

Liposomal dexamethasone combined with CD47 blockade is a promising strategy for overcoming multiple myeloma resistance

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Abstract

Multiple myeloma (MM) is a proliferative malignancy of plasma cells in the bone marrow. Despite advancements in treatment, MM remains incurable, with relapse being virtually inevitable after first-line therapy, ultimately contributing to poor patient prognosis. A major factor in the disease's aggressiveness is the presence of a poorly characterized stem cell-like population, as well as the dynamic interactions between malignant cells and the tumor microenvironment (TME). Standard treatment regimens, including dexamethasone as part of multi-drug protocols, provide symptomatic relief but fail to achieve long-term remission and are often associated with significant side effects.

Among the cellular components of the TME, macrophages have emerged as critical regulators of MM pathogenesis, progression, and resistance to therapy. The interplay between MM cells and macrophages is increasingly recognized as a driving force behind immune evasion and disease persistence. CD47, an immune checkpoint molecule that acts as a “don't eat me” signal by binding to signal regulatory protein alpha (SIRP α) on macrophages, is consistently overexpressed on MM cells. This makes CD47 a promising therapeutic target to restore macrophage-mediated clearance of malignant cells.

In this study, we addressed the limitations of conventional dexamethasone therapy—specifically its poor pharmacokinetics and non-specific tissue distribution—by employing a liposomal encapsulation strategy. Our data demonstrate that liposomal dexamethasone outperforms the free drug formulation in several preclinical settings. We observed that dexamethasone treatment led to an enrichment of the MM stem cell-like compartment, a population associated with therapy resistance, disease relapse, and invasiveness. Concurrently, we observed an upregulation of both pro-phagocytic, “eat me,” e.g. calreticulin (CALR) and anti-phagocytic, “don't eat me,” e.g. CD47 signals. This dual modulation suggests that CD47 upregulation may serve as a resistance mechanism, enabling MM cells to evade immune-mediated clearance.

Interestingly, the effects of dexamethasone on CD47 expression differed between MM subpopulations. While mature, CD138^{pos} cells exhibited increased CD47 expression following treatment, CD138^{neg} stem cell-like cells showed a relative downregulation.

We hypothesize that this differential expression may sensitize the stem-like compartment to immune clearance, while CD47-overexpressing mature cells may provide a protective microenvironment.

To enhance the targeting of both bulk and stem-like MM cells, and to disrupt immune checkpoint-mediated resistance, we evaluated anti-CD47 therapy alone and in combination with liposomal dexamethasone. While anti-CD47 monotherapy exerted a measurable anti-myeloma effect, the combination therapy demonstrated a more pronounced impact, particularly on the stem cell-like population. This combinatorial approach significantly enhanced macrophage-mediated phagocytosis *in vitro*. Additionally, dexamethasone treatment induced an anti-inflammatory macrophage phenotype, consistent with previous findings, potentially facilitating a more proactive macrophage role in malignant cell clearance when used alongside anti-CD47 therapy.

In summary, our data support the benefit of combining anti-CD47 therapy with liposomal dexamethasone as a strategy to overcome drug resistance and immune evasion in multiple myeloma.

Zusammenfassung

Contributions: This abstract has been kindly translated in German by Henrike Jacobi, affiliated with the department of Hematology, Oncology, Hemostaseology and Stem Cell Transplantation, University Hospital RWTH Aachen, Aachen, Germany

Das Multiple Myelom (MM) ist eine proliferative Erkrankung der Plasmazellen im Knochenmark. Trotz aller Behandlungsfortschritte gibt es bisher keine Heilung für das MM und die Prognose ist aufgrund der fast 100-prozentigen Rückfallrate nach der Erstlinientherapie sehr schlecht. Vor allem der Bestand an schlecht charakterisierten stammzellähnlichen Zellen und eine dynamische Interaktion zwischen malignen Zellen und der Tumormikroumgebung (englisch: Tumor micro environment, TME) begründen die hohe Aggressivität des MM. Standardbehandlungen, vor allem mit Dexamethason, meist in Kombination mit anderen Medikamenten, helfen zwar symptomatisch, aber nicht auf lange Sicht und kommen zudem häufig mit erheblichen Nebenwirkungen einher.

Als Bestandteil der zellulären Komponenten des TME, wurden Makrophagen als kritische Regulatoren der MM-Pathogenese, Progression und Therapieresistenz identifiziert. Mehr und mehr wird das Zusammenspiel zwischen MM-Zellen und Makrophagen als treibende Kraft hinter der Immunevasion und Erkrankungspersistenz angesehen. Das Immuncheckpoint-Molekül CD47 fungiert als ein "friss mich nicht"-Signal, indem es an das signal regulatory protein alpha (SIRP α) auf Makrophagen bindet, und ist einheitlich auf MM-Zellen überexprimiert. CD47 ist daher ein vielversprechendes therapeutisches Ziel, um die Makrophagen-Clearance der MM-Zellen wiederherzustellen.

In dieser Arbeit wurden zunächst die Limitationen – besonders die mangelhafte Pharmakokinetik und nicht-spezifische Gewebeverteilung – der konventionellen Dexamethasontherapie mithilfe einer liposomalen Einkapselungsstrategie untersucht. Es konnte gezeigt werden, dass liposomales Dexamethason die freie Form in mehreren präklinischen Experimenten übertrifft.

Diese Arbeit zeigt zusätzlich, dass Dexamethasonbehandlung zu einer Anreicherung der stammzellähnlichen MM-Zellen führt, eine Population, die besonders mit Therapieresistenz, Rückfallrate und Aggressivität assoziiert ist. Zudem konnte sowohl eine Hochregulation pro-phagozytischer "friss mich"-Signale (z.B. Calreticulin) als

auch anti-phagozytischer “friss mich nicht”-Signale (z.B. CD47) beobachtet werden. Diese duale Modulation suggeriert, dass die CD47-Hochregulation ein Resistenzmechanismus der MM-Zellen sein könnte, der ihnen erlaubt, der Immunclearance zu entkommen.

