCACCTTCCCCAACA
HMOX1 rev	AGCTCCTGCAACTCCTCAAA
IL10 fwd	GTGATGCCCCAAGCTGAGA
IL10 rev	ACGGCCTTGCTCTTGTTTT
CD274 fwd	GGCATCCAAGATACAAACTCAA
CD274 rev	CAGAAGTTCCAATGCTGGATTA
PDCD1LG2 fwd	GAGCTGTGGCAAGTCCTCAT
PDCD1LG2 rev	GCAATTCCAGGCTCAACATTA
PDCD1 fwd	CGTGGCCTATCCACTCCTCA
PDCD1 rev	ATCCCTTGTCCCAGCCACTC
ICAM1 fwd	ATGCCCAGACATCTGTGTCC
ICAM1 rev	GGGGTCTCTATGCCCAACAA
VCAM1 fwd	GGGAAGATGGTCGTGATCCTT
VCAM1 rev	TCTGGGGTGGTCTCGATTTTA

* Conventional PCR

3 Results

3.1 Selection of immunomodulating bone marrow MSCs from healthy donors.

Stammzelllabor II (University Hospital Carl Gustav Carus Dresden) manages an expansive bank of frozen primary bone-marrow derived MSCs originating from hundreds of healthy donors. Six donors were initially selected based on sufficiency of material and then further studied for immunomodulating capabilities. The most robust display of the immunosuppressive potential of MSCs has so far been in the interference of T cell proliferation (Di Nicola et al., 2002). However, donor-derived MSCs display a wide range of inhibitory potency in this regard (Von Dalowski et al., 2016) and thus further assessment of the six selected donors was required. The MSCs were co-cultured with allogeneic PBMCs from two healthy donors and the latter were stimulated to proliferate via anti-CD3/CD28 antibody-coated beads. After 5 days, ^{3}H-radiolabelled thymidine was added and the cultures were incubated for an additional 18h before proliferation was assessed as a measure of ^{3}H-thymidine incorporation. The MSCs were irradiated at 30 Gy prior to culturing to inhibit proliferation. Monocultures of irradiated MSCs and unstimulated PBMCs were also prepared to assess background incorporation of ^{3}H-thymidine. **Figure 4** demonstrates that all six donors significantly decreased T cell proliferation, ranging from a 58% decrease (MSC5 against

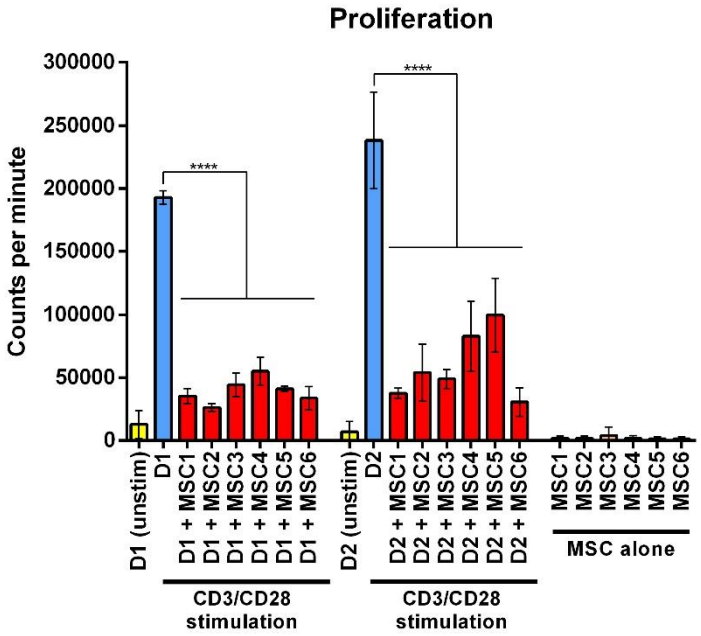


Figure 4. MSCs inhibit unmodified T cell proliferation.
PBMCs from two healthy donors (D1 and D2) were cultured with or without 5x103 irradiated (30 Gy) allogeneic MSCs from six healthy donors (MSC1-6) at a PBMC:MSC ratio of 100:1 and stimulated with anti-CD3/CD28 antibody-coated beads. Cultures of unstimulated PBMCs and MSC monocultures were also prepared. After 5 days, 3H-radiolabelled thymidine was added and the cultures were incubated for an additional 18h. Cells were harvested and 3H-thymidine incorporation was measured via β-counter, with a readout given in Counts per minute. Data are presented as the mean of technical triplicates ± SD. Asterisks represent statistically significant differences (**** $p \leq 0.0001$).

D2) up to 86% (MSC2 against D1) when compared to the positive controls. All six donors were selected for further experiments.

Next, we evaluated the immunomodulatory potential of MSCs on the cytotoxic capabilities of leukemia-reactive $CD8^+$ T cell clones.

3.2 MSC-mediated interference of anti-leukemic clonal $CD8^+$ T cell activity.

In collaboration with the laboratory of Marc Schmitz (University Hospital Carl Gustav Carus Dresden), high-avidity clonal $CD8^+$ cytotoxic T lymphocytes reactive to the AML-associated antigen WT1 (Tunger et al., 2017) and clones reactive to the chronic lymphocytic leukemia (CLL)-associated antigen ROR1 were obtained to investigate the inhibitory potential of bone marrow-derived MSCs against anti-leukemic T cell cytotoxicity (**Figure 5**). HLA-matched target cells (T2 for the HLA-A*02:01 WT1-targeting clone and K562 for the HLA-B*07:02 ROR1-targeting clone) were pulsed with HLA-restricted nonamers $WT1_{126}$ (RMFPNAPYL) or $ROR1_{783}$ (NPRYPNYMF), to be presented on their cell surface conjugated to MHC class 1. The T2 and K562 cells were loaded with ^{51}Cr and co-cultured with the CTL clones and MSCs. Cytotoxicity was reported as a measure of ^{51}Cr release after 4h and is expressed as a percentage of maximum lysis corrected for spontaneous ^{51}Cr release. Irrelevant HIV-derived peptides (Gag-Pol_{896} ILKEPVHGL for T2 and Gag-Pol_{355} GPGHKARVL for ROR1) served as a control for off-target lysis. **Figure 5A** shows that the presence of MSCs did not significantly affect the specific cytotoxic capabilities of the CTLs. Further investigation was conducted in alternative cell culture conditions including, but not limited to, alternate E:T:MSC ratios, a second round of CTL stimulation, pre-incubation of the CTLs with the MSCs before the assay, pre-licensing of the MSCs (see section 3.4), and adjustment of the baseline efficiency of the clones by reducing the concentration of pulsed nonamer. In all instances, MSCs had no discernable effect on the cytotoxicity of either clone (data not shown).

In addition to inhibition of T cell proliferation, MSCs are reported to decrease release of inflammatory cytokines such as IFNγ, TNFα and IL-2 (Laranjeira et al., 2015). As such, this was investigated in the context of anti-leukemic CTL activity. Despite having no discernable effect on cytotoxicity, IFNγ release was significantly decreased after 24h in the presence of all six MSC donors for both CTL clones (average 38.7% inhibition against the WT1-reactive clones and 50.2% against the ROR1-reactive clones) (**Figure 5B**). The decrease of IFNγ by ROR1-reactive CTLs could even be observed as early as 4h of co-culture (average 48.5% inhibition) (**Figure 5C**). Additional inflammatory cytokines (IL-2, IL-4 and TNFα, but not IL-17a) also decreased in the presence of MSCs (average inhibition of 61.1%, 41.2% and 48.8%, respectively). In contrast, when regarding cytotoxic effector molecules, MSCs did not have a significant effect on the concentrations of sFasL, Granzyme A or Perforin within these same cultures, corroborating the cytotoxicity data (**Figure 5D**). Of interest however, Granzyme B and Granulysin concentration noticeably decreased in the presence of all three MSC donors (average inhibition of 46.0% and 34.2% respectively), but perhaps not to the degree that the overall cytotoxic capability of the T cells would be affected in the tested conditions.

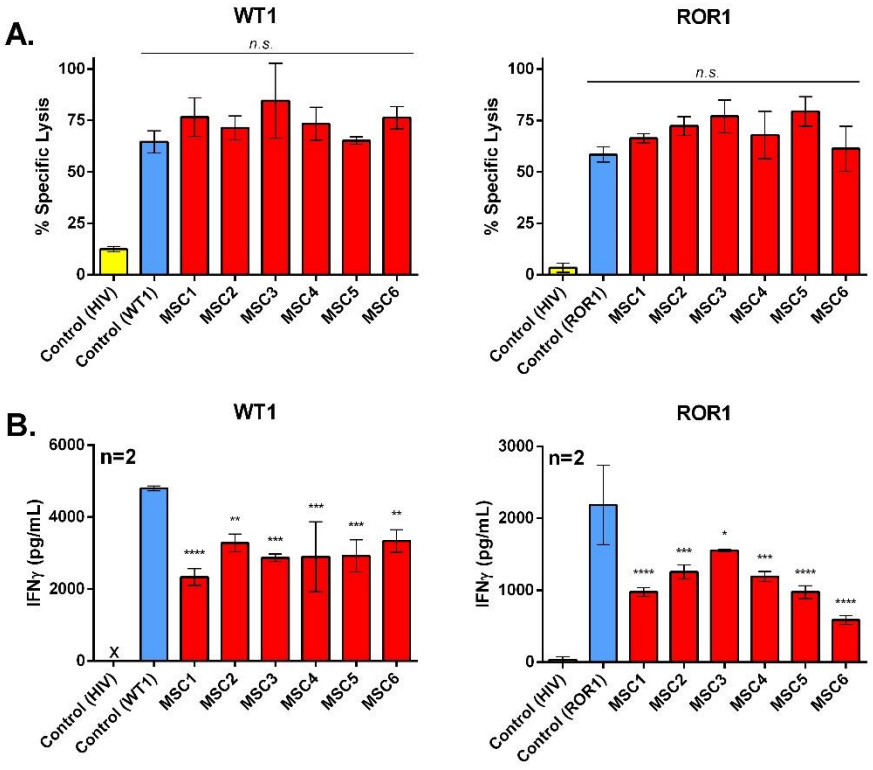


Figure 5. MSCs modulate the inflammatory capabilities of WT1- and ROR1-reactive CD8$^+$ T cell clones without affecting cytotoxicity.

A) Antigen-specific WT1 and ROR1-reactive CTL clones were respectively incubated with 51Cr-loaded target T2 cells pulsed with WT1 peptide or K562 cells pulsed with ROR1 peptide in the presence or absence of 1x104 allogeneic MSCs from six healthy donors (MSC1-6) at a E:T:MSC ratio of 10:1:2. Target cells pulsed with irrelevant HIV Gag-Pol peptides served as a negative control. Efficacy of CTL-mediated lysis of target cells was determined by 51Cr release after 4h as measured by β-counter and is expressed as a percentage of maximum lysis with correction for spontaneous release. Data are presented as the mean of triplicates ± SD. **B)** Cell cultures were prepared as in (A) without 51Cr-loading. After 24h, supernatants were collected and assessed for IFNγ concentration via ELISA. Data are presented as the mean of triplicates ± SD. Asterisks represent statistically significant differences compared to the control. **C and D)** Antigen-specific ROR1-reactive CD8+ CTL clones were incubated with target K562 cells pulsed with ROR1 peptide in the presence or absence of 2.5x104 allogeneic MSCs from 3 healthy donors (MSC1-3) at a E:T:MSC ratio of 2:10:1. Target cells pulsed with irrelevant HIV Gag-Pol peptides served as a negative control (Control-HIV). After 4h, co-culture supernatant was collected and analyzed for the indicated inflammatory cytokines (C) and cytotoxicity effector molecules (D) via ELISA (presented as mean concentration of technical triplicates ± SD). Asterisks represent statistically significant differences (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; n.s. not significant).

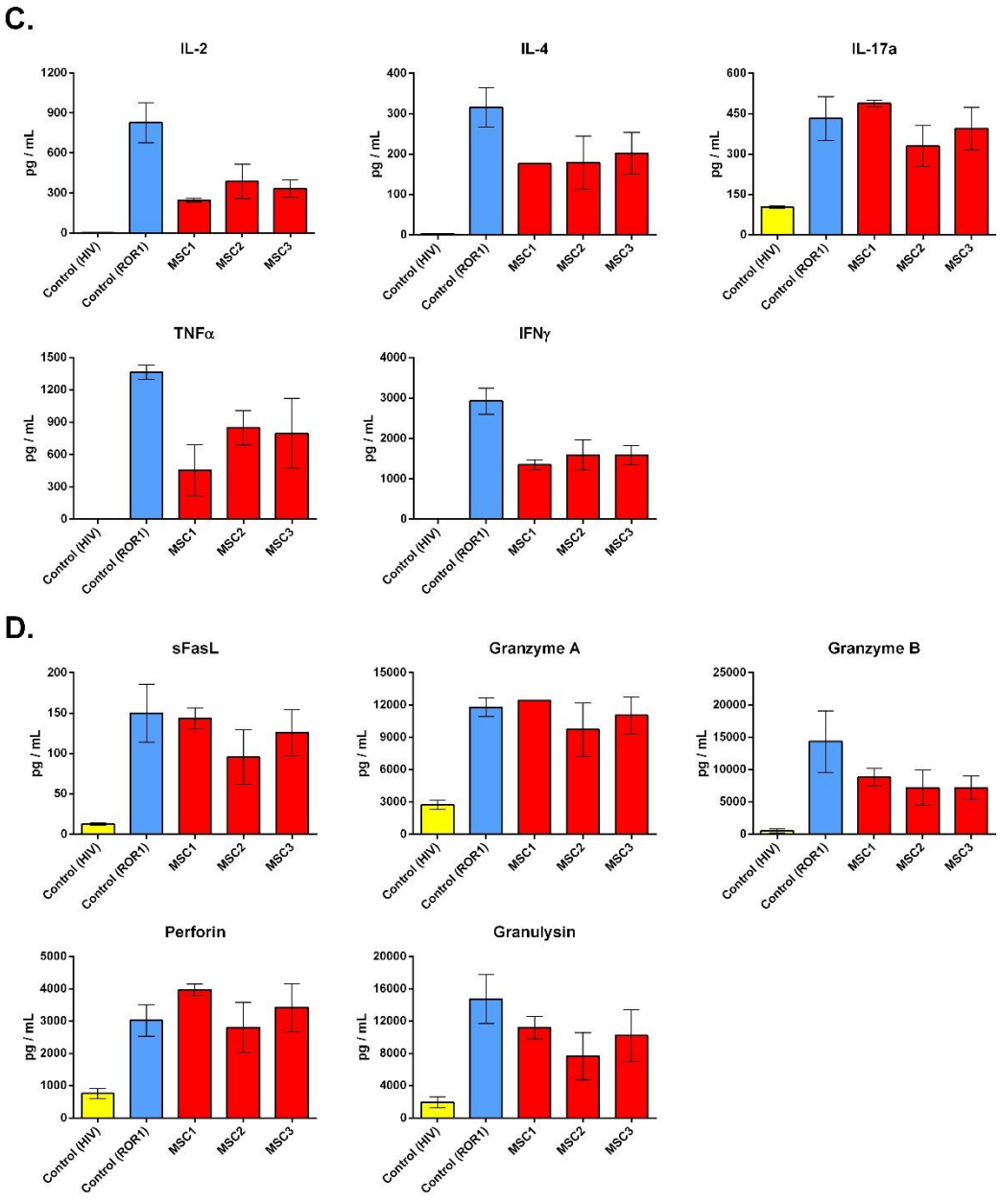


Figure 5 (continued). MSCs modulate the inflammatory capabilities of WT1- and ROR1-reactive CD8$^+$ T cell clones without affecting cytotoxicity.

CAR T cells have recently become an exciting new avenue in the ongoing development of cancer-targeting immunotherapies. However, it remains to be investigated whether the anti-leukemic activity of CAR T cells may be abrogated by the immunomodulatory function of bone marrow-resident MSCs in the context of AML. In the following section, we sought to address this point. Due to all six MSC donors examined so far having similar immunomodulating effects, three were chosen for further investigation (MSC1-3).

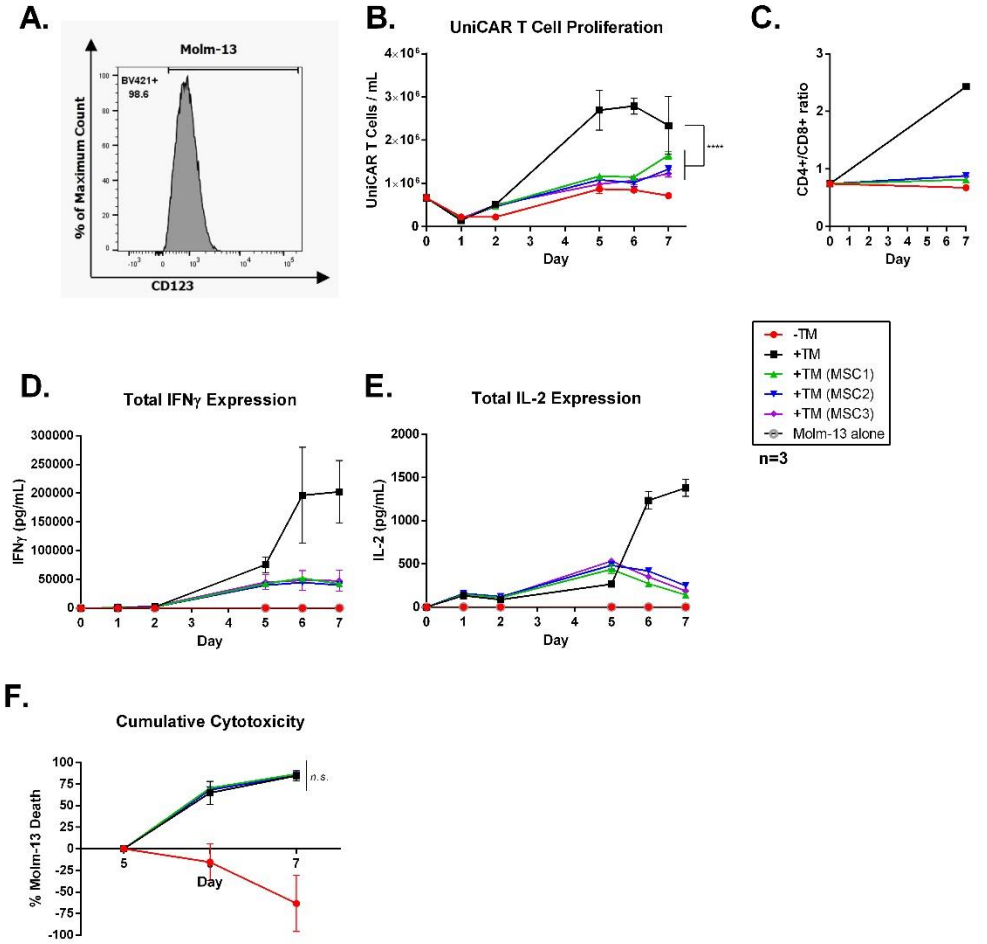


Figure 6. MSCs interfere with the proliferative and inflammatory capabilities of CD123-targeting UniCAR T cells without affecting cytotoxicity in indirect co-cultures.
In the apical chamber of the Transwell cell culture system, eGFP$^+$ UniCAR T cells were redirected with an anti-CD123 target module (+TM, 0.5nM) against eFluor670-labelled MOLM-13 cells in the presence or absence in the basolateral chamber of 7.5x10^3 MSCs from three allogeneic healthy donors (MSC1-3) at a E:T:MSC ratio of 16:16:3. Cultures without TM (-TM) served as a negative control. Additional eFluor670-labelled MOLM-13 cells were added on day 2 (1.2x10^5) and day 5 (variable at a E:T of 1:2). Surviving UniCAR T cells (PI$^-$eGFP$^+$) and MOLM-13 cells (PI$^-$eFluor670$^+$) were quantified via flow cytometry at 1, 2, 5, 6 and 7 days of culture. Shown are representative results of three independent assays. **A)** Flow cytometry analysis of CD123 cell surface expression on MOLM-13. **B)** UniCAR T cell proliferation assessed as PI$^-$eGFP$^+$ cells/mL over time. Data points are presented as the mean of triplicates ± SD. **C)** CD4$^+$ and CD8$^+$ UniCAR T cell populations were assessed by flow cytometry on days 0 and 7. Data is presented as the ratio of CD4$^+$/CD8$^+$ fractions in the DAPI$^-$eFluor670$^-$CD45$^+$eGFP$^+$ cell population. **D and E)** Supernatant was collected on days 1, 2, 5, 6 and 7 and assessed for IFNγ and IL-2 concentration via ELISA. Data are presented as the mean of triplicates ± SD. **F)** MOLM-13 killing kinetics after the third round of UniCAR T cell stimulation on day 5 at a E:T of 1:2. Data points represent cumulative loss of target cells relative to the initial population on Day 5, presented as the mean of technical triplicates ± SD. Negative values indicate MOLM-13 proliferation. Asterisks represent statistically significant differences (**** p≤0.0001; *n.s.* not significant).