Interessanterweise unterschieden sich die Effekte von Dexamethason auf die CD47-Expression je nach MM-Subpopulation. Reife CD138^{pos} Zellen zeigen eine erhöhte CD47-Expression nach der Behandlung, während die stammzellähnlichen CD138^{neg} Zellen eine relative Downregulation zeigten. Unserer Hypothese nach, werden so die stammzellähnlichen Zellen sensibler für die Immunclearance, während die reifen Zellen eine protektive Mikroumgebung schaffen.

Um die Therapieeffekte auf beide MM-Zellarten – die reifen und stammzellähnlichen – zu maximieren, wurde eine anti-CD47-Therapie alleine und in Kombination mit liposomalem Dexamethason untersucht. Schon die anti-CD47-Monotherapie zeigte eine messbare anti-Myelom Wirkung, während mit der Kombinationstherapie zusätzlich zu einer stärkeren Wirkung, auch ein Effekt auf die stammzellähnliche MM-Zellpopulation nachgewiesen werden konnte. *In vitro* steigerte die Kombinationstherapie signifikant die makrophagenvermittelte Phagozytose. Übereinstimmend mit publizierten Daten, induzierte die Dexamethasonbehandlung einen anti-entzündlichen Makrophagenphänotyp, der in einem Ansatz mit anti-CD47-Behandlung möglicherweise zu einer proaktiveren Rolle der Makrophagen in der malignen Immunclearance führt.

Zusammengefasst zeigen unsere Daten, dass die Kombinationstherapie aus anti-CD47 und liposomaler Dexamethasontherapie eine valide Strategie ist, um Resistenz und Immunevasion im Multiplen Myelom zu umgehen.

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1 INTRODUCTION

1.1 MULTIPLE MYELOMA

Multiple myeloma (MM) is second most prevalent hematological malignancy characterized by clonal expansion of plasma cells in the bone marrow (BM). In advanced stages, extramedullary dissemination of malignant cells may occur (Bladé J, 2022). The disease originates from post-germinal center B cells following lineage commitment (Kazandijan D, 2016). A hallmark of the disease is the production of monoclonal proteins (M-proteins) detectable in blood and urine, which contribute to the clinical manifestations of end-organ damage characterized by the hypercalcemia, renal insufficiency, anemia, and bone lesions, collectively referred to as CRAB criteria (Kyle RA, 2005). Despite advances in treatment, MM remains largely incurable, with the majority of patients eventually experiencing relapse and developing resistance to therapy. The disease predominantly affects individuals between the ages of 65 and 75, accounting for approximately 10–15% of hematologic malignancies within this age group and contributed to 21.9% of hematological cancer-related mortality in the United States in 2022 (Siegel RL et al., 2023). Extensive research is ongoing to elucidate the molecular pathogenesis of MM, with particular focus on immune system dysregulation and the tumor microenvironment. Although the advent of novel immunotherapies—including immunomodulatory drugs (IMiDs), monoclonal antibodies, and T cell–redirecting therapies—has significantly improved outcomes, the heterogeneity of the disease remains a major clinical challenge. The selection of optimal therapy, appropriate treatment timing, and personalized approaches remain critical yet complex aspects of MM management (Malard F et al., 2024).

1.1.1 Epidemiology and predisposition

Multiple myeloma accounts for 1% of all cancers and 15% of hematological malignancies (Hungria VT et al., 2017). Its global incidence has risen over the past four decades, particularly among specific age groups, largely due to advancements in diagnostic techniques and increased disease awareness. The highest incidence rates have been reported in Australia and New Zealand (4.86/100,000), North America (4.74/100,000), and Northern Europe (3.82/100,000). Based on Surveillance, Epidemiology, and End Results (SEER) data, the age-adjusted incidence between

2015 and 2019 was 7.1 per 100,000 population (Huang J et al., 2022; Costa LJ et al., 2017). MM is more prevalent in men, with an incidence of 8.8 per 100,000. MM also exhibits one of the most pronounced ethnic disparities among cancers. A study by Waxman and colleagues demonstrated that the incidence of MM is twice as high in African Americans compared to European Americans, and nearly three times higher among those under 50 years of age (Waxman AL et al, 2010). In 2022, the estimated global mortality rate from MM was 1.14 per 100,000 (Huang et al., 2022). Median survival for relapsed MM patients has improved significantly, from 12 months in 2000 to approximately 24 months in more recent years (Kumar et al., 2008). According to the International Agency for Research on Cancer (Ferlay J et al., 2024), five-year prevalence rates range from 1.5–4.3 per 100,000 in Africa and Asia, to 20–31.3 per 100,000 in Europe and the United States, respectively. These improvements in survival align with the introduction of novel therapies, including proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs), such as bortezomib, thalidomide and lenalidomide (Kumrak SK et al., 2008).

The etiology of MM remains elusive, with both genetic and environmental factors thought to contribute to disease development. Established risk factors for MM include age, gender, and ethnic background (Sergentanis TN et al., 2015). Occupational exposures to certain chemicals, such as benzene and toluene (de Roos A et al., 2018), pesticides (Merhi M et al., 2007), and exposure to ionizing radiation exceeding 4 Gy (Preston D et al., 1994), autoimmune diseases, and chronic inflammatory conditions (McShane et al., 2014), have all been associated with an elevated risk of MM. Although MM is not typically classified as a hereditary disorder, rare familial cases have been reported. Notably, individuals with a first-degree relative diagnosed with monoclonal gammopathy of undetermined significance (MGUS) exhibit increased relative risks of developing MGUS, 2.8-fold, MM, 2.9-fold, Waldenström macroglobulinemia, 4.0-fold, and chronic lymphocytic leukemia, 2.0-fold (Lynch HT et al., 2001). Genome-wide association studies (GWAS) have identified over 20 susceptibility loci linked to MM risk, implicating germline genetic variations, particularly in autophagy-related genes and polymorphisms associated with myelocytomatosis oncogene (MYC) activation as potential contributors to disease initiation (Mitchell JS et al., 2016; Clavero E et al., 2023).

1.1.2 Diagnosis, risk stratification and clinical characteristics

Multiple myeloma is part of a spectrum of disorders known as plasma cell dyscrasias, which encompass the asymptomatic premalignant stage of MGUS and smoldering myeloma (SMM) as well as malignant entities, such as plasma cell leukemia and MM itself. In MGUS, the annual risk of progression to MM or a related plasma cell disorder is approximately 1% annually (Landgren O et al., 2013), whereas SMM carries a cumulative 15-year progression rate of 73% (Kyle RA et al., 2007). The International myeloma working group (IMWG) established consensus diagnostic criteria, updated in 2014 and defined MGUS by the presence of <30 g/L of serum M-protein, <10% clonal BM plasma cells in the BM and the absence of MM related end-organ damage. SMM is characterized by either ≥ 30 g/L of serum M-protein or $\geq 10\%$ BM infiltration by clonal plasma cells, again in the absence of end-organ damage. MM defining events are presented as CRAB criteria, as well as the presence of an M-protein, and/or clonal plasma cell infiltration (Rajkumar SV et al, 2014). To aid in prognostication, the International Staging System (ISS) for MM is widely used to stratify risk in newly diagnosed patients (D'Agostino M et al, 2022). Treatment response in MM is routinely evaluated after each cycle of chemoimmunotherapy, primarily through the assessment of serum M- protein levels (Greipp PR et al., 2005). Minimal residual disease (MRD), a key prognostic marker, is assessed in the bone marrow using high-sensitivity techniques, with detection threshold $\geq 10^{-5}$. Sustained MRD negativity, defined as the absence of detectable disease over a 6- to 12-month period has been associated with improved long-term outcomes (Kumar S et al., 2016).

1.1.3 The biology of multiple myeloma

Multiple myeloma is a genetically complex and heterogenous malignancy that arises through a multistep process involving both neoplastic transformation and alterations in the BM microenvironment. During naïve B-cell maturation antigen stimulation within germinal centers triggers class-switch recombination and somatic hypermutation. These processes, while essential for antibody diversification, can introduce DNA damage. If repair mechanisms fail, off-target mutations and chromosomal rearrangements may occur, potentially driving the oncogenic transformation of B cells (Gonzales D et al., 2007).

1.1.3.1 Multiple myeloma genetic background

Multiple primary and secondary genetic events including chromosomal translocations, aneuploidies, gene mutations, and epigenetic aberrations play critical roles in the initiation and progression of MM (fig. 1) (Kumar SK et al, 2017).

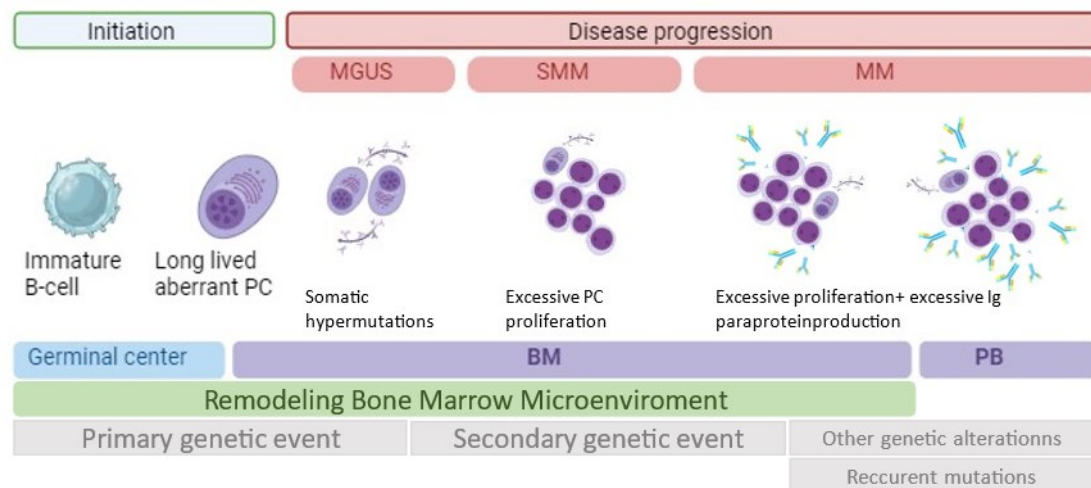


Figure 1. MM development and progression. MM represents the final stage of multistep neoplastic transformation of plasma cells (PCs). In the initial stage, MGUS BM PCs acquire primary genetic alterations that confer a proliferative advantage. This progresses to SMM, characterized by a higher number of clonal PCs in the BM and a moderate increase Ig level. As the disease advances, PCs accumulate secondary genetic mutations that enhance their malignant potential, leading to substantial PC expansion in the BM and excessive Ig secretion into the bloodstream. In later stages, MM may evolve into extramedullary disease. Additional genetic events throughout this progression can further drive MM development and aggressiveness. Adapted from Pinto V, et al, 2020. Created with biorender.com.

Primary initiating events typically involve translocations affecting the immunoglobulin heavy chain (IGH) locus on chromosome 14 and a recurrent set of partner genes, resulting in the overexpression of oncogenes driven by the potent IGH enhancer (table 1). For instance, the t(4;14)(p16;q32) translocation, observed in approximately 10–15% of MM patients, is associated with poor prognosis and reduced overall survival (Morgan GJ et al., 2012). Another major category of initiating events includes hyperdiploidy, characterized by the presence of multiple trisomies. While trisomies are generally associated with favorable outcomes, exceptions linked to poorer prognosis include trisomies involving chr 17, 18, and 21. Conversely, hypodiploidy is associated with adverse outcomes and significantly reduced survival (Chretien ML et al., 2015). Secondary genetic events, which typically arise during disease progression, include copy number abnormalities such as chromosomal deletions and gains that affect key signaling pathways (Walker BA et al., 2015). Some of these aberrations, such as

deletion of the short arm of chromosome 17 (del(17p)), are strongly associated with adverse outcomes. When del(17p) co-occurs with a mutation in the tumor suppressor gene TP53, resulting in biallelic inactivation, its impact on prognosis is even more profound (Corre J et al., 2021). Although, these mutations are often detected at relapse, evidence suggests they may already exist within subclonal populations at the time of diagnosis. Consequently, early identification and characterization of these aggressive subclones are essential for stratifying patients at high risk of disease progression. Representative genetic aberrations are summarized in table 1.