3.3 MSC-mediated bidirectional interference of AML-retargeted UniCAR T cell activity.

3.3.1 MSC-mediated interference of UniCAR T cell activity against AML.

In collaboration with the laboratory of Michael Bachmann and Anja Feldmann (Helmholtz-Zentrum Dresden-Rossendorf), the impact of MSCs on retargetable UniCAR T cells (Cartellieri et al., 2016) was investigated. Initial examination was conducted in the Transwell cell culture system to avoid adhesion of the T cells and AML cells to the MSC layer (see lymphocyte-adhering molecules, section 3.4), potentially biasing the flow cytometry-based assessment of cytotoxicity. MSCs and UniCAR T cells were thus initially limited to paracrine interactions. On day 0, $eGFP^+$ UniCAR T cells in the apical chamber of the Transwell co-culture system were redirected using the anti-CD123 TM against MOLM-13 AML cells tagged with eFluor670, with MSCs resting in the basolateral chamber. To verify MOLM-13 as a suitable target for the anti-CD123 TM, flow cytometry was conducted to confirm cell surface expression of CD123 (**Figure 6A**). CART T cells have been shown to be amenable to repeated cytotoxic challenge *in vitro* (Wang et al., 2019), thus a similar strategy was employed here. Total co-culture time was 7 days, with MOLM-13 cells being replenished on day 2 and day 5. UniCAR T cells and MOLM-13 cells were quantified by flow cytometry on days 1, 2, 5, 6 and 7. The flow cytometry gating strategy can be found in **Supplemental Figure 1**. UniCAR T cell proliferation could initially be observed after 48h of stimulation, and was significantly abrogated at later observed time points in MSC co-cultures compared to positive control samples (average inhibition of 40.0% on day 7), approaching the minimal growth rate of UniCAR T cells lacking TM-mediated stimulation (**Figure 6B**). It has been reported that $CD4^+$ CAR T cells expand at an increased rate compared to their $CD8^+$ counterparts (Wang et al., 2019), and this phenomenon was observed when looking at the changes in proportion of $CD4^+/CD8^+$ UniCAR T cells between day 0 and day 7 in TM-stimulated samples without MSCs (0.74 on day 0 versus 2.43 on day 7), and remained largely unchanged in samples lacking TM (0.68 on day 7) (**Figure 6C**). Intriguingly, the presence of MSCs abrogated the enrichment of $CD4^+$ UniCAR T cells over time, maintaining a steady ratio between the two time points similar to -TM cultures (average of 0.86 on day 7). In addition to inhibiting UniCAR T cell proliferation, MSCs decreased the concentration of IFNγ and IL-2 at later time points, as observed previously with the CTL clones (average inhibition on day 7 of 78.6% for IFNγ, 86.1% for IL-2) (**Figure 6D and 6E**). Finally, the cytotoxic capabilities of the UniCAR T cells were studied by measuring the kinetics of MOLM-13 death after the third round of stimulation. On day 5, MOLM-13 cells were replenished to produce a final E:T ratio of 1:2. In line with the analysis of CTL clones, MSCs did not have an observable effect on the killing capacity of the individual UniCAR T cells against MOLM-13 cells, despite overall lower proliferative and inflammatory capabilities (**Figure 6E**).

The effect of MSCs on UniCAR T cells was also investigated in the context of cytotoxicity against patient-derived $CD33^+CD123^+$ AML blasts. In addition to healthy donor MSCs, MSCs isolated from the bone marrow of a patient suffering from AML were also included (AML-MSC). Similar to what was done previously, Transwell cultures of UniCAR T cells were redirected against AML blasts using the anti-CD123 TM over the course of 7 days in the presence or absence of MSCs. Additional cultures without MSCs or TM were also prepared. As when directed against

MOLM-13, UniCAR T cells showed reduced proliferative (average inhibition on day 7 of 75.0%) (**Figure 7A**) and inflammatory capabilities (average inhibition on day 7 of 31.2% and 89.9% for IFNγ and IL-2, respectively) (**Figure 7B and C**) in the presence of MSCs, including those derived from AML bone marrow, but remained unaffected regarding cytotoxic potential (**Figure 7D**). MSCs also negatively modulated the proliferation of CD33-redirected UniCAR T cells against AML blasts (average inhibition on day 7 of 77.0%) (**Supplemental Figure 2**).

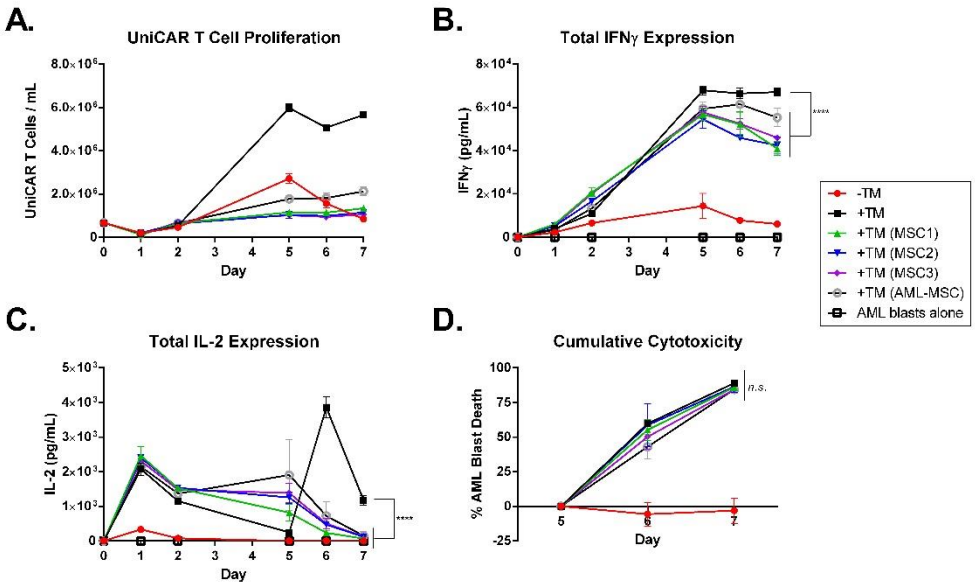


Figure 7. MSCs interfere with the proliferative and inflammatory capabilities of UniCAR T cells targeting primary AML blasts without affecting cytotoxicity in indirect co-cultures.
In the apical chamber of the Transwell cell culture system, eGFP$^+$ UniCAR T cells were redirected with an anti-CD123 target module (+TM, 0.5nM) against eFluor670-labelled CD33$^+$CD123$^+$ AML blasts in the presence or absence in the basolateral chamber of 7.5x10^3 MSCs from three allogeneic healthy donors (MSC1-3) or an allogeneic AML-derived donor (AML-MSC) at a E:T:MSC ratio of 16:16:3. Cultures without TM (-TM) served as a negative control. Additional eFluor670$^+$ blasts were added on day 2 (1.2x10^5) and day 5 (variable at an E:T of 1:1.2). Surviving UniCAR T cells (PI$^-$eGFP$^+$) and blasts (PI$^-$eFluor670$^+$) were quantified via flow cytometry at 1, 2, 5, 6 and 7 days of culture. **A)** UniCAR T cell proliferation assessed as PI$^-$eGFP$^+$ cells/mL over time. Data points are presented as the mean of technical triplicates ± SD. **B and C)** Supernatant was collected on days 1, 2, 5, 6 and 7 and assessed for IFNγ and IL-2 concentration via ELISA. Data are presented as the mean of technical triplicates ± SD. **D)** AML blast killing kinetics after the third round of UniCAR T cell stimulation on day 5 at a E:T of 1:1.2. Data points represent cumulative loss of target cells relative to the initial population on Day 5, presented as the mean of technical triplicates ± SD. Asterisks represent statistically significant differences (**** p≤0.0001; *n.s.* not significant).

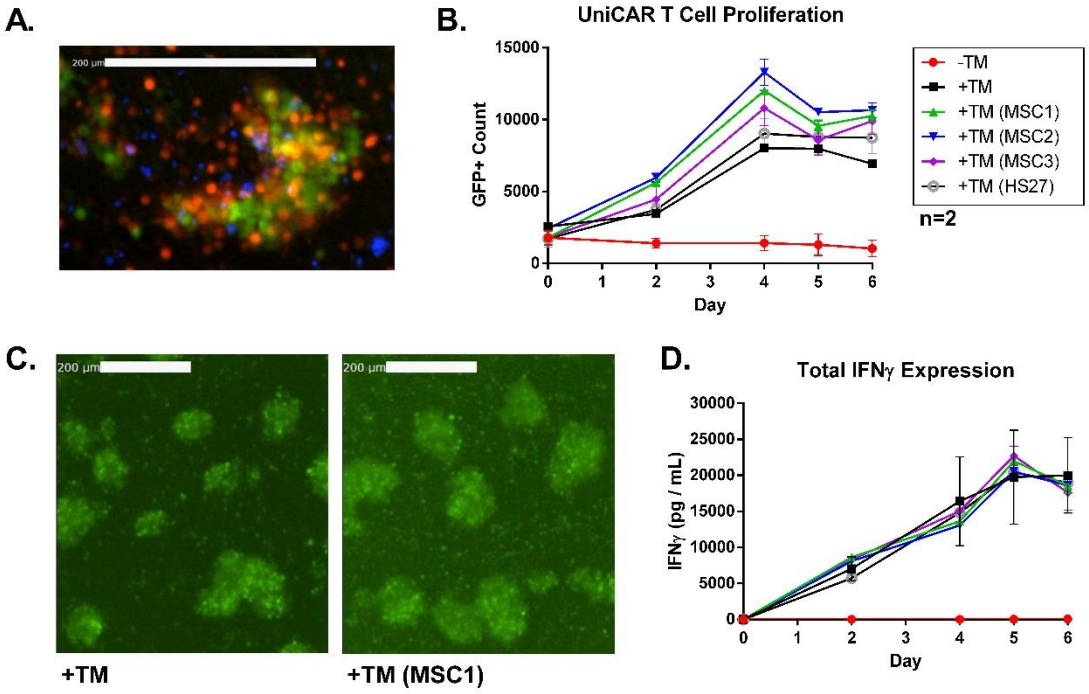


Figure 8. CD123-targeting UniCAR T cells resist MSC interference of proliferation and inflammation in direct co-cultures.
eGFP$^+$ UniCAR T cells were redirected with an anti-CD123 target module (+TM, 0.5nM) against mCherry$^+$ MOLM-13 cells in the presence or absence of 7.5x10^3 MSCs from three allogeneic healthy donors (MSC1-3) or HS27 cells at a E:T:MSC/HS27 ratio of 20:20:3. Cultures without TM (-TM) served as a negative control. Additional mCherry$^+$ MOLM-13 cells were added on day 2 (7.5x10^4) and day 4 (variable at an E:T of 1:10). Surviving UniCAR T cells (DAPI$^-$eGFP$^+$) and MOLM-13 cells (DAPI$^-$mCherry$^+$) were quantified via live fluorescent imaging cytometry at 0, 2, 4, 5 and 6 days of culture. **A)** Representative composited fluorescent image of a culture containing eGFP$^+$ UniCAR T cells (green), mCherry$^+$ MOLM-13 cells (red) and DAPI$^+$ necrotic cells (blue). **B)** UniCAR T cell proliferation assessed as eGFP$^+$ counts over time. Data points are presented as the mean of technical triplicates ± SD. **C)** Representative eGFP$^+$ images of samples containing anti-CD123 TM, with (right) and without (left) MSCs. **D)** Supernatant was collected on days 2, 4, 5 and 6 and assessed for IFNγ concentration via ELISA. Data are presented as the mean of triplicates ± SD.

In addition to releasing anti-inflammatory cytokines, MSCs can also express a range of cell surface immune checkpoint molecules such as PD-L1 and PD-L2 (Davies et al., 2017) (see section 3.3.4). These factors can naturally only interact with T cells in a contact-dependent setting, and thus were previously blocked in the Transwell-based assays. Quantitative live fluorescent imaging was chosen in order to permit measurement of target and UniCAR T cells in direct contact co-cultures while also circumventing the previously stated potential bias related to flow cytometry introduced by adhesion of cells to the MSC layer. UniCAR T cells find themselves suitable for such an approach as the transduced CAR vector also labels them with eGFP (Cartellieri et al., 2016). An mCherry-expressing MOLM-13 cell line was selected as the target, with DAPI serving as a marker for dead cells. **Figure 8A** shows an example overlaid image of the eGFP, mCherry and DAPI channels. On day 0 of the assay, UniCAR T cells were once again redirected against MOLM-13 using the anti-CD123 TM in the presence or absence of MSCs. Additional cultures without MSCs or TM were also prepared. In addition, the MSC-like bone marrow fibroblast cell

line HS-27 was also included in the study as a potential inhibitor of T cells. Total culture time was 6 days, with further administration of MOLM-13 on day 2 and day 4. Cultures were imaged on days 0, 2, 4, 5 and 6, with software-based quantification of surviving UniCAR T cells ($DAPI^-$ $eGFP^+$) and MOLM-13 cells ($DAPI^-mCherry^+$). UniCAR T cells did not proliferate in the absence of TM, as expected. However, in contrast to data obtained from indirect co-cultures, MSCs and HS-27 did not negatively impact TM-dependent UniCAR T cell expansion (**Figure 8B**). Visual inspection of the images confirmed the algorithmic quantification of the UniCAR T cells, with the number and size of $eGFP^+$ T cell clusters being approximately equivalent in +TM samples, regardless of the presence of MSCs or HS27 (**Figure 8C**). In addition to imaging, supernatant from the co-cultures was also collected over the course of the assay and assessed for IFNγ concentration. Once again, in contrast to previous indirect co-culture data, neither the presence of MSCs nor HS-27 had any discernible effect (**Figure 8D**).

One potential explanation for these disparate observations is that in addition to killing AML cells, UniCAR T cells may also act in an off-target manner against MSCs, which would only be possible in a direct contact setting. We sought to address this point in the following section.

3.3.2 UniCAR T cell-mediated apoptosis of MSCs.

To answer the question of UniCAR T cells potentially being toxic to MSCs, UniCAR T cells were cultured in direct contact of a CellTraceViolet-labelled allogeneic MSC layer along with MOLM-13 cells, with or without anti-CD123 TM. As a control, the MSCs were also cultured alone. Cells were harvested after 24h and 48h, and the MSCs were analyzed by flow cytometry for the presence of cell surface phosphatidylserine, a marker of apoptosis, with fluorophore-conjugated Annexin V, and for necrosis with the DNA intercalator 7-AAD. The gating strategy can be found in **Supplemental Figure 3**. After 24h, no observable increase in apoptosis was detected compared to the background control (**Figure 9A, left**). However, after 48h, MSCs became significantly apoptotic specifically in the presence of TM-redirected UniCAR T cells (46.5% versus 4.0%), with only slight increase above background in co-cultures lacking TM (13.2% versus 4.0%) (**Figure 9A, right**). To investigate whether apoptosis induction of MSCs was dependent on direct contact with TM-redirected UniCAR T cells, additional co-cultures were prepared in the Transwell culture system, as performed previously. In addition to the anti-CD123 TM, the anti-CD33 TM was also included. Cell surface analysis of CD33 expression on MOLM-13 can be found in **Supplemental Figure 4**. In **Figure 9B**, we could observe that after 48h, apoptosis induction was specific to direct contact co-cultures with TM-redirected UniCAR T cells (41.5% for anti-CD33 TM and 57.6% for anti-CD123 TM versus 12.1%), as the proportion of apoptotic MSCs is similar to background in indirect cultures (10.2% for anti-CD33 TM and 10.7% for anti-CD123 TM versus 12.1%). Apoptosis levels were also slightly elevated in direct cultures without TM (31.3% versus 12.1%). Apoptosis induction by UniCAR T cells redirected by both the anti-CD123 TM and the anti-CD33TM implies that the target antigen is not a factor in this activity. Indeed, MSCs were found to be negative for cell surface expression of both CD33 and CD123 as determined by flow cytometry (**Figure 10**).

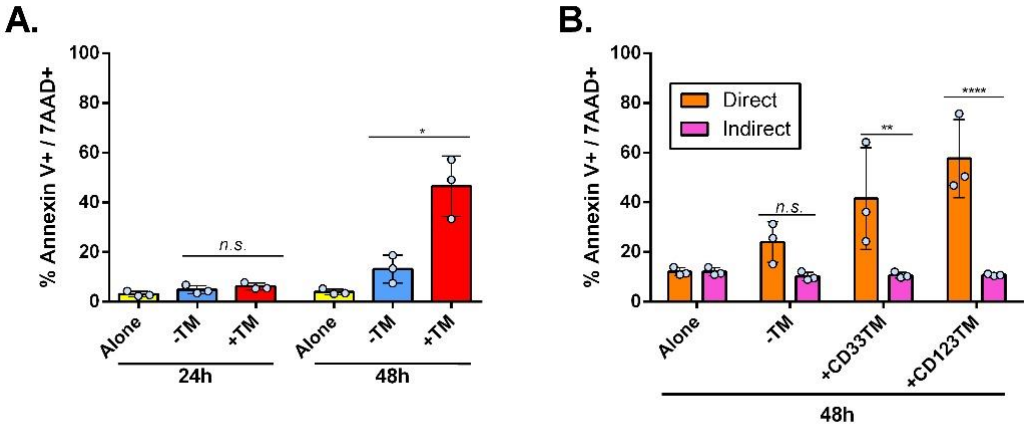


Figure 9. UniCAR T cells induce MSC apoptosis in direct co-cultures.
A) UniCAR T cells were redirected with an anti-CD123 target module (+TM, 0.5nM) against MOLM-13 cells in the presence or absence of 5x104 CellTraceViolet-labelled MSCs from three allogeneic healthy donors at an E:T:MSC ratio of 5:5:1 or 10:10:1. Cultures without TM (-TM) or MSCs alone served as negative controls. MSCs were collected after 24h and 48h and assessed for apoptosis via Annexin V and 7-AAD flow cytometry. Apoptotic MSCs were defined as the CellTraceViolet+AnnexinV+7-AAD+ cell fraction and reported as a percentage of total CellTraceViolet+ cells. Data are presented as the mean of biological triplicates ± SD. **B)** As in (A), additionally with samples containing an anti-CD33 TM (+CD33TM, 0.5nM) and indirect co-cultures established with MSCs plated into the basolateral chamber of the Transwell system. The E:T:MSC ratio used was 10:10:1 and samples were assessed after 48h. Data are presented as the mean of biological triplicates ± SD. Asterisks represent statistically significant differences (* $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$; n.s. not significant).

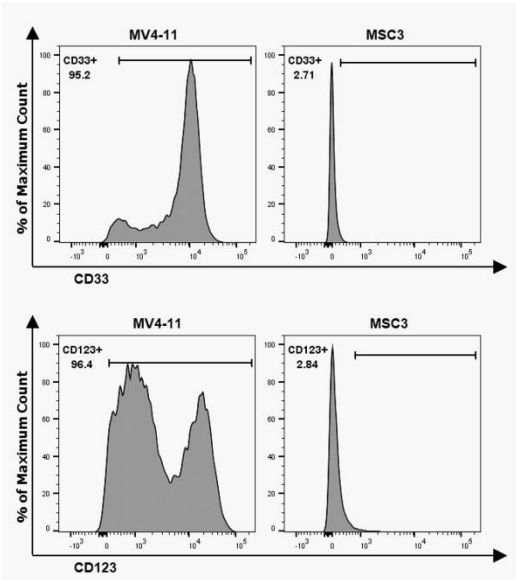


Figure 10. MSCs do not express the AML markers CD33 and CD123.
Flow cytometry analysis of CD33 and CD123 cell surface expression on MSCs from a healthy donor (MSC3). MV4-11 cells served as a positive control.