Table 1. Representative genetic aberrations according to the disease stage progression.
Adapted from Pinto V, et al, 2020.

Primary genetic events			Secondary genetic events		Additional genetic alterations		
IgH translocations	t(4;14)	t(8;14)	Deletions	del(17p)	Recurrent mutations	NRAS	DIS3
	t(11;14)	t(14;16)		del(1p)		KRAS	PB1
	t(6;14)	t(14;20)		del(13q)		BRAF	CDKN2C
						TRAF	FAM46C
						CYLD	EGR1
						TP53	MAX
						IRF4	
Aneuploidies: Hyperdiploidy	chr 3,5,7,9, 11, 15, 19, 21		Gains	gain (1q) amp 1q21	Other genetic mutations	Deregulated MYC expression Abnormal miRNA expression Secondary rearrangements	
			Monosomies	chr 13, 14, 17			

1.1.3.2 Bone marrow microenvironment

Bone marrow tumor microenvironment (TME) plays a crucial role in promoting MM progression by facilitating immune evasion and contributing to drug resistance. It comprises both noncellular components, such as extracellular matrix (ECM) proteins, secreted growth factors, and cytokines, and a diverse array of cellular elements (fig. 3). These include stromal cells, osteoblasts, osteoclasts, endothelial cells, lymphocytes, dendritic cells, monocytes, macrophages, and myeloid-derived suppressor cells (MDSCs), all of which collectively support MM development and survival (Giannakoulas, N et al., 2021).

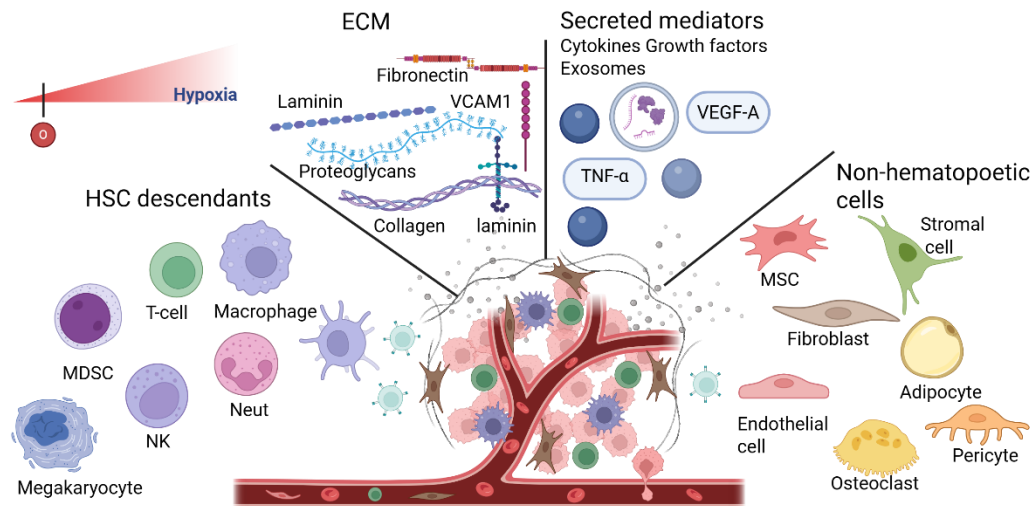


Figure 3. BM microenvironment in MM. Main components of bone marrow microenvironment contributing to tumor growth, evasion and drug resistance can be broadly categorized into four main components: 1. Immune cells (HSC descendants) consisting of macrophages, T-cells, dendritic cells, neutrophils, NK cells, myeloid-derived suppressor cells (MDSC) and megakaryocytes. 2. Bone resident cells of non-hematopoietic origin, including osteoclasts, osteoblasts, stromal cells, mesenchymal stromal cells, fibroblasts, etc. 3. Secreted mediators that include cytokines, growth factors and exosomes. 4. Components of ECM such as fibronectin, laminin, VCAM-1, proteoglycans. Created with biorender.com.

Stromal cells and ECM are essential for establishing the premetastatic niche, which attracts infiltrating malignant plasma cells through cell-cell contacts, such as C-X-C motif chemokine ligand 12 (CXCL12) – C-X-C motif chemokine receptor 4 (CXCR4) interactions (Martínez-Moreno M et al., 2016). The interaction between MM cells and TME stimulates the secretion of numerous growth factors and cytokines, including interleukin (IL) 6, insulin-like growth factor 1 (IGF-1), B-cell activating factor (BAFF), a proliferation inducing ligand (APRIL), tumor necrosis factor alpha (TNF- α) and vascular endothelial growth factor (VEGF). These factors promote MM cell growth, proliferation, and migration (Hideshima T et al., 2016). MM cells also engage with ECM components, such as fibronectin, vascular cell adhesion molecule 1 (VCAM1), and proteoglycans, via adhesion molecules such as CD38 and heparan sulfate proteoglycan CD138. These interactions enhance MM cell survival and proliferation and contribute to the development of cell adhesion-mediated drug resistance (Neri P and Bahlis NJ., 2012). Once the MM-supportive microenvironment is established, malignant cells reprogram resident BM cells. Endothelial cells, macrophages, stromal cells, and other BM components participate in forming the vascular niche, which supports MM proliferation and survival, and shields cells from cytotoxic agents. Pro-angiogenic factors such as VEGF family members, monocyte chemoattractant protein-1 (MCP-1), TNF- α , and IL-