An initial investigation was conducted into the possibility that TMs may bind to MSCs in an antigen-independent manner, thusly rendering them secondary targets for UniCAR T cell cytotoxicity (**Supplemental Figure 5**). UniCAR T cells were once again redirected with the anti-CD33 TM or anti-CD123 TM against MOLM-13 cells in the presence of CellTraceViolet-labelled MSCs in contact-dependent or -independent cultures. MSCs were also cultured alone as a background control. After 24h and 48h (direct cultures) or just 48h (indirect and MSC alone cultures), the cells were collected and incubated in a high concentration of anti-CD33 TM or anti-CD123 TM, followed by TM-recognizing primary mouse anti-human La5B9 and secondary goat anti-mouse IgG-PE conjugate. The cells were also incubated with only the primary and secondary antibodies or with only the secondary antibody. Surviving MSCs were assessed for TM binding as a function of PE fluorescence by flow cytometry ($CellTraceViolet^{+}7\text{-}AAD^{-}PE^{+}$). **Supplemental Figure 5A** presents the gating strategy. In **Supplemental Figure 5B**, we could observe that both the anti-CD33TM and anti-CD123TM seemed to bind to MSCs at the 24h time point. However, TM-binding seemed to be negative for MSCs cultured alone and for MSCs harvested from indirect co-cultures. In addition, the anti-CD33 TM did not seem to bind to MSCs in 48h direct co-cultures. In regards to the anti-CD123 TM in 48h direct cultures, PE MFI was above background for both incubations of MSCs with and without the TM, implying binding at the level of primary anti-La5B9. Further investigation needs to be performed into off-target binding of TMs to MSCs.

MSCs have so far been shown to be astute mediators of AML-targeting T cells in regards to proliferative and inflammatory potential. In the next section, we investigated the possible MSC-induced transformation of UniCAR T cells into a more senescent phenotype.

3.3.3 Induction of AML-redirected UniCAR T cell senescence by MSCs.

Senescence is an aberrant phenotype identified in tumour-infiltrating T cell populations from a variety of cancers, including those of the bone-marrow (Liu et al., 2020). They have a low proliferative capacity and can be identified by loss of CD28 and gain of CD57, among other markers. Before exploring the question of whether MSCs could induce the senescence of AML-redirected UniCAR T cells, initial investigation was conducted with unmodified T cells from healthy donor PBMCs. PBMCs have already been established has having a lower proliferative capacity when co-cultured with MSCs (**Figure 1)**. We next investigated whether MSCs could induce an enrichment of $CD28^{-}$ and $CD57^{+}$ T cells within the global $CD4^{+}$ and $CD8^{+}$ populations, as well as within further subcompartments of T cell memory (naïve, stem cell memory, central memory, effector memory and terminal effector), as defined by cell surface expression of CD45RA, CCR7 and CD45RO. T cells that did not fall into these definitions of memory were categorized as *other*. After six days of direct and indirect contact co-culture with CD3/CD28-stimulated PBMCs, cells were harvested and analyzed by flow cytometry. An example of the flow cytometry gating strategy can be found in **Supplemental Figure 6**. **Supplemental Figure 7** summarizes the final memory distribution of the T cells. The majority of $CD4^{+}$ T cells were distributed into the central memory fraction (59.9% - 76.4%, across all samples), followed by effector memory (11.0% - 33.1%). Central memory was also the largest subpopulation within the $CD8^{+}$ fraction (34.1% - 56.6%), followed by both stem cell memory (11.4% - 23.1 %) and effector

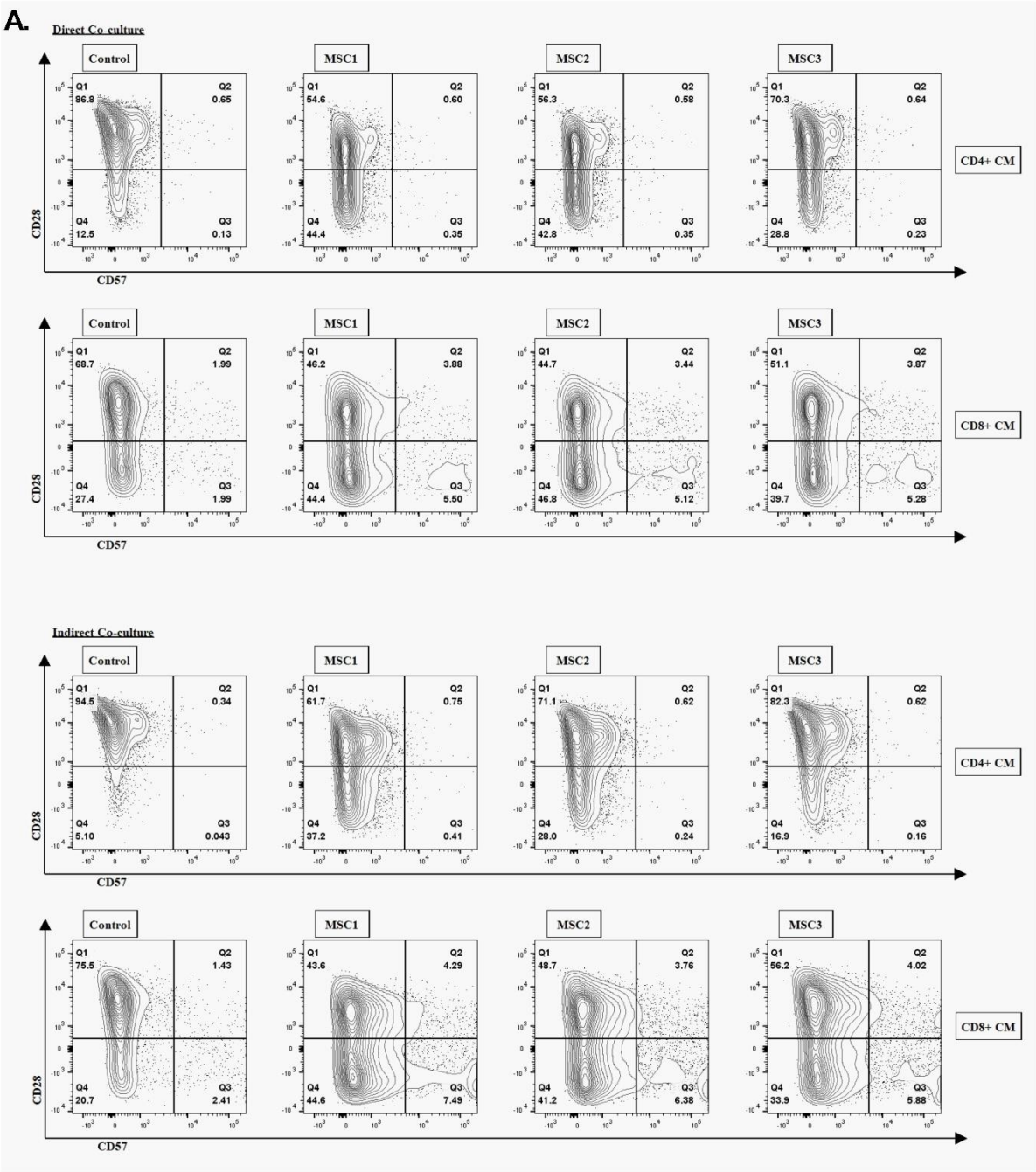


Figure 11. MSCs induce senescence of unmodified T cells, as characterized by loss of CD28 and gain of CD57.

memory (11.8% - 22.5%). Intriguingly, the presence of MSCs may be an influential factor in the distribution of the $CD8^+$ T cells, with late-stage effector memory and terminal effector populations being several fold higher in MSC co-cultures compared to the controls (effector memory: 12.3% - 22.5% versus 11.8% - 12.1%; terminal effector: 4.4% - 14.0% versus 2.2% - 2.7%), implying an more aged T cell population. Contact-dependent or -independent culture conditions did not seem to significantly affect the final memory distribution of the T cells. With regards to induction of

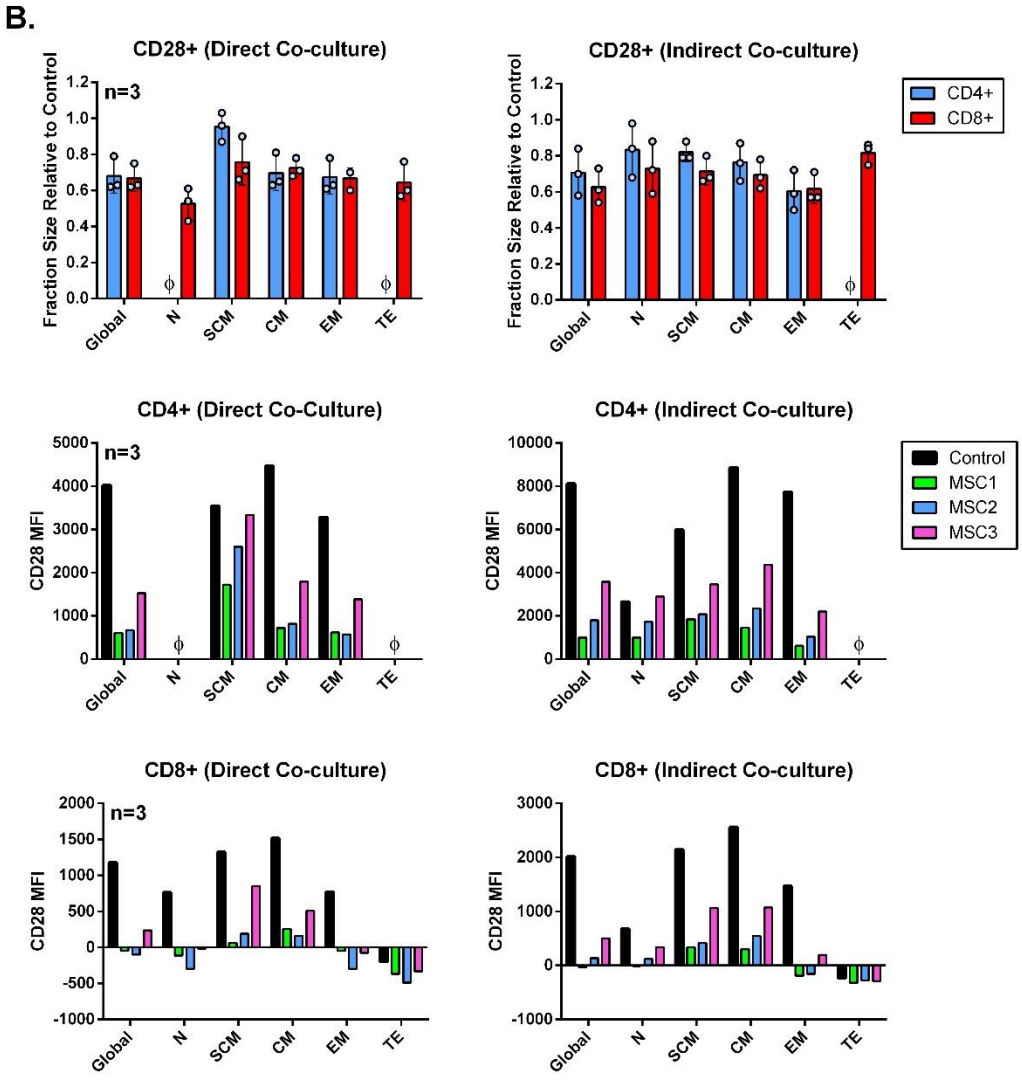


Figure 11. (continued). MSCs induce senescence of unmodified T cells, as characterized by loss of CD28 and gain of CD57.

senescence (i.e. loss of CD28 and gain CD57) within the global and memory populations of $CD4^+$ and $CD8^+$ T cells, MSC-mediated loss of CD28 could be observed on T cells within both the $CD4^+$ and $CD8^+$ fractions, irrespective of memory stage and direct or indirect co-culture conditions (**Figure 11**). **Figure 11A** shows example dot plots for CD28 and CD57 cell surface expression within the central memory compartment of $CD4^+$ and $CD8^+$ T cells in contact-dependent (above) and -independent (below) cultures. **Figure 11B, above** shows the MSC-mediated decrease in the population size of $CD28^+$ cells within the global $CD4^+$ and $CD8^+$ T cell populations for both contact-dependent ($CD4^+$: 32.0% decrease in size compared to control, averaged across all MSC donors; $CD8^+$: 33.3%) and -independent cultures ($CD4^+$: 29.3%; $CD8^+$: 37.3%). This decrease could also be observed within the individual memory stages, though the magnitude of the difference varied between subgroups, for both direct co-cultures ($CD4^+$: 4.6% to 32.7% average decrease; $CD8^+$: 24.3% to 47.3%) and indirect co-cultures ($CD4^+$: 16.7% to 39.7%; $CD8^+$: 18.3%

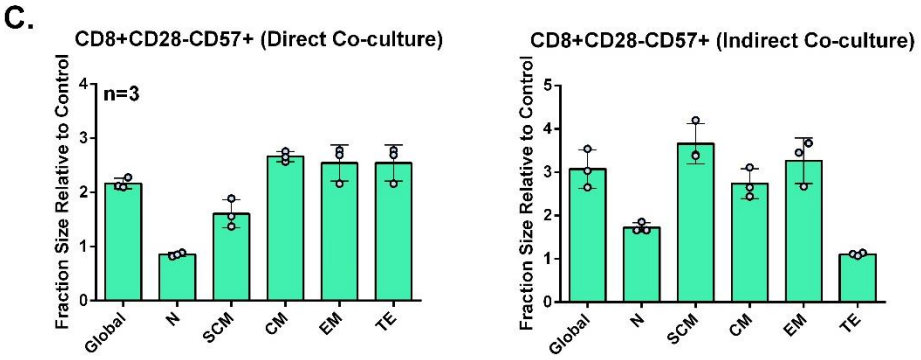


Figure 11. (continued). MSCs induce senescence of unmodified T cells, as characterized by loss of CD28 and gain of CD57.
Healthy donor PBMCs were cultured with or without $5x10^4$ allogeneic MSCs from 3 healthy donors (MSC1-3) at a PBMC:MSC ratio of 5:1 and stimulated with anti-CD3/CD28 antibody-coated beads. In addition to direct co-cultures, indirect Transwell cultures were prepared with the apical chamber containing the PBMCs and beads, and the basolateral chamber containing the MSCs. After 6 days, cells were harvested and analyzed by flow cytometry. T cell senescence ($CD28^-$ and $CD28^-CD57^+$) was assessed within the global $CD4^+$ and $CD8^+$ T cell populations (Global; $DAPI^-CD45^+CD4^+$ and $DAPI^-CD45^+CD8^+$), as well as within further T cell memory subpopulations: Naïve (N; $CD45RA^+CCR7^+CD45RO^-$), Stem Cell Memory (SCM; $CD45RA^+CCR7^+CD45RO^+$), Central Memory (CM; $CD45RA^-CCR7^+CD45RO^+$), Effector Memory (EM; $CD45RA^-CCR7^-CD45RO^+$) and Terminal Effector (TE; $CD45RA^+CCR7^-CD45RO^-$). **A)** Example dot plots assessing CD28 and CD57 cell surface expression within the central memory populations of $CD4^+$ and $CD8^+$ T cells for direct co-cultures (above) and indirect co-cultures (below). **B)** Above: Fraction size of $CD28^+$ cells within the global and memory stages of $CD4^+$ (blue) and $CD8^+$ (red) T cell population in direct (left) and indirect (right) MSC co-cultures relative to the control. Data are presented as the mean of biological triplicates ± SD. φ indicates lack of sufficient number of events for assessment. Below: Median fluorescence intensity (MFI) of CD28 within the global and memory stages of $CD4^+$ (above) and $CD8^+$ (below) T cell populations for direct (left) and indirect (right) control cultures (black) and MSC co-cultures (MSC1; green, MSC2; blue, MSC3; purple). φ indicates lack of sufficient number of events for assessment. **C)** Fraction size of $CD28^-CD57^+$ cells within the global and memory stages of $CD8^+$ T cell population in direct (left) and indirect (right) MSC co-cultures relative to the control. Data are presented as the mean of biological triplicates ± SD.

to 38.3%). MSC-mediated loss of CD28 could also be observed when assessing the median fluorescence intensity (MFI) across these populations (**Figure 11B, below**). When defining senescent T cells as $CD8^+CD28^-CD57^+$, we measured an average MSC-mediated enrichment of 2.2-fold (direct co-cultures) and 3.1-fold (indirect co-cultures) relative to the control within the global $CD8^+$ population (**Figure 11C**). Within the $CD8^+$ memory subpopulations, the average fold change ranged from 0.9 to 2.7 for direct cultures, and 1.7 to 3.7 for indirect cultures.

UniCAR T cells were also investigated for the senescence-associated loss of CD28 and gain of CD57. $eGFP^+$ UniCAR T cells were redirected with an anti-CD123 TM against MOLM-13 cells in the presence or absence of MSCs from three allogeneic healthy donors in contact-dependent co-cultures, or two donors in contact-independent Transwell co-cultures. MOLM-13 cells were replenished within the cultures on day 2, and cell harvesting and analysis by flow cytometry was carried out on day 5. An example of the gating strategy can be found in **Supplemental Figure 8**. As above with the unmodified T cells, before assessing CD28 and CD57 levels, UniCAR T cells were divided into $CD4^+$ and $CD8^+$ compartments, and then further subdivided into memory populations based on cell surface expression of CD45RA, CCR7 and CD45RO. **Supplemental Figure 9** summarizes the memory distribution of the UniCAR T cells.

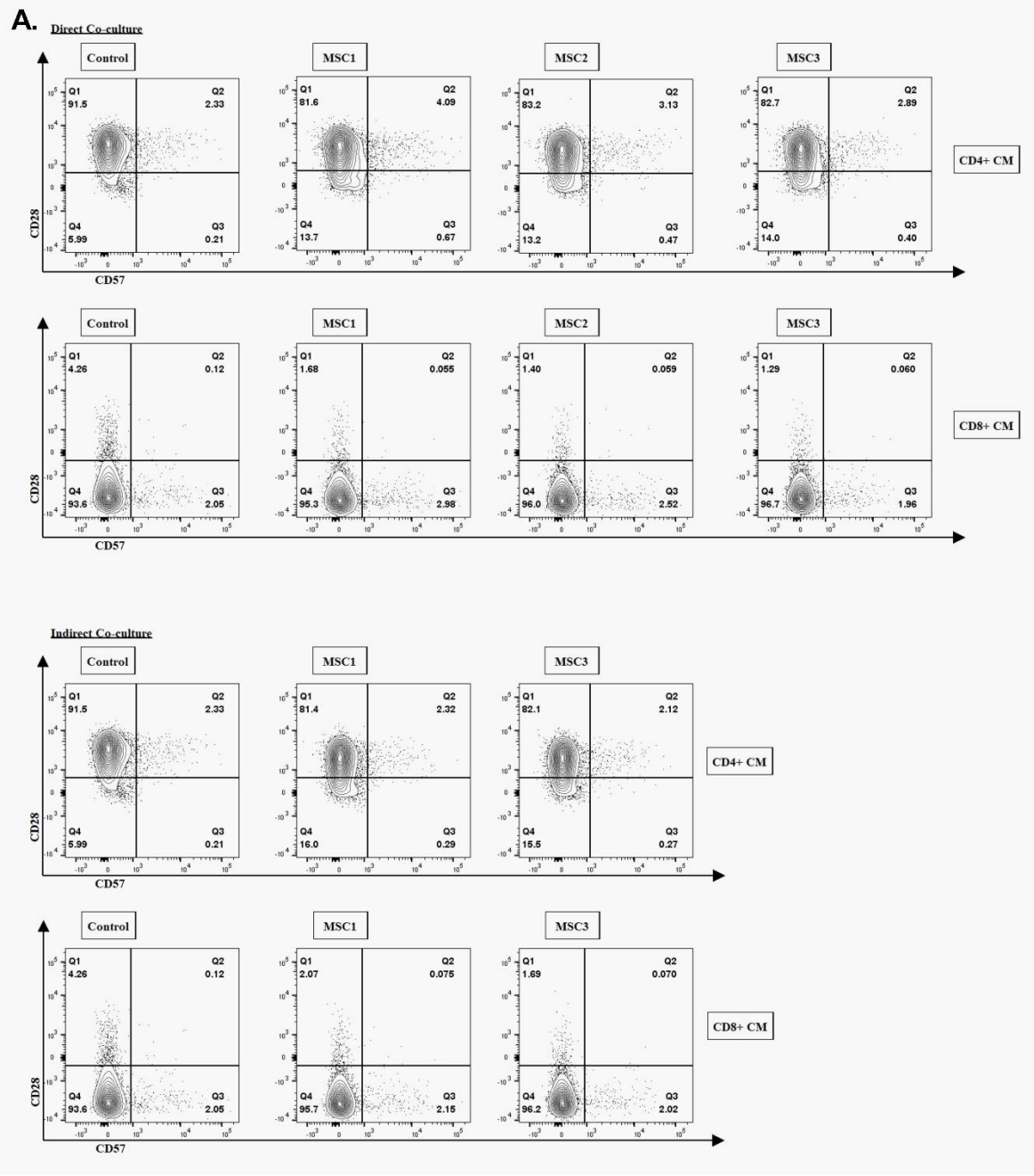


Figure 12. MSCs induce senescence of UniCAR T cells, as characterized by loss of CD28.