8 further promote neovascularization and disease progression (Jephcote C et al., 2020). An imbalance in the osteoblast-to-osteoclast ratio favors osteoclast activation and bone resorption. MM cell dormancy is closely associated with proximity to osteoblasts; however, remodeling of the endosteal niche by osteoclasts leads to reactivation and displacement of dormant MM cells (Lawson MA et al., 2015). Additionally, osteoclasts contribute to immunosuppression by increasing anti-apoptotic protein expression (Holthof LC et al., 2020). MM-derived exosomes further modulate the microenvironment, stimulating premetastatic niche formation and enhancing drug resistance (Wang J et al., 2014, Wortzel I et al., 2019). Immune cells within the BM microenvironment also contribute to MM progression by enabling immune evasion. IL-6 and transforming growth factor-beta (TGF- β) impair conventional CD4⁺ T-cell proliferation, fostering a T helper (Th) 17-skewed response. Th17 cells secrete IL-17 and IL-10, which suppress immune surveillance and promote osteoclastogenesis, thereby contributing to MM associated bone disease (Prabhala RH et al., 2010; Noonan K et al., 2010). The expansion of regulatory T cells (Tregs), MDSCs, and tumor-associated macrophages (TAMs) maintains an immunosuppressive microenvironment. TAMs, which are recruited via CXCR4–CXCL12 signaling, express characteristic markers such as CD163 and CD206. These cells fail to produce TNF- α in response to lipopolysaccharide (LPS), have impaired phagocytic and antigen-presenting capacities, and secrete immunosuppressive cytokines such as IL-6 and IL-10, collectively leading to inhibition of T-cell and natural killer (NK) cell activity (Berardi S et al., 2013, Beider K et al., 2014). Furthermore, NK cell dysfunction is commonly observed during MM progression, characterized by reduced cytotoxicity, decreased expression of degranulation markers, and upregulation of inhibitory receptors such as programmed cell death protein 1 (PD-1) (Pazina T et al., 2021). Lastly, dendritic cells display impaired antigen presentation due to the influence of IL-6 and TGF- β in the local microenvironment (Leone P et al., 2015).

1.1.3.3 Tumor associated macrophages

Macrophages are highly plastic immune cells capable of sensing and responding to microenvironmental cues in a tissue-specific manner. As key players in immune surveillance, they recognize a wide range of stimuli, including antigens of various origins, immune complexes, cell-derived mediators, and signals associated with apoptotic or necrotic cell death. In response, macrophages undergo polarization into

one of two opposing functional states: the pro-inflammatory, classically activated M1 phenotype, or the anti-inflammatory, alternatively activated M2 phenotype. Furthermore, macrophages exhibit remarkable phenotypic plasticity, allowing them to adapt and shift between functional states in response to changing microenvironmental signals. (Martinez FO, 2014; Sica A, 2012; Novak ML, 2013). M2 macrophages are characterized by distinct gene and protein expression profiles. In response to various external stimuli, they polarize into different subtypes: M2a, M2b, M2c, and M2d, each with specialized immunoregulatory functions (fig. 4) (Ferrante CJ, 2012).

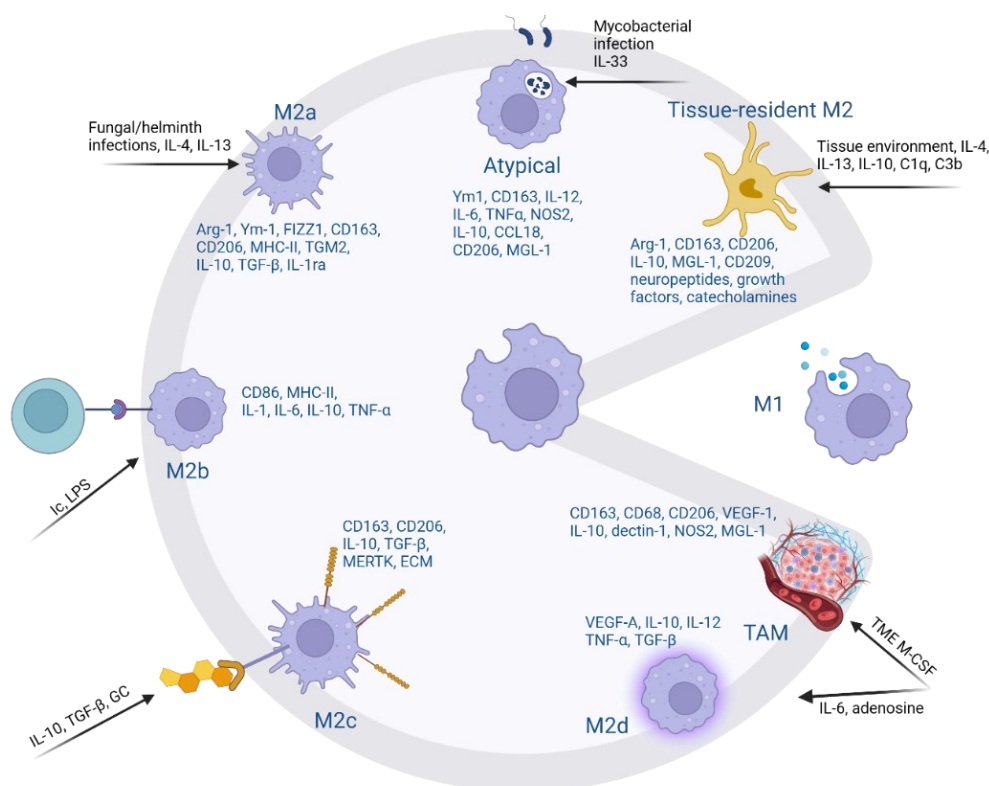


Figure 4. Overview of macrophage polarization states. Adapted from *Röszer T*, 2015. The figure shows most prevalent M2 activation states, with markers associated with distinct phenotypes. The upstream signal inducers are labeled with arrows. Abbreviations are defined in the text.