In contrast to unmodified T cells, most UniCAR T cells fell into the effector memory compartment (40.0% - 51.2%), followed by central memory (28.6% - 45.1%). No significant differences could be observed between the $CD4^+$ and $CD8^+$ T cells, nor in T cells between culture conditions. Concerning MSC-mediated induction of senescence, UniCAR T cells found themselves to be rather resistant to loss of CD28 and gain of CD57 in comparison to the unmodified PBMCs. **Figure 12A** shows example dot plots for the assessment CD28 and CD57 within the central memory

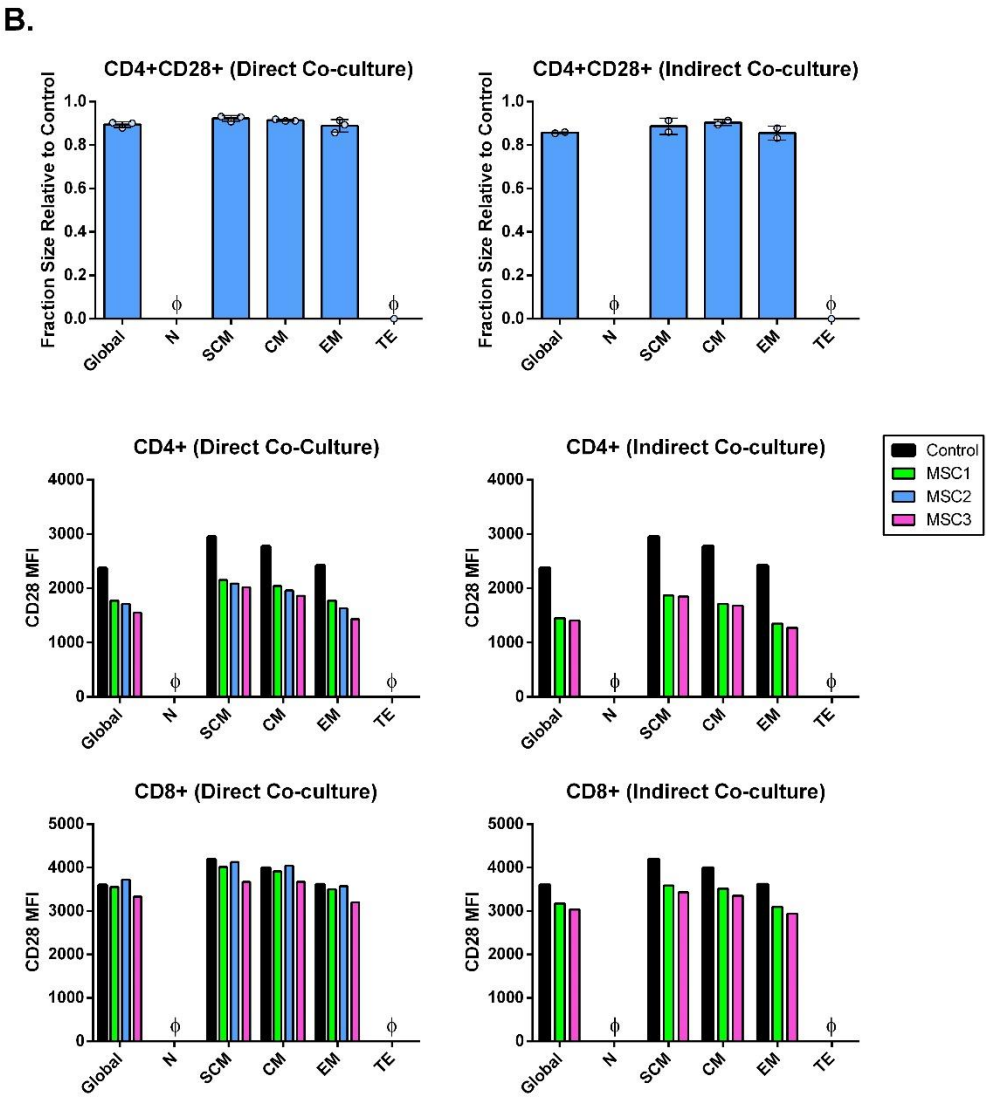


Figure 12. (continued). MSCs induce senescence of UniCAR T cells, as characterized by loss of CD28.

compartment of $CD4^+$ and $CD8^+$ T cells in contact-dependent (above) and -independent (below) cultures. First considering the $CD4^+$ fraction, MSC-mediated decrease in the proportion of $CD28^+$ UniCAR T cells was relatively modest in comparison to the unmodified T cells, but could still be observed within both direct and indirect cultures (**Figure 12B, above**), with a decrease of 10.6% (direct co-cultures) and 14.3% (indirect co-cultures) in comparison to the control, averaged across all MSC donors. Within the $CD4^+$ memory subpopulations, the average decrease ranged from 7.7% to 11.2% in direct co-cultures and 9.6% to 14.4% in indirect co-cultures. MSC-mediated loss of the CD28 MFI could also be readily observed within the global and memory $CD4^+$ compartments (**Figure 12B, below**). Regarding the $CD8^+$ compartment, most cells were surprisingly $CD28^{lo/-}$, even within the control cultures (**Figure 12A**). Despite this, decrease of the CD28 MFI, though slight, could still reliably be observed in an MSC-dependent manner (**Figure 12B, below**), particularly in the indirect co-cultures. However, no significant senescence-associated enrichment

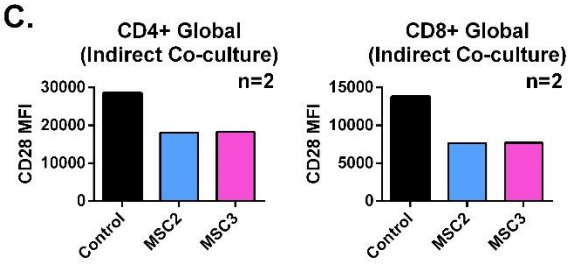


Figure 12. (continued). MSCs induce senescence of UniCAR T cells, as characterized by loss of CD28. eGFP$^+$ UniCAR T cells were redirected with an anti-CD123 target module (0.5nM) against MOLM-13 cells in the presence or absence of 2.5x10^5 MSCs from three allogeneic healthy donors (MSC1-3) in direct co-cultures or two allogeneic healthy donors (MSC1 and MSC3) in indirect Transwell co-cultures at a E:T:MSC ratio of 5:5:1. Additional MOLM-13 cells were added (3.75x10^6) after 2 days. On day 5, cells were harvested and analyzed by flow cytometry. T cell senescence (CD28$^-$ and CD28$^-$CD57$^+$) was assessed within the global CD4$^+$ and CD8$^+$ T cell populations (Global; DAPI$^-$eGFP$^+$CD45$^+$CD4$^+$ and DAPI$^-$eGFP$^+$CD45$^+$CD8$^+$), as well as within further T cell memory subpopulations: Naïve (N; CD45RA$^+$CCR7$^+$CD45RO$^-$), Stem Cell Memory (SCM; CD45RA$^+$CCR7$^+$CD45RO$^+$), Central Memory (CM; CD45RA$^-$CCR7$^+$CD45RO$^+$), Effector Memory (EM; CD45RA$^-$CCR7$^-$CD45RO$^+$) and Terminal Effector (TE; CD45RA$^+$CCR7$^-$CD45RO$^-$). **A)** Example dot plots assessing CD28 and CD57 cell surface expression within the central memory populations of CD4$^+$ and CD8$^+$ UniCAR T cells for direct co-cultures (above) and indirect co-cultures (below). **B)** Above: Fraction size of CD28$^+$ cells within the global and memory stages of the CD4$^+$ T cell population in direct (left) and indirect (right) MSC co-cultures relative to the control. Data are presented as the mean of biological triplicates (direct) biological doublets (indirect) ± SD. Below: Median fluorescence intensity (MFI) of CD28 within the global and memory stages of CD4$^+$ (above) and CD8$^+$ (below) UniCAR T cell populations for direct (left) and indirect (right) control cultures (black) and MSC co-cultures (MSC1; green, MSC2; blue, MSC3; purple). φ indicates lack of sufficient number of events for assessment. **C)** In the apical chamber of the Transwell cell culture system, eGFP$^+$ UniCAR T cells were redirected with an anti-CD123 TM (+TM, 0.5nM) against eFluor670-labelled MOLM-13 cells in the presence or absence in the basolateral chamber of 7.5x10^3 MSCs from two allogeneic healthy donors (MSC2 and MSC3) at a E:T:MSC ratio of 16:16:3. Additional eFluor670-labelled MOLM-13 cells were added on day 2 (1.2x10^5) and day 5 (variable at E:T of 1:2). On day 7, surviving UniCAR T cells (DAPI$^-$eGFP$^+$CD45$^+$) were assessed for CD28 cell surface expression by flow cytometry within the global CD4$^+$ (left) and CD8$^+$ (right) subpopulations. Results are presented as median fluorescence intensity (MFI) of CD28.

of CD57 could be observed. Additional investigation into CD28 expression in the context of UniCAR T cell proliferation and cytotoxicity assessment in the presence of MSCs (section 3.2) was also conducted (**Figure 12C**). Here, decrease of the CD28 MFI could once again be observed within both the global CD4$^+$ and CD8$^+$ fractions in contact-independent cultures with MSCs.

In the next section, we characterized how the inflammatory microenvironment generated by AML-engaged UniCAR T cells leads to the activation of MSC immunosuppressive activity.

3.3.4 Licensing of MSCs in the context of UniCAR T cell anti-leukemic activity.

It has long been the prevailing theory that the majority of the immunomodulatory properties of MSCs must be engaged by external inflammatory stimuli, in a process often referred to as "licensing" (Krampera, 2011). Before characterizing the polarization of MSCs from a relative benign state into active anti-immunity agents as a result of the UniCAR T cell response against AML, we first studied this phenomenon in the context of unmodified T cell-mediated

inflammation. For this purpose, a mixed-lymphocyte reaction (MLR) was performed using allogeneic PBMCs collected from two healthy donors, providing a resulting conditioned medium (MLR-CM) laden with inflammatory cytokines (IFNγ: 15349 pg/mL; TNFα: 914 pg/mL). MSCs were incubated with 50% MLR-CM for 48h and assessed for expression of anti-inflammatory genes (**Figure 13A**), immune checkpoint genes (**Figure 13B**) and T cell adhesion genes (**Figure 13C**). PTGS2 (fold increase of 43.5 averaged across tested donors), IDO1 (790.9), TNFAIP6 (14.5), IL10 (6.7), CD274 (129.7), PDCD1LG2 (6.5), ICAM1 (235.8) and VCAM1 (2.4) all displayed a robust increase of expression in response to the MLR-CM treatment, while TGFB1, HGF, HO and PDCD1 remained relatively stable (fold change between 0.75 and 1.5). It should be noted that IL10 and PDCD1 both had overall very low expression levels relative to GAPDH (IL10: 1.0×10^{-5}, averaged across all samples; PDCD1: 2.1×10^{-4}), likely resulting in diminutive protein expression. Indeed, PD-1 barely registered on the surface of MSCs (**Figure 13D**) and IL-10 could not be detected in activated CTL clone or UniCAR T cell-treated MSC supernatant (data not shown). MSC licensing was further assessed by flow cytometry for the cell surface expression of immune checkpoint molecules and adhesion molecules (**Figure 13D**). When treated with inflammatory 50% MLR-CM for 48h, the proportion of positive MSCs increased significantly for GITRL (2.0 fold increase averaged across all donors), ICOSL (4.8), HVEM (7.2), PD-L1 (7.4), PD-L2 (4.5) and ICAM-1 (3.9), while 4-1BBL, GAL9 and PD-1 were essentially undetectable above background. The inhibitory checkpoint molecules B7H3, CD155 and VISTA were found on the surface of 100% of the MSCs, regardless of treatment. However, MFI assessment showed that median cell surface expression of CD155 and VISTA increased significantly in response to MLR-CM (average fold increase of 1.9 and 1.8, respectively), though B7H3 remained unchanged (**Figure 13D, below**). In parallel to direct immunosuppressive properties, licensed MSCs have also been cited as potential antigen-presenting cells akin to dendritic cells (Van Megen et al., 2019). MSCs were harvested from non-contact cultures of CD3/CD28-stimulated PBMCs and analyzed by flow cytometry for cell surface expression of the MHC Class II molecule HLA-DR, and the CD28 ligands CD80 and CD86. HLA-DR was indeed detectable on the surface of 93.1% - 99.4% of tested MSCs, but neither CD80 nor CD86 were significantly detectable above background levels (**Supplemental Figure 10**).

MSCs licensed by activated UniCAR T cells were similarly studied for cell surface expression of immune checkpoint ligands and T cell adhesion molecules. The fraction of positive MSCs increased significantly for PD-L1 (average fold increase of 5.8), PD-L2 (4.0), ICAM-1 (8.6), VCAM-1 (1.3) and LFA-3 (93.0) upon co-culture with activated UniCAR T cells (**Figure 14A**). As negative-regulating ligands of the activated T cell receptor PD-1, PD-L1 and PD-L2 are of particular interest as PD-1 increased significantly on the cell surface of UniCAR T cells over time (even without the presence of TM-mediated redirection), for both $CD4^+$ and $CD8^+$ T cell populations (average fold increase 4.6 and 22.0, respectively) (**Figure 14B**). Increase of gene expression as examined by qPCR for several notable MSC immunosuppressive factors in response to UniCAR T cell activity against AML can be found in **Supplemental Figure 11**.

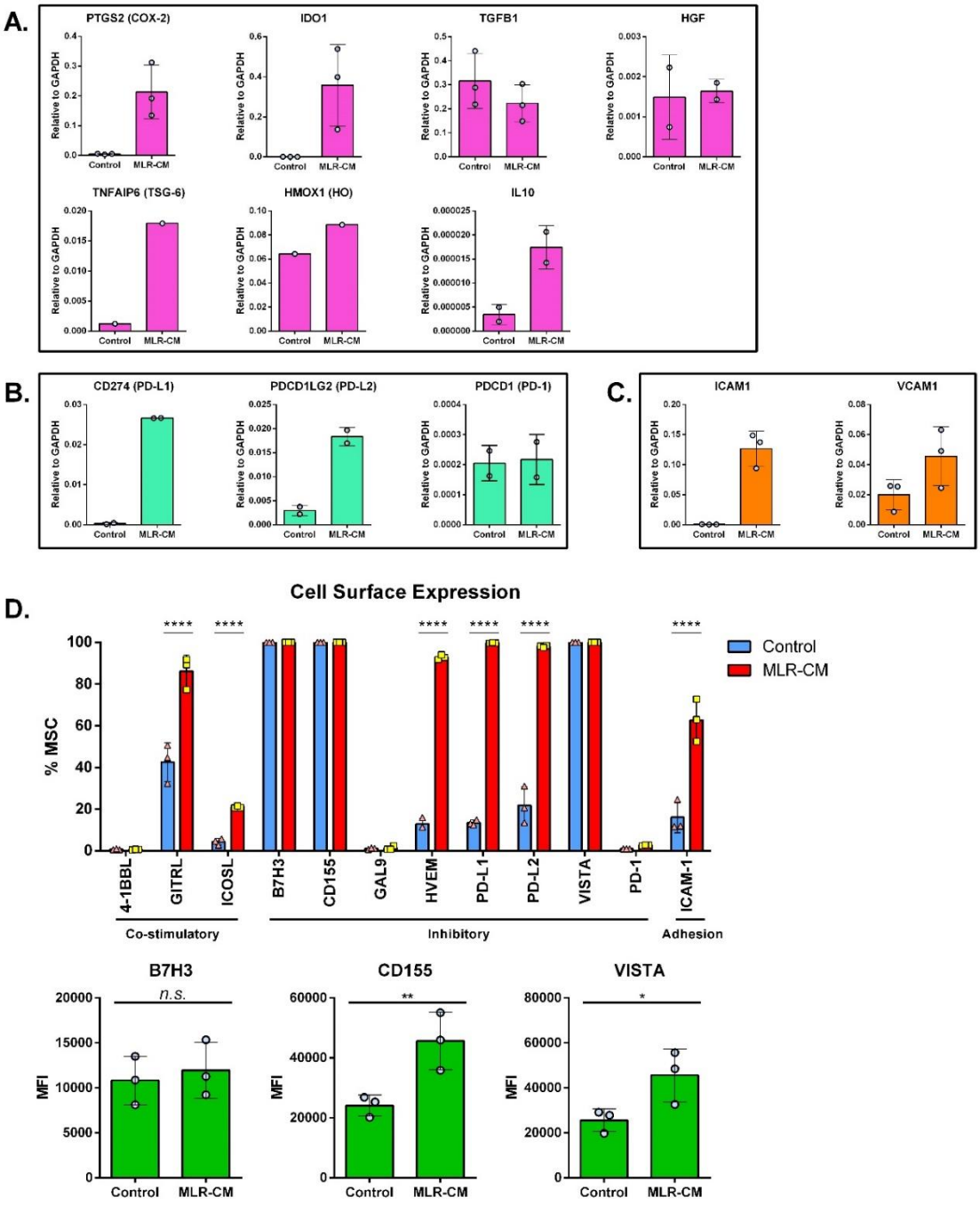


Figure 13. Inflammatory stimuli of activated unmodified T cells induce the expression of anti-inflammatory, immune checkpoint and lymphocyte adhesion molecules in MSCs.

A-C) Healthy donor MSCs were incubated in normal media (Control) or 50% media conditioned from a mixed lymphocyte reaction (MLR-CM). mRNA was isolated after 48h and reverse-transcribed into cDNA libraries. Expression of the indicated anti-inflammatory genes (A), immune checkpoint genes (B) and cell adhesion genes (C) was assessed by qPCR. Data are reported as the mean expression levels relative to that of GAPDH for 1-3 biological replicates ± SD. **D)** MSCs were prepared as in (A-C). After 48h, cells were harvested and analyzed for cell surface expression of the indicated markers by flow cytometry. Data are presented as the mean % of positive MSCs (above) and median fluorescence intensity (MFI; below) of biological triplicates ± SD. Asterisks represent statistically significant differences (* $p \leq 0.05$; ** $p \leq 0.01$; **** $p \leq 0.0001$; n.s. not significant).