M2a macrophages, induced by IL-4 and IL-13, show reduced phagocytic and antimicrobial activity due to low nitric oxide production. (Martinez FO, 2013; Hesse M, 2001). M2b macrophages, stimulated by immune complexes and LPS, modulate adaptive immunity through B-cell interaction. (Wang Q, 2010; Ferrante CJ, 2013; Anderson CF, 2002). M2c macrophages, activated by IL-10, TGF-β, or glucocorticoids, exhibit strong anti-inflammatory and pro-fibrotic properties, including apoptotic cell

clearance (Shapouri-Moghaddam A, 2018; Zizzo G, 2012). M2d macrophages, also known as tumor-associated macrophages (TAMs), are driven by IL-6 and adenosine (Wang Q, 2010; Ferrante CJ, 2013). They are recruited to the tumor microenvironment through chemotactic signals such as C-C motif chemokine ligand 12 (CCL12) and monocyte-colony stimulating factor (M-CSF). Here, they release elevated levels of anti-inflammatory IL-10 and reduced levels of pro-inflammatory cytokines IL-12, IL-1 β , TNF α , and IL-6. TAMs also contribute to tumor angiogenesis by releasing pro-angiogenic mediators such as VEGF (Mantovani A, 2004; Condeelis, 2006; Ferrante CJ, 2013). Given their immunosuppressive and pro-tumorigenic functions, TAMs are increasingly recognized as potential therapeutic targets and prognostic biomarkers in malignancies. Several clinical studies have demonstrated that high BM infiltration by M2-polarized macrophages is associated with poor prognosis and reduced response to chemotherapy and correlates with unfavorable clinical outcome and increased relapse rate (Zheng Y et al., 2009, Chen X, 2017). TAMs constitute approximately 10% of infiltrating cells in the MM tumor microenvironment, with a promoting role in MM cell survival and proliferation through the secretion of key cytokines, notably IL-6 and IL-10, and distinct expression of CD163 and CD206, reflecting their phenotype. (Petty AJ et al., 2019; Kim J et al., 2012; Kumari N et al., 2016). TAMs also contribute to angiogenesis indirectly, by releasing pro-angiogenic factors including VEGF, FGF2, chemokines, and matrix metalloproteinases (MMPs), and directly, by either vasculogenic mimicry or through integration into tumor vasculature (Aras S et al., 2012, Scavelli C et al., 2008 Kim J, 2009). Immune suppression mediated by inhibition of cytotoxic T-cell activity, T-cell proliferation and activation through cytokine secretion and immune checkpoint inhibition represents an important mechanism driving MM progression (Fransen LE et al., 2019, Zheng, Y et al., 2009, Winkler IG et al., 2010). Unlike healthy macrophages, TAMs exhibit impaired phagocytic capacity toward MM cells (Kim D et al., 2012). MM cells overexpress cluster of differentiation 47 (CD47), an immune checkpoint molecule that protects them from macrophage-mediated phagocytosis (Kathora S et al., 2021). CD47 is a surface glycoprotein of the immunoglobulin superfamily that interacts with ligands including integrin, thrombospondin 1, and signal regulatory protein α (SIRP α or CD172a). The CD47–SIRP α interaction transmits a “don’t eat me” signal, inhibiting phagocytosis. Emerging evidence identifies CD47–SIRP α as a critical immune checkpoint in MM and other malignancies (Jiang Z et al., 2021). Consequently, monoclonal antibodies targeting

this axis are under active preclinical investigation and represent a major focus of this work. Beyond immune evasion, TAMs promote MM cell survival and drug resistance through both signaling and direct cell contact. Co-culture studies have demonstrated that macrophages protect MM cells from melphalan-induced apoptosis via P-selectin glycoprotein ligand-1 (PSGL-1)/P-selectin and ICAM-1/CD18 interactions (Zheng Y et al., 2013). Additionally, macrophage-derived IL-1 β and proinflammatory surface markers contribute to bortezomib resistance (Beyar-Katz O et al., 2019), while macrophage-induced B-cell activating factor (BAFF) expression on MM cells activates the NF- κ B pathway, further inhibiting bortezomib-induced apoptosis (Chen J et al., 2017). Furthermore, co-culture with macrophages increases MM cell viability under dexamethasone or lenalidomide treatment, underscoring their role in therapeutic resistance (Zheng Y et al., 2009; Beider K et al., 2014). Overall, elucidating the mechanisms through which macrophages confer protection and promote survival of MM cells offers valuable insights for developing targeted therapeutic strategies in MM.

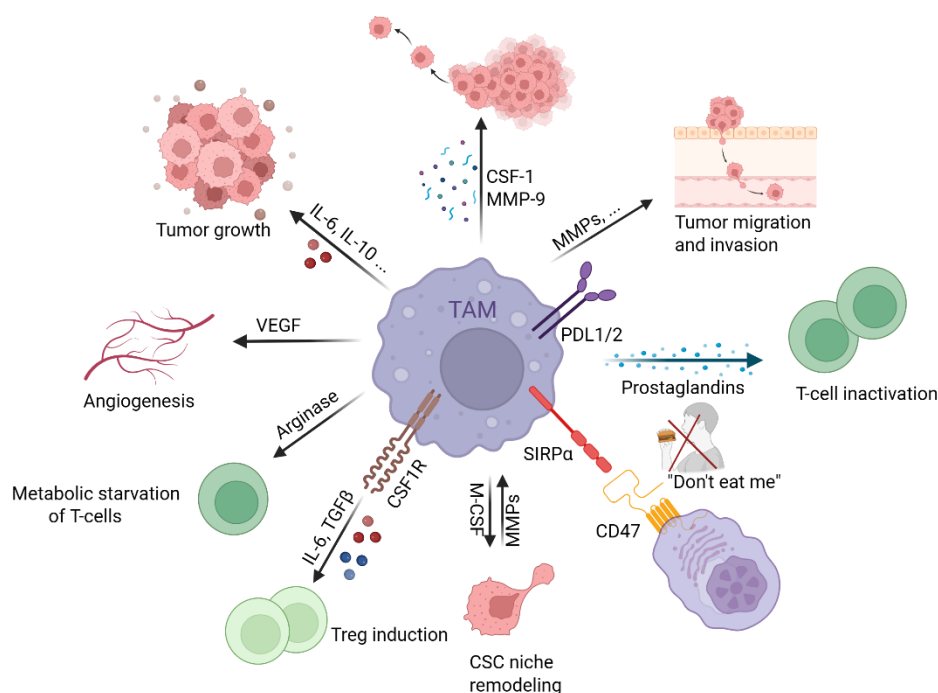


Figure 5. Main tumor supportive roles of TAMs. Adapted from Chen Y et al, 2019. Specific secretion profile and/or surface protein interaction of TAM trigger different processes of tumor development. Figure is representing the impact of TAMs on tumor growth, invasion, metastasis, angiogenesis, cancer stem-cell induction, T-cell inactivation, Treg induction, T-cell starvation, and immune checkpoint inhibition via CD47-SIRP α interaction.

1.1.3.4 Cancer stemness in Multiple Myeloma

MM is a post-germinal center B-cell malignancy characterized by the accumulation of clonal plasma cells. Despite recent therapeutic advances, MM remains incurable, as most patients eventually relapse or develop treatment resistance. Increasing evidence points to a distinct subpopulation of tumor cells with the capacity to initiate and sustain tumor growth. These cells exhibit features of self-renewal, pluripotency, unlimited proliferative potential, and resistance to chemotherapy and radiotherapy. Referred to as cancer stem cells (CSCs), they are believed to drive tumor heterogeneity and contribute to relapse and drug resistance. A subpopulation of poorly differentiated progenitor cells is believed to underlie relapse and therapeutic resistance in MM. These cells exhibit CSC-like properties and are commonly referred to as MM stem cell-like cells (Matsui W et al., 2008, Franqui-Machin R et al., 2015). However, a consensus on the definitive phenotype of MM CSCs remains elusive. Clonogenic MM stem-like cells have been shown to exhibit an immunophenotype similar to memory B cells (Davies FE et al., 2000). Most malignant B-cell lymphomas, chronic lymphocytic leukemia, and MM are thought to originate from germinal or post-germinal center B cells, given their shared somatic hypermutation and immunoglobulin class-switch recombination status (Gonzales D et al., 2007). Syndecan-1 or CD138, a marker expressed by most MM plasma cells, is specific to terminally differentiated plasma cells and is absent in early B-cell stages and proliferative plasmablasts (Chilosi M et al., 1999, Calame et al., 2001). MM cell lines, such as RPMI 8226 and NCI-H929, harbor a minor, <5% CD138^{neg} subpopulation with enhanced clonogenicity and stem-like features compared to the CD138^{pos} population (Matsui W et al., 2004, Tarte et al., 2002). Several putative MM stem cell phenotypes have been described, including CD138^{neg}CD19^{pos}CD27^{neg} (Matsui W et al., 2004), CD138^{pos}CD34^{pos}B7⁻H1^{pos} (Kuranda K et al., 2010), and CD38^{high}CD45^{neg} (Kim D et al., 2012). In addition, side population (SP) cells, characterized by their ability to efflux Hoechst 33342 dye via ABC transporters, have been identified in MM and other malignancies. These cells are implicated in drug resistance, tumor recurrence, and stemness (Baeten JT et al., 2019). MM SP cells typically reside in a dormant, G0/G1 phase, express pluripotency-associated genes such as SOX2, OCT4, NANOG, KLF4, and exhibit close interactions with the TME (Wen,J et al., 2015, Yang Y et al., 2015). Notably, non-side population (NSP) cells can convert into SP cells under hypoxic conditions through TGF- β signaling (Jakubikova J et al., 2011). Aldehyde dehydrogenase (ALDH), a member of the

NAD(P)⁺-dependent enzyme family involved in the metabolic detoxification of aldehydes from substrates such as ethanol and cytotoxic drugs, is a key mediator of drug resistance in MM CSCs (Vasilou et al., 2004). ALDH plays a central role in CSCs regulation of differentiation, apoptosis, proliferation, and chemoresistance (Xu X et al., 2015). In MM, the dominant isoform ALDH1 is associated with enhanced stemness, drug resistance, and increased tumorigenicity in NOD/SCID mouse models (Zhou W et al., 2014). CD138^{neg} ALDH1^{pos} subpopulations in human MM cell lines demonstrate greater clonogenic potential compared to CD138^{pos} ALDH1^{pos} counterparts (Reghunathan et al., 2013). Key signaling pathways implicated in MM stemness and therapeutic resistance include Wnt, Hedgehog (Hh), Notch, and PI3K/Akt/mTOR. The Wnt pathway promotes MM cell proliferation, migration, invasion, drug resistance, and CSC maintenance (Yang Y et al., 2013). Hh signaling contributes to sustaining the undifferentiated CSC state (Peacock JD et al., 2007). Retinoic acid receptor alpha 2 (RAR α 2), overexpressed in both CD138^{pos} plasma cells and MM CSC activates Wnt and Hh pathways, thereby enhancing self-renewal, proliferation, migration, and drug resistance (Derksen PW et al., 2004, Yang et al., 2013). Notch signaling is constitutively active in MM, driving proliferation, apoptosis resistance, and osteoclastogenesis. The PI3K/Akt/mTOR pathway supports tumor growth and contributes to the inactivation of tumor suppressor genes (Guo W et al., 2021). CD24, a cell adhesion molecule involved in antigen-dependent B-cell activation and proliferation, has been implicated in cancer invasion and metastasis. A recent study identified CD24 expression as a marker of tumor-initiating cells and found it to be associated with poor prognosis in multiple myeloma (Gao M et al., 2020). Bruton's tyrosine kinase (BTK), a therapeutic target in MM, positively regulates OCT4, SOX2, NANOG, MYC stemness genes via the Akt/Wnt/ β -catenin axis, promoting self-renewal, clonogenicity, and drug resistance (Yang Y et al., 2015). Although CSCs exhibit high tumorigenic potential and activation of stemness pathways, direct evidence for the existence of a single tumor-initiating cell within MM tumors remains lacking. Nevertheless, continued investigation of these cells is crucial for advancing our understanding of MM CSC-specific functional properties and for guiding the development of more effective therapeutic strategies.

1.1.4 Therapeutic strategies in Multiple Myeloma

Treatment of newly diagnosed MM patients is guided by eligibility for high-dose therapy and autologous stem cell transplantation (ASCT), which in turn depends on the patient's functional status, age, and comorbidities. The goal of first-line therapy, with or without ASCT, is to achieve deep response while minimizing the toxicity. Treatment response is categorized by the International Myeloma Working Group (IMWG) into minimal, partial, very good partial, complete, and stringent complete response (CR). Patients achieving CR may be further stratified based on MRD status, with MRD negativity correlating with improved overall survival (OS) (Kumar S et al., 2016, Paiva B et al., 2015). For ASCT candidates, exposure to stem cell-toxic agents such as melphalan and IMiDs should be limited to no more than four cycles to preserve optimal stem cell yield. In fit patients, triplet regimens are preferred over doublets due to superior response rates, progression-free survival (PFS), and OS (Kumar S et al., 2012).