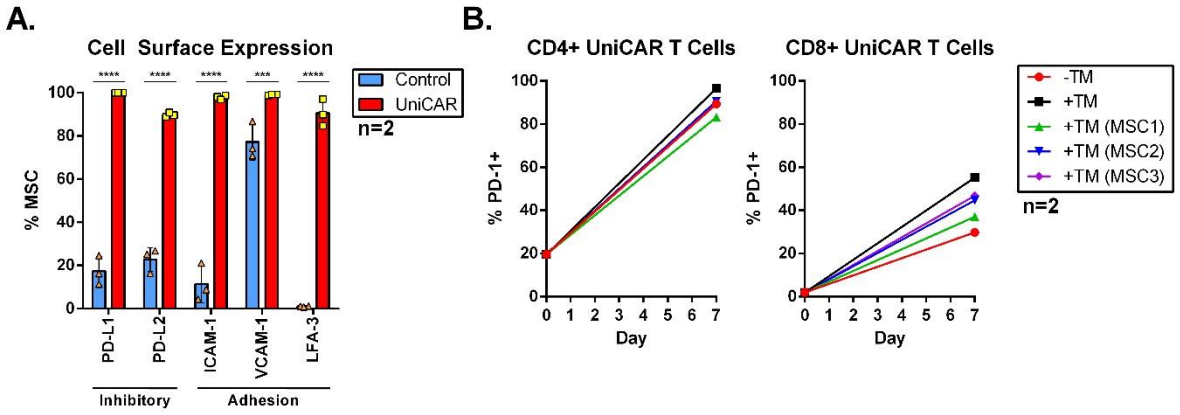


Figure 14. AML-activated UniCAR T cell inflammatory stimuli induce the cell surface expression of immune checkpoint and lymphocyte adhesion molecules in MSCs.
In the apical chamber of the Transwell cell culture system, eGFP$^+$ UniCAR T cells were redirected with an anti-CD123 target module (+TM, 0.5nM) against eFluor670-labelled MOLM-13 cells in the presence or absence in the basolateral chamber of 7.5x10^3 MSCs from three allogeneic healthy donors (MSC1-3) at a E:T:MSC ratio of 16:16:3. Cultures without TM (-TM) served as a negative control. Additional eFluor670-labelled MOLM-13 cells were added on day 2 (1.2x10^5) and day 5 (variable at E:T of 1:2). On day 7, cells were collected and analyzed for cell surface expression of the indicated markers by flow cytometry. Shown are representative results of two independent assays. **A)** Cell surface expression of the indicated markers on DAPI$^-$ MSCs of the basolateral chamber. Data are presented as the mean % of positive MSCs from biological triplicates ± SD. Asterisks represent statistically significant differences (*** p≤0.001; **** p≤0.0001). **B)** Cell surface expression on day 0 and day 7 of PD-1 on surviving CD4$^+$ (left) and CD8$^+$ (right) UniCAR T cells of the apical chamber (DAPI$^-$eGFP$^+$CD45$^+$). Data are presented as the mean % of PD-1 positive UniCAR T cells.

In order to fully assess the change in gene expression resulting from a sustained inflammatory stimulus in the context of UniCAR T cell engagement against AML, whole transcriptomic analysis of MSCs was performed. On day 0, MSCs from two healthy donors (MSC2 and MSC3) were co-cultured in triplicate in the basolateral chamber of the Transwell culture system, along with apical UniCAR T cells retargeted against MOLM-13 cells using the anti-CD123 TM. MOLM-13 cells were replenished in the apical chamber on day 2 and day 5. On day 7, the chambers were separated and total RNA was isolated from the MSCs. RNA was also isolated from control MSCs cultured alone. IFNγ concentration was assessed within the supernatants of the treated samples (MSC2: average of 7824 ± 523 pg/mL; MSC3: 6968 ± 71 pg/mL) and control samples (below detectable levels). RNA sequencing was performed by the Deep Sequencing facility at the Center for Molecular and Cellular Bioengineering. Pearson correlation between the samples identified four clusters of association: two highly correlative and two moderately correlative (**Figure 15A**). As expected, the technical replicates were almost all perfectly matched ($r \geq 0.998$), followed by the biological replicates within a given treatment ($r = 0.984$ to 0.993). Next came correlation between treatment conditions within a given donor ($r = 0.772$ to 0.805), followed finally by correlation between the treated samples from one donor and the untreated samples from the other ($r = 0.734$ to 0.753). Sample association was also assessed by principle component analysis (PCA) (**Figure 15B**), aligning the samples into four clusters by technical replicate group. Treatment condition provided the greatest discrimination between the samples (PC1: 95% variance), with donor origin providing relatively little distance (PC2: 4% variance).

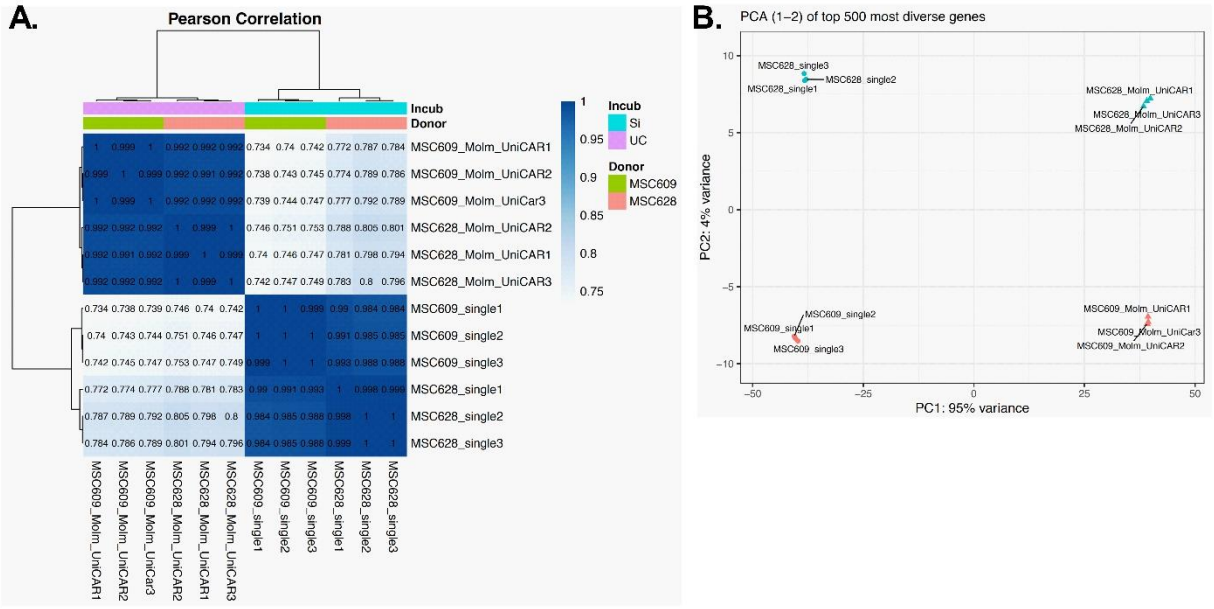


Figure 15. AML-activated UniCAR T cell inflammatory stimuli significantly redirect the MSC transcriptome.
In the Transwell basolateral chamber, $1x10^5$ MSCs from two healthy donors (MSC2/MSC609, MSC3/MSC628) were co-cultured in triplicate with apical UniCAR T cells retargeted against MOLM-13 cells with the CD123 TM (0.5nM) at a E:T:MSC ratio of 5:5:1. Additional MOLM-13 cells were added after 2 days ($1.5x10^6$) and 5 days ($1.0x10^6$). Additional MSCs were cultured alone as a control. After 7 days, the chambers were separated and the MSCs underwent RNA isolation and were analyzed by whole transcriptome sequencing. Samples were assessed by **(A)** Pearson correlation, **(B)** PCA (1 and 2) based on the top 500 most diverse genes and by **(C)** deviation from the average for the top 100 genes with the highest variance.

Applying a false discovery rate of 5%, 17424 differentially expressed genes were identified when comparing UniCAR-treated MSCs to the control, of which 8934 were upregulated and 8490 were downregulated. **Figure 15C** clusters the samples based on the deviation from the mean for the top 100 genes of highest variance, listed on the right. While most of these genes segregate the samples based on treatment, THNSL2 and PSG4 hint at the phenomenon of inter-donor variability. Indeed, when only assessing the UniCAR T cell-treated samples, Pearson correlation produced two distinct clusters: the first for the technical replicates ($r \geq 0.999$) and the second for the donors ($r = 0.991$ or 0.992) (**Figure 16A**). PCA of these samples also discriminated between the two MSC donors (PC1: 97% variance) (**Figure 16B**). Finally, the samples could be clustered by donor based on the top 100 genes of highest variance across all samples (**Figure 16C**). With a false discovery rate of 5%, 5236 genes were found to be differentially expressed between the treated donors, with 2627 upregulated genes and 2609 downregulated genes.

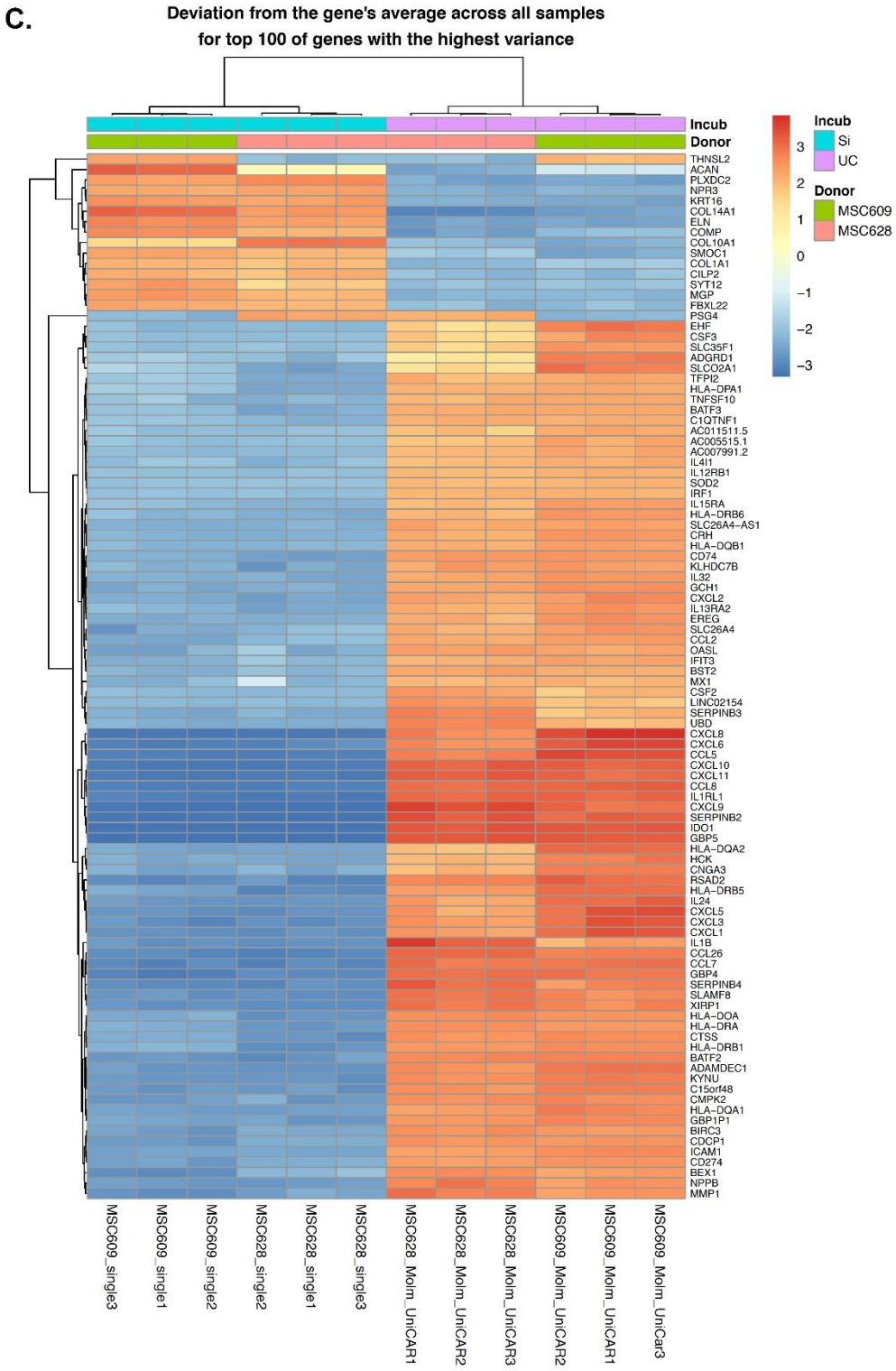


Figure 15. (continued). AML-activated UniCAR T cell inflammatory stimuli significantly redirect the MSC transcriptome.

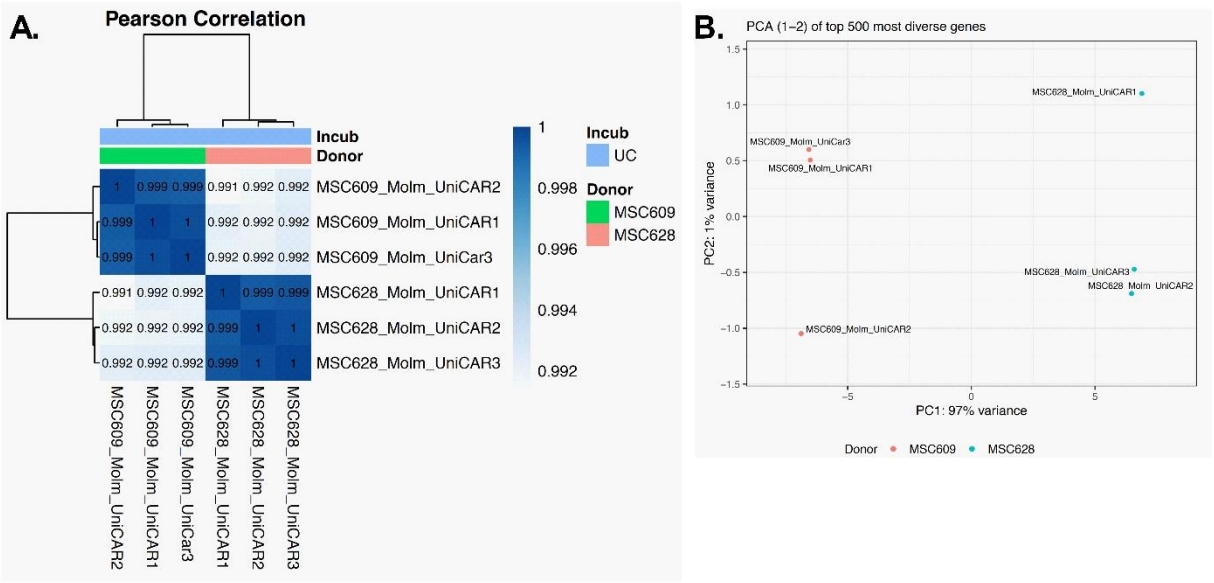


Figure 16. The MSC transcriptome is subject to inter-donor variability in the context of UniCAR T cell interference.
Transcriptomic differences of the UniCAR T cell-treated samples of two MSC donors (MSC2/MSC609, MSC3/MSC628) were assessed by **(A)** Pearson correlation, **(B)** PCA (1 and 2) based on the top 500 most diverse genes and by **(C)** deviation from the average for the top 100 genes with the highest variance.

As expected, an initial assessment of the genes enriched in MSCs upon UniCAR T cell co-culture identified the same adhesion and immunosuppressive molecules that were previously found upregulated by qPCR and flow cytometry in the context of unmodified and UniCAR T cell inflammation. Additional genes reported as contributors to MSC-mediated immunosuppression were also found upregulated, including Gal-1 (1.7 fold increase), Gal-3 (2.9), arginase 2 (2.9) and CD73 (4.1). Furthermore, the genes encoding human nitric oxide synthase (NOS1, NOS2 and NOS3) were not expressed in any of the samples, confirming reports that NO-mediated suppression of T cells remains a species-restrictive mechanism so-far exclusive to murine MSCs (Hurwitz et al., 2020). At a glance, the top upregulated genes could broadly be grouped into several functional categories, including immunomodulators (such as IDO1, CD274 and IL6), MHC class II-related molecules (HLA-DRA, CD74), chemoattractants (CXCL9, CXCL10), interleukin receptors (IL1RL1, IL15RA), inflammation-related transcription factors (BATF2, IRF1), as well as apoptosis modulators (BIRC3, TNFSF10). A cursory examination of the top enriched genes in the control samples, thus likely downregulated in response to inflammatory stimuli, identified many components of the ECM (such as COL1A1, ELN and ACAN), ECM remodelers (HITRA1, PRSS23), integrins and other ECM-adhesion molecules (ITGBL1, THBS2), as well as cytoskeletal molecules (KRT16, LMOD1).

To assign further biological relevance to these transcriptional changes, the differentially expressed genes were further grouped into curated gene sets from the Molecular Signatures Database (MsigDB) by computational methods (Gene Set Enrichment Analysis (GSEA) and Ensemble of Gene Set Enrichment Analyses (EGSEA)). First considering gene sets that were both

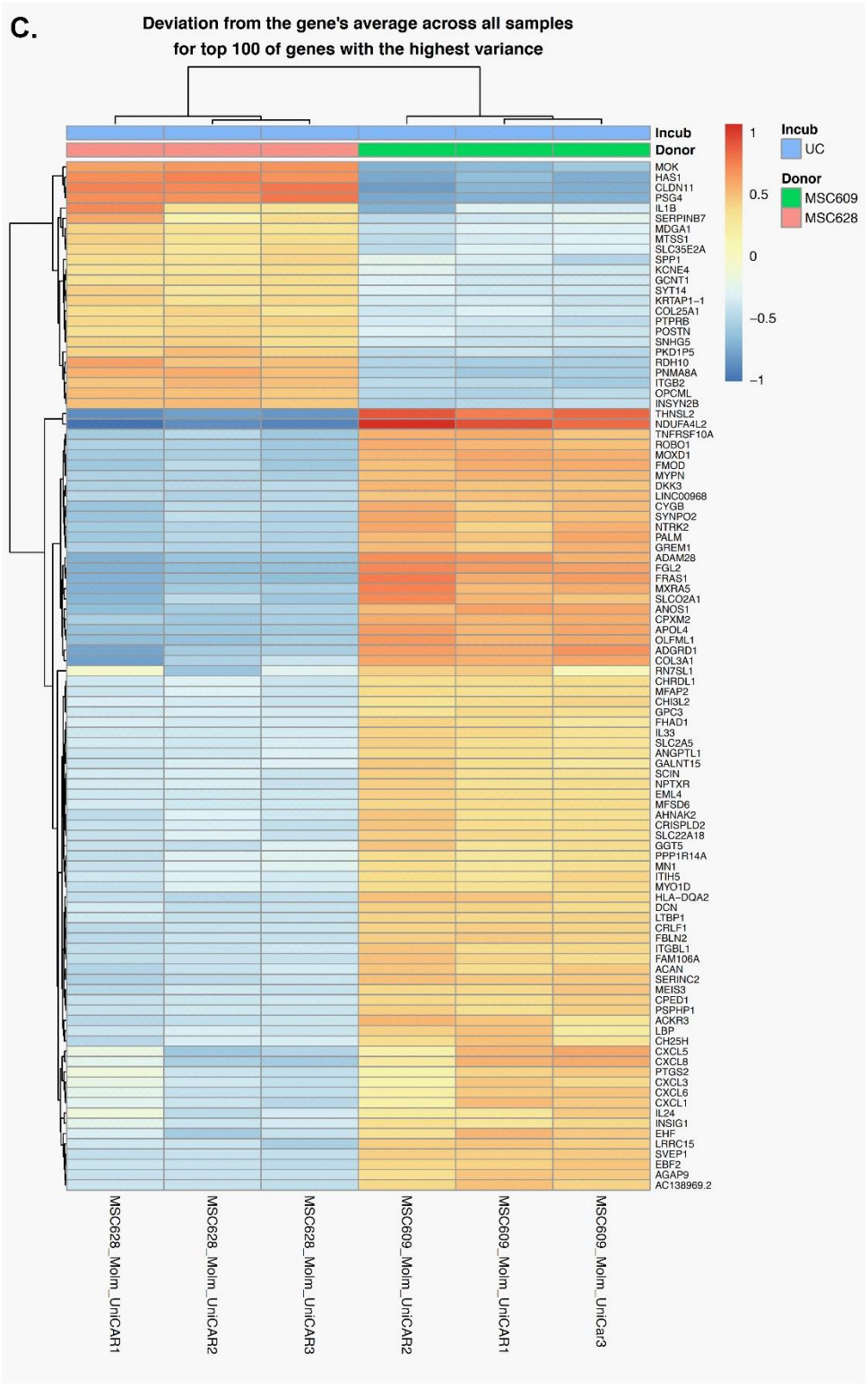


Figure 16. (continued). The MSC transcriptome is subject to inter-donor variability in the context of UniCAR T cell interference.

upregulated upon treatment and shared between the two donors, we found as expected enrichment in signatures relating to the general inflammatory response and response to specific inflammatory signals, including IFNγ, TNFα, IL-1, IL-18, IFNα and TGFβ. Regarding common downstream signal transduction pathways, members and target genes of the interconnected Jak-STAT, MAPK and PI3K-Akt-mTOR pathways were upregulated. In connection to these, Myc and Ras-targeting gene sets were also identified. Likely in response to the activation of these pathways, biological processes relating to general regulation of immunity, cytokine production, intracellular transport, protein secretion and exocytosis were all enriched in the co-cultured samples. Interestingly, a subset of upregulated genes were found to be highly correlative with a set of genes enriched in regulatory T cells in comparison to conventional T cells (Toker et al., 2013) (160 and 159 out of 193 genes, for MSC2 and MSC3 respectively), hinting at shared immunomodulatory functions. Gene sets relating to antigen processing and presentation were also highlighted. In addition, signatures associated with cell cycle regulation and cell division, cytoskeleton modulation, DNA replication and metabolism, as well as organelle fission and transportation, were found enriched in treated MSC samples. Furthermore, many gene sets that can be associated with energy production were also upregulated, such as catabolism of macromolecules, oxidative phosphorylation, glycolysis, sugar metabolism and the lactic acid cycle. Finally, enrichment in several sets relating to stress response, DNA damage response and repair, as well as apoptosis were also identified.

Next, by looking at the signatures enriched in the controls in comparison to the UniCAR T cell-treated samples, we could identify families of genes that were ostensibly downregulated upon treatment. Generally speaking, considerably fewer gene sets could be identified in this manner, which is perhaps indicative of MSCs having a less active phenotype in homeostatic conditions. Of the signaling pathways and related targets that were enriched in control samples, Wnt-β catenin, Hedgehog, Notch and BMP signatures were found to be upregulated. Interestingly, gene sets relating to the TGFβ signaling pathway were once again highlighted, hinting at multi-layered regulation and activity. From a phenotypic perspective, several gene families relating to morphogenesis, development and differentiation were enriched, including adipogenesis and osteogenesis-associated signatures. Processes related to cell-cell and cell-ECM adhesion were also enriched in control samples. Finally, a set of genes found downregulated in regulatory T cells in comparison to conventional T cells (Toker et al., 2013) was enriched in control samples, i.e. downregulated in licensed MSCs, once again hinting at shared immunomodulatory mechanisms (128 and 125 out of 196 genes, for MSC2 and MSC3 respectively).

4 Discussion

With a current five-year survival rate of less than 30% (SEER, 2019), management of acute myeloid leukemia remains a monumental challenge. Without discounting progress made with targeted treatment, this state of affairs is likely a reflection of induction and consolidation treatment regimens remaining largely unchanged over the past few decades and underlines a desperate need for novel therapeutic options. Fortunately, the last few years have seen what is sure to be a revolution in the treatment of cancer as lymphocyte-redirecting immunotherapies enter clinical trials. However, despite many resounding success stories, victory against AML continues to be elusive. As light is shed onto mechanisms of cancer-mediated immune escape, a new appreciation has developed regarding the reciprocal interactions within the local tumour microenvironment in suppressing the host immune response, and by extension immunity-based therapies. In the case of the AML niche, multiple cellular constituents have been demonstrated to contribute towards the bone marrow becoming an immune sanctuary. Mesenchymal stromal cells are emblematic of this activity with the ability to express a wide range of soluble and cell surface immune modulating molecules. This thesis was an exploration into how MSCs modulate the activity of leukemia-targeting cytotoxic T lymphocytes, so that we may eventually alleviate some of the obstacles preventing a durable immunotherapeutic treatment of AML.

4.1 Limitations of the study

Important caveats need to be taken into consideration when assessing the data presented in this thesis. All assays were conducted in 2-dimensional in vitro co-cultures, an approach that is not only plagued with reproducibility issues between laboratories (Begley and Ellis, 2012), but also differs considerably from the clinical setting. MSCs constitute less than 0.01% of the bone marrow (Rasini et al., 2013), thus our co-cultures likely artificially exaggerate the immunosuppressive contribution of MSCs in comparison to the original physiological context. Additionally, the leukemic niche is a complex, multi-dimensional cellular network in a constant state of flux, evolving in response to variations in O2 and nutrient availability, treatment and disease progression. AML itself can present with a wide variety of driver mutations that impact prognosis, subtly changing the local environment and affecting treatment response. A closer approximation would be the use of xenograft models, but even these are ultimately vague facsimiles (Libby, 2015; Galipeau and Sensébé, 2018), culminating in a severe challenge in the translation of positive pre-clinical data to actual beneficial therapy, particularly in the field of oncology (Hutchinson and Kirk, 2011). One needs only to compare the cytotoxic potential of anti-leukemic CAR T cells in in vitro killing assays (and in vivo models) to the ultimate outcome of their administration in the clinical context to understand that co-cultures are only a basic starting point and cannot by themselves be a stand-in for the reality of a heterogeneous, variable biological setting.

With this in mind, efforts were made in our study to hopefully provide robust and reproducible data. Primary MSCs originating from healthy volunteers were initially selected from a bank of frozen material. Without ignoring the issues stated above associated with in vitro research, it is the general consensus that primary cells, in comparison to immortalized stromal cell

lines, would permit a closer approximation of their immunosuppressive capabilities within the bone marrow microenvironment. It has been demonstrated however that donor variability is a significant factor in determining MSC immunopotency (Von Dalowski et al., 2016), which we were able to observe to a degree as well. To factor in donor heterogeneity and uncover universal commonalities in the immunomodulatory potential of MSCs, multiple donors were selected to be studied in parallel. In addition, MSCs isolated from the bone marrow of AML patients have been shown to be molecularly distinct from their healthy counterparts (von der Heide et al., 2017; Kornblau et al., 2018; Azadniv et al., 2019). Thus, AML-derived MSCs were also included in our assays when available. Similarly, patient-derived AML blasts substituted AML cell lines as the target in our cytotoxicity assays when possible. Finally, UniCAR T cells were prepared freshly on an assay-to-assay basis with different T cell donors, which allowed for both mimicking the clinical preparation of CAR T cells and controlling for inter-donor heterogeneity.

4.2 MSC interfere with anti-leukemic T cell-mediated inflammation

Interference of anti-leukemic T cell effectors was explored herein with WT1 and ROR1-targeting CD8+ cytotoxic T lymphocyte clones, as well as with CD33 and CD123-redirected UniCAR T cells. In accordance with previous reports, the presence of MSCs significantly abrogated the antigen-specific T-cell mediated release of the inflammatory molecules IFNγ, TNFα, IL-2 and IL-4, though IL-17a remained unaffected. Interestingly, for both the CTL clones and the UniCAR T cells, this observation did not translate to a reduction in cytotoxicity, confirming similar findings with WT1-reactive T cells (Ramasamy et al., 2008). In their report, Ramasamy *et al*. discuss the possibility that MSC mediation of cytotoxicity could simply be a function of longer co-culture times. However, we show in week-long killing assays with UniCAR T cells that suppressed inflammation is completely decoupled from cytotoxic potential.

The role of inflammation in defining the AML immune micromilieu is incredibly complex. On the one hand, these aforementioned inflammatory cytokines, among a multitude of cell-specific roles, globally mediate the activation and proliferation of innate and adaptive immune effector cells. From this, it is conceivable that MSC-mediated suppression of inflammation would thus ultimately contribute to a weaker, less durable anti-leukemic immune response. The reverse side of the coin is that these very same inflammatory signals are what engage the potent immunosuppressive activity within the bone marrow, leading to the expression of mediators of immune escape such as IDO-1 and PD-L1, and ultimately characterizing the bone marrow as immune privileged. From a clinical perspective, MSC-mediated suppression of inflammation may also be viewed as a double-edged sword. After having stratified the AML bone marrow into distinct gene expression signatures, Vadakekolathu *et al*. associated high IFNγ-signaling with resistance to chemotherapy (Vadakekolathu et al., 2020). In contrast, recent studies have shown that immunotherapies on the other hand benefit from a highly inflammatory contexture. Several groups have demonstrated across a multitude of solid cancers that an inflammatory gene signature is predictive of PD-1 blockade therapy response (Ayers et al., 2017; Cristescu et al., 2018; Ott et al., 2019). In murine models, Zemek *et al*. have demonstrated that pre-treatment of initially non-responding tumours with an inflammatory cocktail including IFNγ sensitized them to combined

PD-1 and CTLA-4 blockers (Zemek et al., 2019). In the context of AML, Rutella *et al*. correlated the IFNγ gene expression signature as a predictor for positive response to CD3/CD123 bispecific antibody therapy (Rutella et al., 2018). In consideration of these findings, it would thus seem likely that MSC-mediated suppression of inflammation would lead to non-responsiveness of AML patients to immunotherapy. In the case of UniCAR T cells, this was demonstrated in contact-independent co-cultures, reinforcing the notion that MSC-released molecules, such as PGE2, kynurenine and TGFβ, are sufficient to abrogate T cell activity. It has been demonstrated that blocking some these molecules *in vitro* reverses MSC inhibition of T cells (Ryan et al., 2007), indicating that they would likewise be attractive targets to reverse MSC-mediated suppression of anti-leukemic inflammation.

4.3 MSCs interfere with of UniCAR T cell proliferation.

In extended co-cultures, MSCs were shown to be able to abrogate the proliferative potency of the UniCAR T cells, and these observations remained consistent across tested healthy and patient-derived MSC donors, as well as multiple T cell donors. This activity would likely contribute towards weaker anti-leukemic immunity from tumour-infiltrating UniCAR T cells, as it is the common view that a sustained, long-term immune response requires effective cell proliferation. It has been reported that AML patients with an immune-depleted bone marrow signature possessed a significantly higher tumour burden (Vadakekolathu et al., 2020). In another recent study, Lamble *et al*. stratified bone marrow T cells from AML patients based on proliferative capacity and correlated the "non-proliferators" with higher blast counts (Lamble et al., 2020).

Additionally, we have observed that MSCs abrogate the enrichment in CD4+ UniCAR T cells over their CD8+ counterparts. It has been previously reported that while CD8+ CAR T cells are able to provide a robust anti-tumourigenic response in the short-term, CD4+ CAR T cells are required for long-term sustained activity and surveillance (Adusumilli et al., 2014; Wang et al., 2018). Indeed, it has been demonstrated that CD4+ CAR T cells possessed higher proliferative, degranulation and inflammatory capacities, expressed fewer exhaustion markers, and a high CD4+/CD8+ ratio ultimately culminated in more efficacious cytotoxicity and higher survival in animal models. It thus follows that the MSC-mediated reduction in CD4+ UniCAR T cells would impede a persistent, long-term anti-leukemic response.

4.4 UniCAR T cells induce apoptosis of MSCs

When MSCs, UniCAR T cells and target AML cells were co-cultured in direct contact conditions, the previously observed immunosuppressive capacity of MSCs in non-contact cultures was lost. We later established that this was likely the result of MSCs undergoing off-target apoptosis mediated by stimulated UniCAR T cells. Contact-dependent loss of MSC activity as a result of lymphocyte-induced toxicity has been previously described with allogeneic PBMCs (Chinnadurai et al., 2016). Additionally, reports by Augello *et al*. and others have shown that

MSCs do not induce an allogeneic response without the presence of a lymphocyte-stimulating agent (Augello et al., 2005). This parallels our own observations that without TM stimulation, UniCAR T cells did not induce MSC apoptosis. TM-dependency could insinuate that MSCs express the targeted AML-associated antigens, but cell surface expression of CD33 and CD123 was negative, thus apoptosis induction could not be a result of on-target UniCAR T cell activity. One hypothesis was that the cytotoxic synapse could form should the TMs bind to MSCs in an off-target manner, however an initial investigation has produced mixed results. MSCs harvested from 24h direct co-cultures did seem to be able to bind to the anti-CD33 and anti-CD123 TMs, but this could not be observed for either MSCs co-cultured indirectly or MSCs cultured alone, nor for the surviving MSCs from the 48h direct co-cultures. One could propose that at 24h, before the onset of apoptosis, TM-binding occurs upon cell contact-specific changes to the MSCs, after which they are eliminated by the 48h time point, however further investigation would be required. Interestingly, in the case of the 48h direct co-culture with anti-CD123 TM, the surviving MSCs were found positive for the La/SS-B La5B9 antigen, independently of TM-binding. This could be due to shuttling of La/SS-B to the cell surface from the nucleus under conditions of cellular stress (Bachmann et al., 1990). In our gene expression analysis, we found in MSCs that several stress-related pathways were engaged during co-culture with UniCAR T cells. Another possibility is that after on-target killing of AML cells, La/SS-B could be released into the local environment and coat the cell surface of neighbouring MSCs, as demonstrated by Koritska *et al*. with murine A9 cells becoming positive for human La/SS-B after co-culture with irradiated HeLa cells (Koristka et al., 2013). In either case, cells under these conditions could technically be recognized by the La5B9 antigen-targeting UniCAR in the absence of TM binding. These hypotheses are entirely speculative and further investigation would be required. Finally, because UniCAR T cells continue to express their endogenous TCR in addition the CAR, it is possible that an allogeneic reaction is induced resulting from an HLA haplotype mismatch once the TM is administered and the UniCAR T cells have been robustly activated. Secondary toxicity resulting from allogeneic adoptive cell therapies is of primordial concern in current regimens that include treatments such as allogeneic HSCT, even despite efforts to minimize immunological incompatibilities between the donor and the recipient. However, this form of off-target killing due HLA haplotype mismatch would be less of an issue in the current clinical administration of UniCAR T cells, as they are produced from the apheresis of the patient's own peripheral blood (Wermke et al., 2021).

4.5 MSCs induce senescence of anti-leukemic T cells

Senescent CD8+ lymphocytes have been identified within the peripheral blood (Beatty et al., 2009; Knaus et al., 2018) and bone marrow (Rutella et al., 2021) of AML patients. They are associated with impaired anti-leukemic toxicity, reduced proliferation, unresponsiveness to checkpoint blockade therapies and shorter overall survival. Among other cell surface markers, they are characterized as having gain of CD57 and loss of CD28. Given the abrogated proliferative capacity of activated T cells when exposed to MSCs, the question was raised as to whether MSCs could induce the aberrant phenotype of T cell senescence. Unmodified T cells and to a lesser degree UniCAR T cells demonstrated loss of CD28 in MSC co-cultures in both CD4+ and CD8+

fractions, independently of T cell differentiation status. Enrichment of CD8+CD28-CD57+ cells could also be observed in the unmodified T cell and MSC co-cultures, though they remained at a low percentage relative to the total CD8+ population. In addition, this induction of senescence-associated markers, much like with inhibition of proliferation, was observed in both contact and non-contact settings, implying the sufficiency of MSC paracrine interactions in inducing these changes. These findings are intriguing in their implications; loss of CD28 expression would severely impair activation of T lymphocytes by antigen-presenting cells, for instance. However, other markers would need to be investigated to truly establish that MSCs induce T cell senescence, such as loss of CD27 and gain of KLRG-1.

Although the accumulation of senescent TILs has been noted in a variety of cancers, the means by which tumours induce senescence are still being investigated. One likely mechanism is replicative senescence resulting from chronic antigen exposure, triggered by the DNA damage response as a result of telomere-shortening, as has been observed in the context of ageing and chronic viral infection (Vallejo, 2005). Ye *et al*. were the first to demonstrate that regulatory T cells are likely contributors, inducing CD27 and CD28 loss in both CD4+ and CD8+ cells, along with an inhibited proliferative capacity (Ye et al., 2012). Since then, mechanistic insights have slowly been emerging, such as senescence induction through Treg-mediated cAMP production within effector T cells (Ye et al., 2014). This mechanism is potentially shared by MSCs via T cell adenylate cyclase activation through extracellular adenosine produced by the CD73-CD39 receptors common on both MSCs (Saldanha-Araujo et al., 2011) and Tregs (Deaglio et al., 2007), as well as through PGE2 release (Valitutti et al., 1993). T cell senescence was also shown to be induced by Tregs through metabolic competition (Liu et al., 2018), drawing a parallel to the increased glycolysis of MSCs under inflammatory conditions (Liu et al., 2019), which we also confirm in our transcriptomic analysis. We have also observed that in MSC co-cultures, the memory pool of CD8+ PBMCs (though not of UniCAR T cells) became enriched in effector memory and terminal effector cells, in both the contact and non-contact setting. At these stages, T cells express the senescence-associated phenotype of CD27lo/-CD28lo/-CD57+KLRG-1+ (Ahlers and Belyakov, 2010; Gattinoni et al., 2017). This could hint at a paracrine-dependent mechanism with which MSCs modulate T cell differentiation. In addition to TCR stimulation, cytokines play an important role in controlling T cell memory (Hope et al., 2019). TGFβ, constitutively expressed by MSCs, is one such cytokine, though the role it plays in shaping memory is somewhat paradoxical. On the one hand, TGFβ inhibition was found to promote the central memory phenotype over effector memory (Takai et al., 2013). In another study however, TGFβ receptor null T cells led to a higher percentage of KLRG-1+ effectors after acute bacterial infection in murine models (Ma and Zhang, 2015). Further investigation is required into the role of TGFβ as a modulator of memory and the interplay of its function in the wider context of MSC-mediated activity.

The most notable divergence between MSC-mediated T cell impairment and senescent TILs lies with the latter retaining the capacity to produce high levels of pro-inflammatory cytokines, the so-called senescence-associated secretory phenotype (SASP) (Liu et al., 2020). Indeed, even Treg-mediated senescent T cells continue to produce high levels of IFNγ and TNFα (Ye et al., 2012). While multiple suppressive mechanisms are shared between MSCs and Tregs, such as TGFβ

production and the aforementioned CD73-CD39-adenosine axis, licensed MSCs express additional immune regulators typically associated with antigen-presenting cells and myeloid-derived suppressor cells (MDSCs), such as immune checkpoint ligands, IDO-1 and COX-2. Indeed, the COX-2 produced cytokine PGE2 is sufficient to suppress T cell IFNγ gene expression and release (Snijdewint et al., 1993; Katamura et al., 1995) It is thus feasible that induction of T cell senescence is compounded by MSC-mediated suppression of the SASP through other effector molecules.

4.6 Anti-leukemic T cell activity activates the immunosuppressive program of MSCs

We confirm the widely accepted paradigm that the immunosuppressive activity of MSCs is largely in response to inflammatory stimuli, leading to the expression of numerous immune checkpoint molecules and immune-mediating cytokines. We found that the mRNA levels of HGF and TGFβ were constitutively expressed, but Ryan *et al*. have demonstrated that inflammation mediates their release (Ryan et al., 2007). Our findings differ from previous publications with regards to bone marrow-derived MSC expression of IL-10 (Ryan et al., 2007) and galectin-9 (Gieseke et al., 2013; Ungerer et al., 2014; Kim et al., 2015), of which we could not detect on either the mRNA or protein level. This may be due to inter-donor variability or differing culture conditions. We are the first to report that MSCs express the immune checkpoint molecule VISTA, which is also expressed in the AML niche on immunosuppressive MDSCs (Deng et al., 2019), as well as other myeloid and lymphoid hematopoietic cells. VISTA (also known as PD-1H) is an attractive target for blockade therapy in leukemia, as Kim *et al*. have demonstrated in vivo that murine AML growth is slower in VISTAnull mice, and even further diminished upon treatment with a VISTA antibody (Kim et al., 2017). The immune checkpoint molecule HVEM has been recently characterized in intrahepatic cholangiocarcinoma-infiltrating MSCs (Gan et al., 2021), and we are the first to characterize it in bone marrow-derived MSCs. However, Chinnadurai *et al*. have previously reported that HVEM (TNFR) could not be detected in IFNγ-licensed of MSCs (Chinnadurai et al., 2014). This may be due to IFNγ being insufficient for MSCs to reach their full immunosuppressive potential, as others have reported that inflammatory mediators such as IL-1β (Redondo-Castro et al., 2017; Liu et al., 2021) and TNFα (Dorronsoro et al., 2014) also play crucial roles. Much like VISTA, there has been growing consideration of HVEM as a therapeutic target, with Lasaro *et al*. describing the improved anti-tumour immunity in in vivo adenocarcinoma models upon blockade of the BTLA-HVEM axis (Lasaro et al., 2011). Additionally, Hobo *et al*. have demonstrated that HVEM is highly expressed on tumour cells in AML and other hematological malignancies, and that CD8+ T cells generated from HSCT patients had enhanced ex vivo activity upon blockade of the HVEM-BTLA pathway (Hobo et al., 2012).

In addition to the study of specific modulators of immunity, we conducted whole transcriptomic analysis of the differentially expressed genes between resting MSCs and MSCs under conditions of AML-targeting UniCAR T cell activity. The examination of the top differentially expressed genes and related pathways paints a surprising contrast between the role of MSCs in homeostatic conditions and under anti-leukemic T cell-mediated inflammation.

Without inflammatory stimuli, our data characterizes MSCs as important mediators of the bone marrow ECM, through the expression of collagens and similar molecules, as well as ECM-remodeling proteases. In addition, under these conditions, pathway analysis suggests that MSCs possess distinct osteogenic and adipogenic potentials, carefully balanced through Wnt, BMP, Hedgehog, Notch and TGFβ signaling (James, 2013; Takam Kamga et al., 2021), a balance which may be lost in inflammatory conditions. Indeed, Fasslrinner *et al*. have shown that MSCs lose differentiation potential and undergo osteogenesis when treated with inflammatory mixed-lymphocyte conditioned medium (Faßlrinner et al., 2012). Under inflammatory stimuli, our data suggest that these matrix and differentiation-related properties are reduced in favour of potent immunosuppressive functions mediated by the activation of Jak-STAT, MAPK and PI3K-Akt-mTOR pathways, leading to the expression of immune checkpoint ligands, anti-inflammatory cytokines, as well as lymphocyte adhesion molecules and chemokines. In addition, MSCs become more metabolically active, confirming similar reports (Liu et al., 2019), through increase in glycolysis and oxidative phosphorylation signatures. This is also in line with glucose deprivation as a mechanism to trigger T cell senescence (Liu et al., 2018). MSCs also activate transcriptional programs to induce proliferation, confirming reported observations (Faßlrinner et al., 2012). Finally, we have also observed increase in stress and apoptotic related gene expression. Vallabhaneni *et al*. have shown that stressed MSCs induced by nutrient-deprivation provided increased support to osteosarcoma cells in comparison to MSCs in normal conditions (Vallabhaneni et al., 2016). In addition, apoptosis and clearance by host macrophages was shown in vivo to be an important immunomodulatory mechanism in the therapeutic MSC treatment of GvHD (Galleu et al., 2017; de Witte et al., 2018). It is thus feasible that stress-related pathways contribute towards leukemic support, though further investigation is required.

4.7 Outlook

In summary, our data demonstrate that MSCs respond to inflammatory stimuli, expressing a large number of immunomodulators that impact the proliferative and inflammatory capacity of AML-targeting cytotoxic T cells, and in so doing promote an aberrant, senescence-associated phenotype. The next step will be to ascertain the contribution of each mediator expressed by MSCs and identify those that are particularly relevant in suppressing anti-leukemic CAR T cell proliferation and inflammation. A systematic siRNA-based knock down approach has already been established by our group for MSC and AML cell co-cultures to investigate MSC support of AML chemotherapy resistance (Fusenig, 2021), and a similar strategy may be used here. MSCs would be transfected with siRNA targeting a gene of interest before incubating with UniCAR T cells and AML cells, with irrelevant siRNA (i.e luciferase) as a control. The readout would be proliferation and inflammatory cytokine release, as established in this thesis. The targets would be genes that are highly expressed under inflammation based on our transcriptomic data, as well as modulators that have already been established in the literature as potent mediators of T cell activity, with a particular focus on paracrine molecules to be in line with our observations regarding contact-independent UniCAR T cell suppression. Preliminary investigation of knock down efficiency and durability would be done with MLR-CM treated MSCs and assessed by qPCR or western blot.

Further knock down monitoring will also be conducted at the termination of the cytotoxicity assays by isolating the basolateral MSCs in the Transwell co-cultures. As an alternative to siRNA, many compounds have been described as efficient blockers of various MSC molecules, such as 1-methyl L-tryptophan for IDO-1 and aspirin for COX-2, leading to decreased MSC potency (Ryan et al., 2007). These could be later investigated in xenograft models to develop a combinatorial approach with CAR T cell therapy.

MSCs have been identified in the tumour niche of AML patients (Corradi et al., 2018), but their specific contribution in suppressing anti-leukemic immunity in the larger context of the leukemic microenvironment has yet to be elucidated. They are relatively rare in comparison to other bone marrow constituents, but this is likely counterbalanced by the fact that MSCs can promote and recruit the other regulatory cells of the niche, such as Tregs, MDSCs and M2 macrophages, in addition to directly mediating immunity (Pleyer et al., 2016; Müller et al., 2021). When it comes to clinical translation, a more holistic approach may be worth considering, focusing on individual suppressive mechanisms activated by the wider leukemic microenvironment, as many of these molecules are shared across multiple cell types. One target that may be relevant in the clinical setting is IDO-1. It is one of the 18 genes related to T cells or IFNγ response that Ayers *et al*. and others have described in a tumour expression profile that was shown to have strong prognostic significance for successful PD-1 blockade therapy across multiple cancers (Ayers et al., 2017; Cristescu et al., 2018; Ott et al., 2019). In our own gene expression data, IDO1 has a fold change expression of over 80000 in MSCs under UniCAR T cell-mediated inflammation. In fact, almost all of the genes of the signature that are not specific to T cells (i.e. the genes for chemokines, immune checkpoints, and MHC molecules) are expressed by MSCs according to our data. Moreover, inhibition of IDO-1 is sufficient to reverse MSC-mediated abrogation of T cell proliferation (Ryan et al., 2007). IDO-1 inhibition may synergize with immunotherapy in AML, perhaps most obviously with the aforementioned PD-1 blockade. In addition to the immunotherapy-related evaluations (inflammatory cytokines, blast count, adverse events, etc.) IDO-1 activity can be monitored in patients with plasma kynurenine levels (Beatty et al., 2017). However, the current inhibitors under clinical investigation were found to possess a number of limitations and off-target toxicities, partly due to their nature as tryptophan analogs (Günther et al., 2019), thus the discovery of alternative IDO-1 drugs may first be warranted. COX-2 is a particularly interesting target, as in addition to regulating immunity, it was found to play a role in tumour cell growth, survival, metastasis and angiogenesis in a number of cancers (Hashemi Goradel et al., 2019). Furthermore, COX-2-inhibiting therapies have already been approved and are widely used in pain and inflammation management. Similar to IDO-1, intravenous administration could lead to improved response when used in combination with immune-based therapies currently under investigation. Efficiency of COX-2 inhibition can be monitored by plasma PGE2 levels (Perkins et al., 2001; Kuo et al., 2016), though careful management would be required as inflammation-related toxicities could be exacerbated, in addition to other hazards associated with COX-2 inhibition such as cardiovascular hypertension and stroke (Arora et al., 2020).

In summary, we have herein demonstrated that bone marrow MSCs reduce the proliferative and inflammatory capacities of AML-targeting T lymphocytes. This is achieved as a response to

inflammation, by which MSCs polarize towards an anti-inflammatory phenotype and express immune-mediating soluble factors, lymphocyte-attracting chemokines and adhesion molecules, as well as immune checkpoint ligands. Thusly, MSCs are potent inhibitors of T cell-mediated anti-leukemic activity and suppressors of sustained immunotherapeutic response (**Figure 17**).

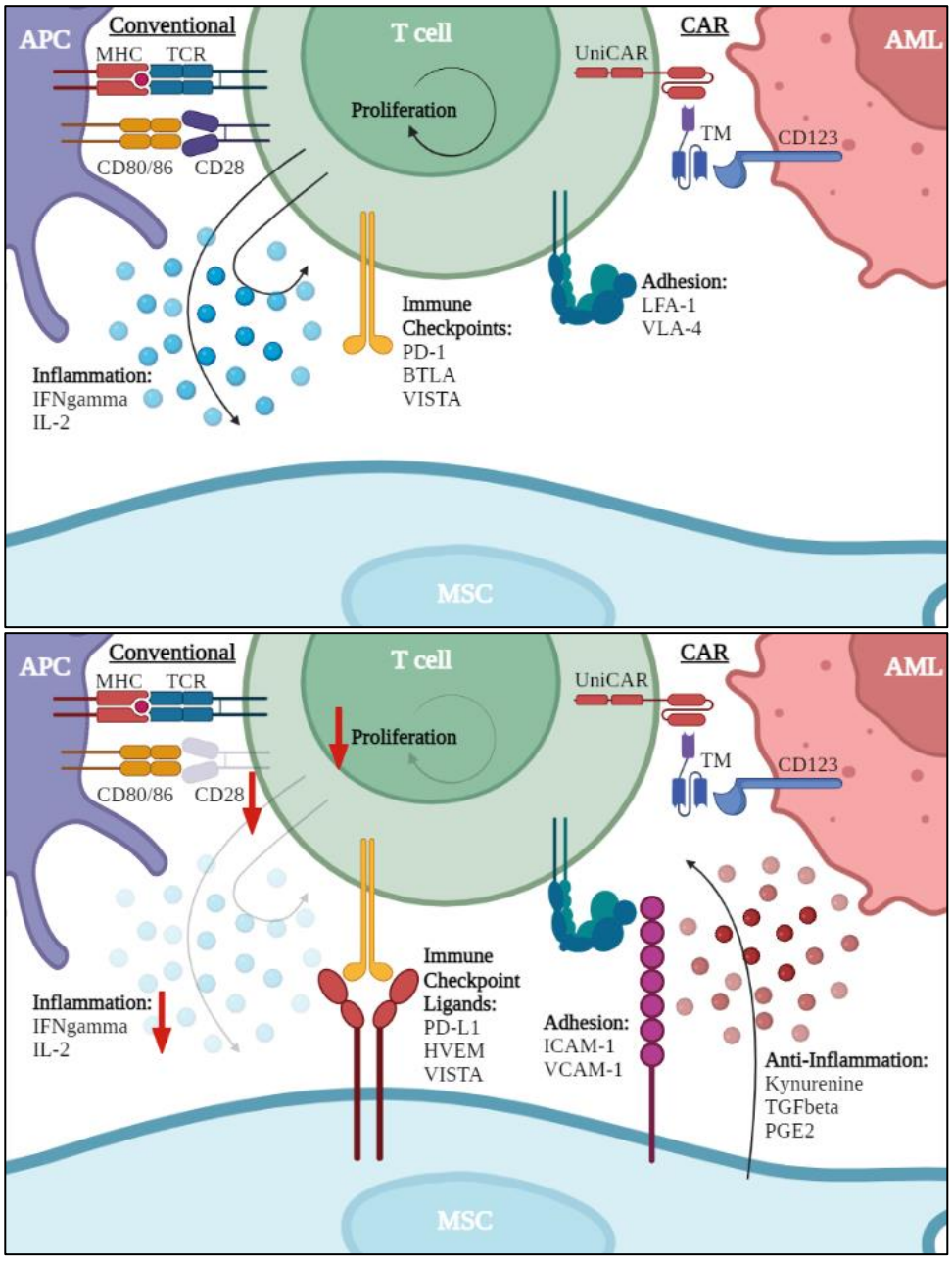
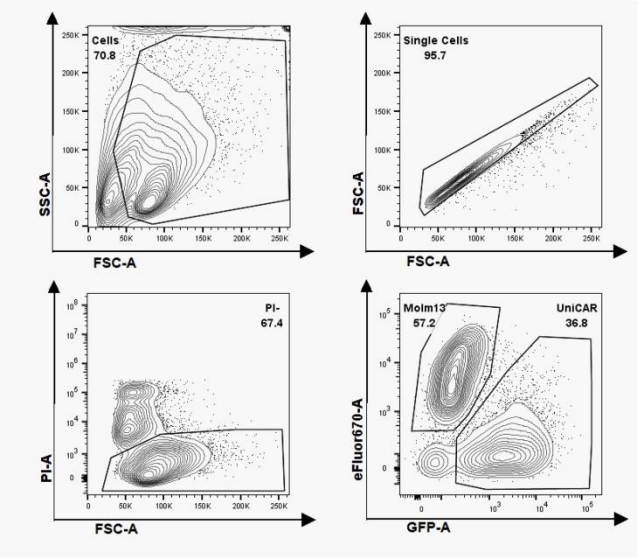
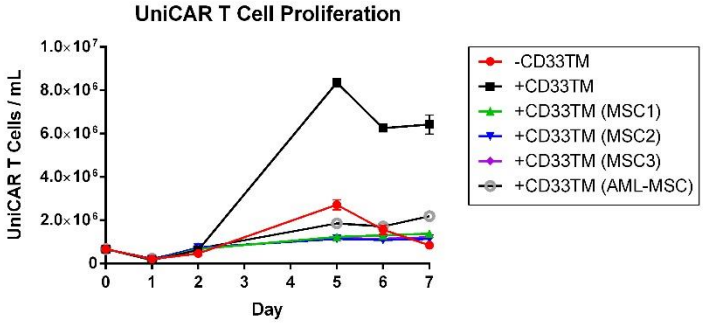


Figure 17. Bidirectional crosstalk between MSCs and anti-leukemic T cells.
AML-infiltrating T cells become activated through conventional antigen presentation or through target recognition via the CAR, leading to proliferation, expression of immune checkpoint molecules, as well as release of inflammatory cytokines. These inflammatory mediators in turn polarize resident MSCs towards an immunosuppressive phenotype, leading to the expression of T cell adhesion molecules and chemokines, immune checkpoint ligands and anti-inflammatory cytokines, which contribute towards inhibiting T cell proliferation and inflammation. (Figure created with Biorender).

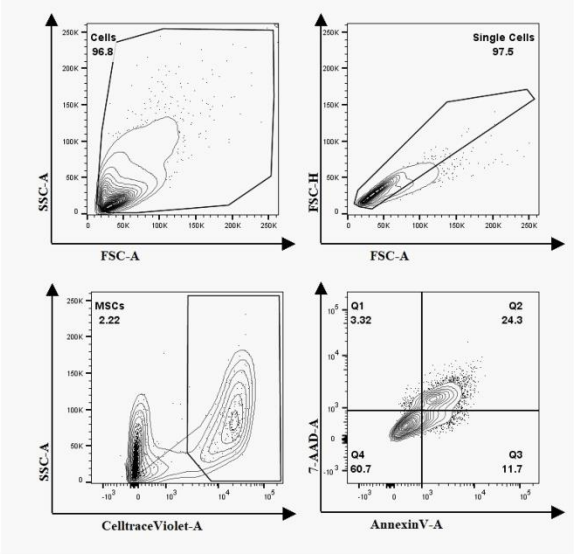
5 Appendix



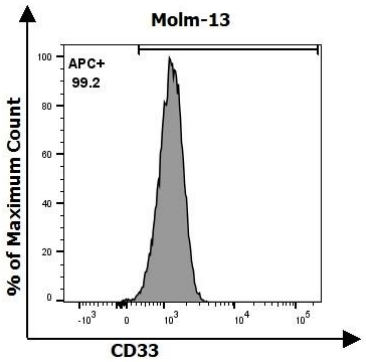
Supplemental Figure 1. Flow cytometry gating strategy for UniCAR T cell cytotoxicity assessment.



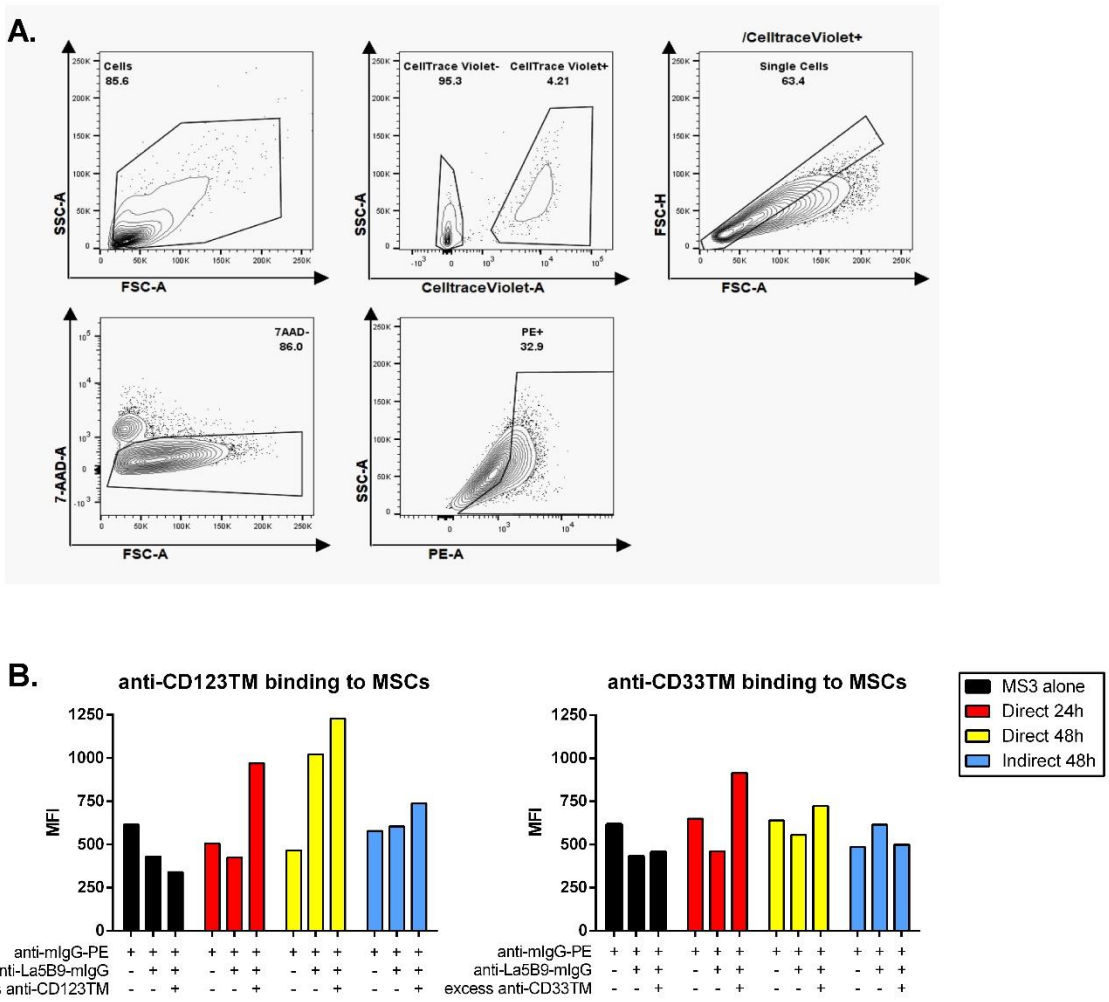
Supplemental Figure 2. MSCs interfere of CD33-targeting UniCAR T cell proliferation.
In the apical chamber of the Transwell cell culture system, eGFP$^+$ UniCAR T cells were redirected with an anti-CD33 TM (+TM, 0.5nM) against eFluor670-labelled CD33$^+$CD123$^+$ AML blasts in the presence or absence in the basolateral chamber of 7.5x10^3 MSCs from three allogeneic healthy donors (MSC1-3) or an allogeneic AML-derived donor (AML-MSC) at a E:T:MSC ratio of 16:16:3. Cultures without TM (-TM) served as a negative control. Additional eFluor670-labelled blasts were added on day 2 (1.2x10^5) and day 5 (variable at E:T of 1:1.2). Surviving UniCAR T cells (PI$^-$eGFP$^+$) were quantified via flow cytometry at 1, 2, 5, 6 and 7 days of culture. UniCAR T cell proliferation was assessed as PI$^-$eGFP$^+$ cells/mL over time. Data points are presented as the mean of triplicates ± SD.



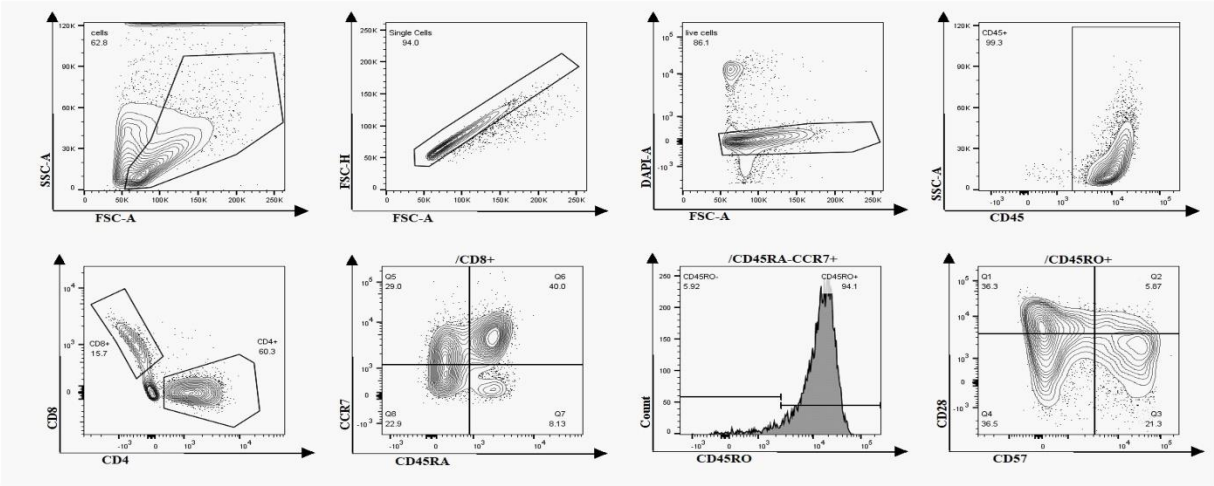
Supplemental Figure 3. Flow cytometry gating strategy for MSC apoptosis assessment.



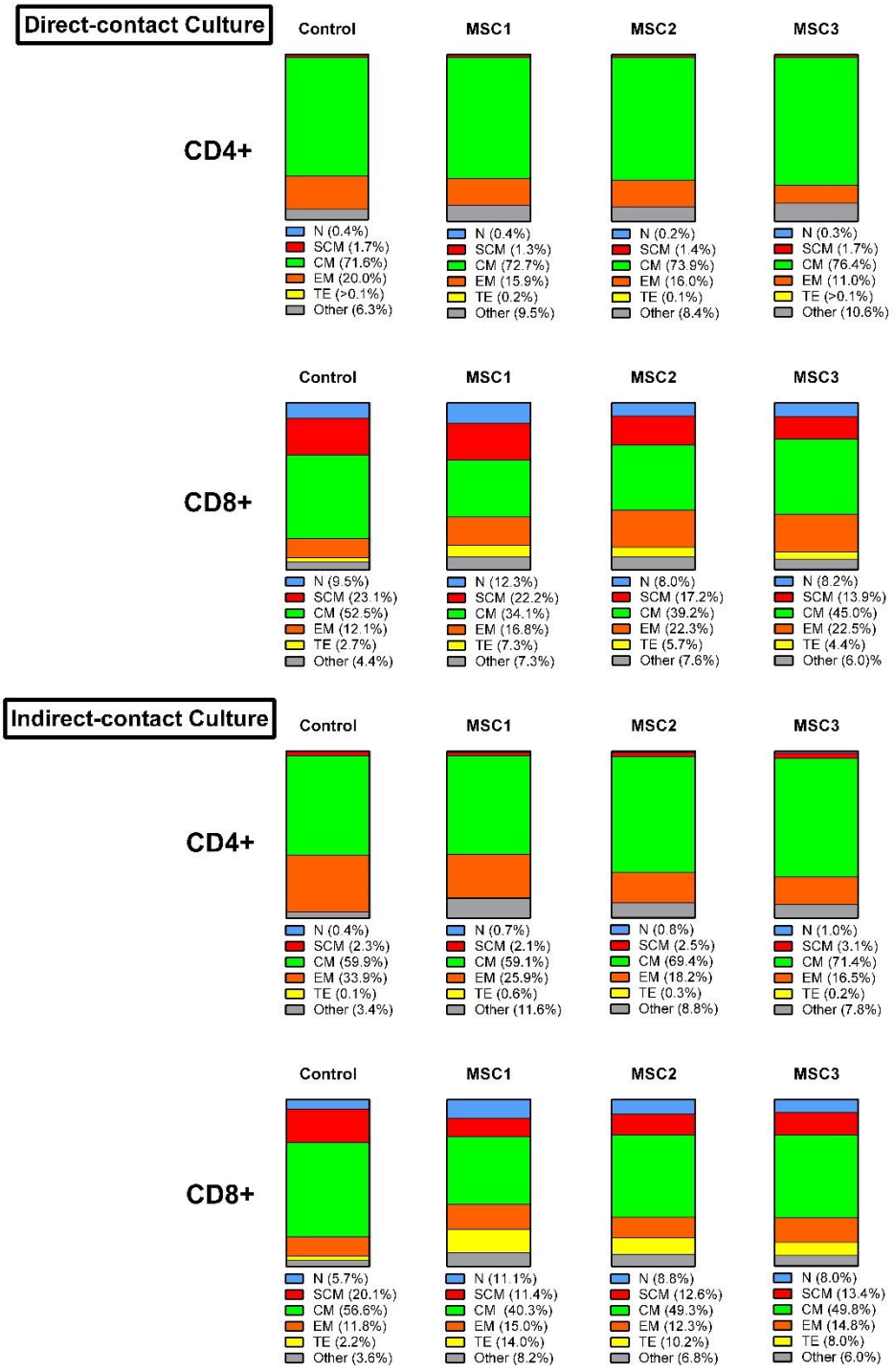
Supplemental Figure 4. Flow Cytometry Analysis of CD33 expression on MOLM-13.



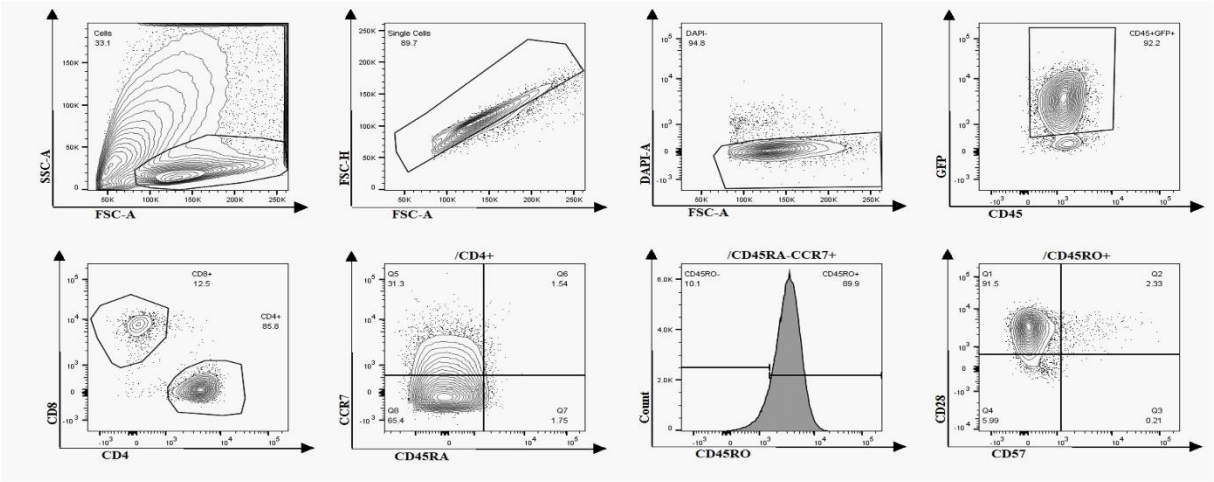
Supplemental Figure 5. TMs can bind unspecifically to MSCs in the context of UniCAR T cell interference. UniCAR T cells were redirected with an anti-CD33 TM or an anti-CD123 TM (+TM, 0.5nM) against MOLM-13 cells in the presence of $5x10^4$ CellTraceViolet-labelled healthy donor MSCs in direct or indirect Transwell cultures at a E:T:MSC ratio of 10:10:1. As a control, MSCs were also cultured alone. After 24h and 48h (direct cultures) or just 48h (indirect and MSC alone cultures), cells were collected and incubated with 315nM (630X) anti-CD33TM or 313.5nM (627X) anti-CD123TM, followed by primary mouse anti-humanLa5B9 and secondary goat anti-mouseIgG-PE conjugate, as indicated. Surviving MSCs were assessed for TM binding as a function of PE fluorescence by flow cytometry ($CellTraceViolet^+7\text{-}AAD^-PE^+$). **A)** Gating strategy for assessing PE-labelling of MSCs. **B)** Data are reported as median fluorescence intensity of PE for anti-CD33 TM (right) and anti-CD123 TM (left).



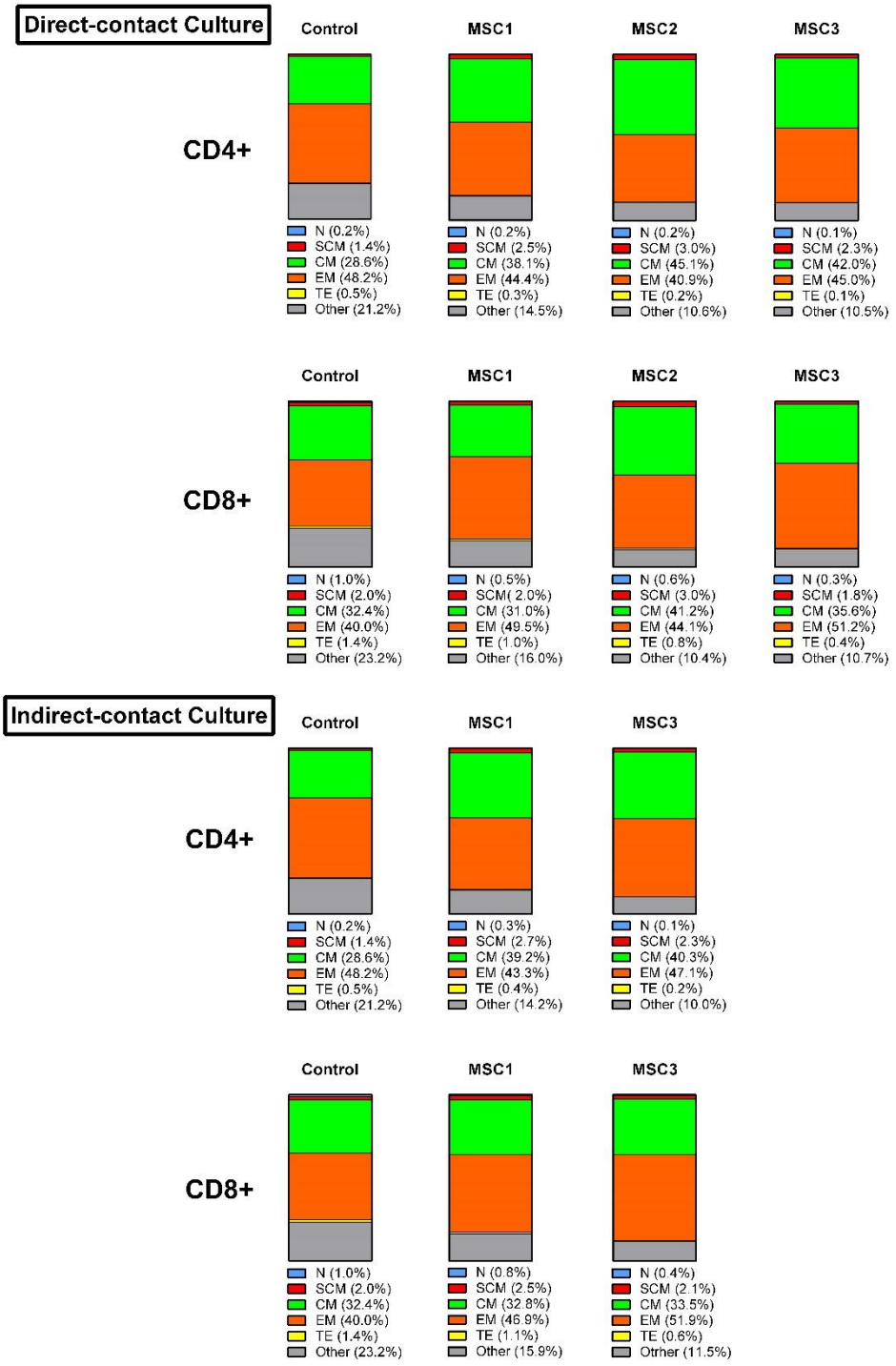
Supplemental Figure 6. Flow cytometry gating strategy for assessment of unmodified T cell senescence. Example shown for $CD8^+$ central memory T cells.



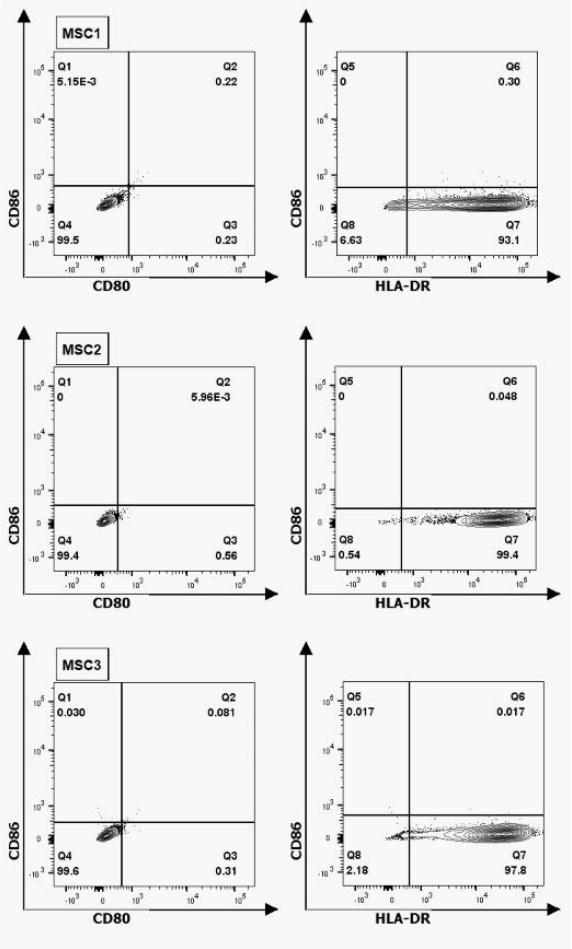
Supplemental Figure 7. MSCs affect the memory distribution of unmodified CD8$^+$ T cells.
Memory distribution was assessed within the global CD4$^+$ and CD8$^+$ T cell populations (DAPI$^-$CD45$^+$CD4$^+$ and DAPI$^-$CD45$^+$CD8$^+$): Naïve (N; CD45RA$^+$CCR7$^+$CD45RO$^-$), Stem Cell Memory (SCM; CD45RA$^+$CCR7$^+$CD45RO$^+$), Central Memory (CM; CD45RA$^-$CCR7$^+$CD45RO$^+$), Effector Memory (EM; CD45RA$^-$CCR7$^-$CD45RO$^+$) and Terminal Effector (TE; CD45RA$^+$CCR7$^-$CD45RO$^-$). *Other* refers to cells that could not be characterized within these compartments. Data are presented as the % of the global CD4$^+$ and CD8$^+$ populations for direct co-cultures (above) and indirect co-cultures (below).



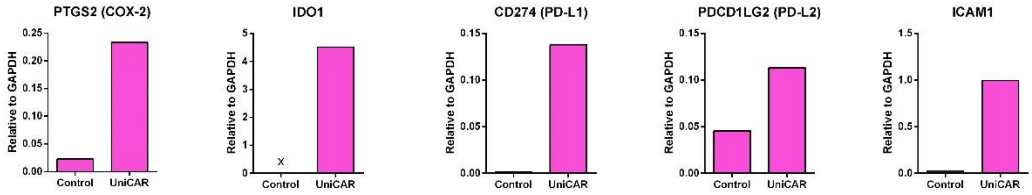
Supplemental Figure 8. Flow cytometry gating strategy for assessment of UniCAR T cell senescence.
Example shown for $CD4^+$ central memory T cells.



Supplemental Figure 9. MSCs do not affect the memory distribution of UniCAR T cells.
Memory distribution was assessed within the global CD4+ and CD8+ T cell populations (DAPI-eGFP+CD45+CD4+ and DAPI-eGFP+CD45+CD8+): Naïve (N; CD45RA+CCR7+CD45RO-), Stem Cell Memory (SCM; CD45RA+CCR7+CD45RO+), Central Memory (CM; CD45RA-CCR7+CD45RO+), Effector Memory (EM; CD45RA-CCR7-CD45RO+) and Terminal Effector (TE; CD45RA+CCR7-CD45RO-). Other refers to cells that could not be characterized within these compartments. Data are presented as the % of the global CD4+ and CD8+ populations for direct co-cultures (above) and indirect co-cultures (below).



Supplemental Figure 10. MSCs express the antigen-presenting cell marker HLA-DR, but not CD80 or CD86. With the Transwell cell culture system, indirect co-cultures of CD3/CD28-stimulated healthy donor PBMCs (apical) and $5x10^4$ allogeneic MSCs from 3 healthy donors (MSC1-3; basolateral) were established at a PBMC:MSC ratio of 5:1. After 48h, cell surface expression of CD80, CD86 and HLA-DR was assessed on MSCs by flow cytometry. Data are presented as dot plots.



Supplemental Figure 11. AML-activated UniCAR T cell inflammatory stimuli induce the gene expression of anti-inflammatory, immune checkpoint and lymphocyte adhesion molecules in MSCs.
In the apical chamber of the Transwell cell culture system, UniCAR T cells were redirected with an anti-CD123 TM (+TM, 0.5nM) against MOLM-13 cells in the presence in the basolateral chamber of $1x10^5$ healthy donor MSCs at a E:T:MSC ratio of 5:5:1. Additional MOLM-13 cells were added on day 2 ($1.5x10^6$) and day 5 ($1x10^6$). As a control, MSCs were also cultured alone. On day 7, mRNA was isolated from the MSCs and reverse-transcribed into cDNA libraries. The expression of the indicated genes were assessed by qPCR and the data are reported as expression levels relative to that of GAPDH.

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7 Declarations

Anlage 1: Erklärung zur Eröffnung des Promotionsverfahrens

Technische Universität Dresden

Medizinische Fakultät Carl Gustav Carus

Promotionsordnung vom 24. Oktober 2014

1. Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

2. Bei der Auswahl und Auswertung des Materials sowie bei der Erstellung des Manuskripts habe ich Unterstützungsleistungen von folgenden Personen erhalten: **Martin Bornhäuser**

3. Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe eines kommerziellen Promotionsberaters bzw. einer kommerziellen Promotionsberaterin in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

4. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

5. Die Inhalte dieser Dissertation wurden in folgender Form veröffentlicht: **Nicht zutreffend**

6. Ich bestätige, dass es keine zurückliegenden erfolglosen Promotionsverfahren gab.

7. Ich bestätige, dass ich die Promotionsordnung der Medizinischen Fakultät der Technischen Universität Dresden anerkenne.

8. Ich habe die Zitierrichtlinien für Dissertationen an der Medizinischen Fakultät der Technischen Universität Dresden zur Kenntnis genommen und befolgt.

9. Ich bin mit den an der Technischen Universität Dresden geltenden „Richtlinien zur Sicherung guter wissenschaftlicher Praxis, zur Vermeidung wissenschaftlichen Fehlverhaltens und für den Umgang mit Verstößen“ einverstanden.

Russell Towers

Dresden, den

Anlage 2: Bestätigung über Einhaltung der aktuellen gesetzlichen Vorgaben

Hiermit bestätige ich die Einhaltung der folgenden aktuellen gesetzlichen Vorgaben im Rahmen meiner Dissertation:

- Das zustimmende Votum der Ethikkommission bei Klinischen Studien, epidemiologischen Untersuchungen mit Personenbezug oder Sachverhalten, die das Medizinproduktegesetz betreffen

Aktenzeichen der zuständigen Ethikkommission: **EK206082008, EK98032010, EK307082018**

- Die Einhaltungen der Bestimmungen des Tierschutzgesetzes

Aktenzeichen der Genehmigungsbehörde: **Nicht zutreffend**

- Die Einhaltung des Gentechnikgesetzes

Projektnummer: **Az.: 55-881 1.72189**

- Die Einhaltung von Datenschutzbestimmungen der Medizinischen Fakultät und

des Universitätsklinikums Carl Gustav Carus.

Russell Towers

Dresden, den