1.1.4.1 Induction therapy for ASCT-Eligible patients

For fit patients with newly diagnosed MM, induction with a triple regimen includes IMiDs, PIs and corticosteroid, typically lenalidomide-bortezomib-dexamethasone (RVd) (Durie BG et al., 2017). Double regimens bortezomib-dexamethasone (Vd) or lenalidomide-dexamethasone (Rd) are used for those with limited tolerance (Rajkumar SV et al., 2010; Harousseau JL et al., 2010). In frail or ASCT-ineligible patients attenuated RVd (O'Donnell EK et al., 2018) or daratumumab-lenalidomide-dexamethasone (DRd) (Fancon T et al., 2019) improve outcomes. Carfilzomib-based regimens (KRd) show promise in high-risk patients (Dimopoulos MA, 2015). Ongoing clinical trials, such as CASSIOPEIA and GRIFFIN are investigating quadruplet regimens including daratumumab (Moreau P et al., 2019, Voorhees P et al., 2018). In addition, bone-modifying agents, denosumab or zoledronic acid are recommended for all newly diagnosed patients.

1.1.4.2 Consolidation and maintenance therapy

Consolidation therapy increases depth of response following induction therapy and typically involves ASCT and/or single or multidrug regimens after ASCT (Kapor P et al., 2013). Maintenance therapy typically involving lenalidomide or bortezomib is used in both ASCT-eligible and ASCT-ineligible patients (Nooka AK et al., 2014) and has

been shown to prolong PFS and OS, particularly in high-risk patients (Palumbo A et al., 2015).

1.1.4.3 Relapsed and refractory Multiple Myeloma

Deeper therapeutic responses in relapsed or refractory multiple myeloma are associated with improved relapse-free survival (RFS). Treatment selection is guided by several factors, including patient performance status, disease aggressiveness, cytogenetic profile at relapse, prior therapies, and the depth and duration of previous responses. In general, triplet regimens incorporating at least one novel agent class are preferred in cases of relapse following lenalidomide or bortezomib exposure (Dingli D et al., 2017). For patients who deferred initial transplantation, ASCT may still be a viable option at relapse (Branagan A et al., 2020). Detailed treatment algorithms have been published and are available in Branagan et al., 2020.

1.1.4.4 Emerging treatment strategies

The therapeutic paradigm of MM has evolved markedly with the introduction of novel treatment strategies aimed to improving response rates, overcoming mechanisms of drug resistance, and selectively targeting defined molecular pathways. The clinical integration of monoclonal antibodies against CD38 and signalling lymphocytic activation molecule F7 (SLAMF7), in combination with IMiDs and PIs, has resulted in improved PFS among patients with relapsed or refractory MM. In recent years, novel immunotherapeutic strategies targeting surface antigens such as B-cell maturation antigen (BCMA), G protein-coupled receptor class C group 5 member D (GPRC5D), Fc receptor homolog 5 (FcRH5), CD138, CD48, CD56, and CD74 have emerged as promising approaches in the management of MM. Cellular therapies, including chimeric antigen receptor (CAR) T-cell and CAR natural killer (CAR NK) cell therapies, have gained considerable clinical interest, particularly in the context of relapsed or refractory MM. Notably, two CAR T-cell products—idecabtagene vicleucel and ciltacabtagene autoleucel—have received FDA approval for this indication. Monoclonal antibodies such as daratumumab, elotuzumab, and isatuximab have been approved for clinical use since 2015, marking a pivotal advancement in MM therapy. Subsequently, antibody–drug conjugates (ADCs), including belantamab mafodotin, AMG 224, and MEDI2228, have been developed. Among these, belantamab mafodotin

has received regulatory approval for the treatment of relapsed or refractory MM following at least four prior lines of therapy.

Bispecific antibodies, including teclistamab and elranatamab, targeting BCMA on myeloma cells and CD3 on T lymphocytes, respectively have demonstrated overall response rates exceeding 60% in patients with relapsed or refractory disease. Notably, bispecific antibodies have also shown efficacy in patients who have previously received anti-BCMA therapies and CAR T-cell therapy (Utku Aygüneş, 2024). Among the most promising strategies for activating antitumor immunity is immune checkpoint blockade. Immune checkpoints are inhibitory pathways that maintain homeostasis by preventing excessive immune activation and protecting normal tissues from immune-mediated damage (Pardoll DM, 2012). It is well established that numerous malignancies, including MM, exploit these pathways by overexpressing checkpoint ligands to evade immune surveillance. Blockade of these interactions can reverse the immunosuppressive TME and enhance antitumor immune responses (Sun J et al., 2019).

Notable examples of T-cell checkpoint inhibition include disruption of the interaction between PD-1 and its ligand PD-L1, as well as between cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and B7-1/B7-2 (Buchbinder et al., 2016). In addition to T cells, macrophages also express immune checkpoint receptors such as SIRP α , which interacts with CD47 expressed on various mature cell types. Engagement of the SIRP α –CD47 axis initiates a signaling cascade that suppresses phagocytosis, functioning as a "don't eat me" signal (Weiskopf et al., 2017). CD47 overexpression has been identified as a hallmark of several cancers, including MM (Zhao et al., 2018). Currently, a variety of therapeutic approaches are under investigation to inhibit the CD47–SIRP α immune checkpoint, including the development of anti-CD47 antibodies, anti-SIRP α antibodies, and soluble SIRP α fusion proteins (Yang et al., 2019).

Dexamethasone, an essential component of nearly all MM treatment regimens, is associated with significant off-target adverse effects including systemic immunosuppression, osteoporosis, and diabetes (Deshantri et al., 2016), as well as unfavorable pharmacokinetics that necessitate high and frequent dosing (Metselaar et al., 2005).

Liposomes have emerged as one of the most extensively studied and widely used nanocarriers for targeted drug delivery across a range of diseases. Their inherent advantages, such as biocompatibility, low toxicity, high drug-loading capacity, and tunable release profiles, confer their suitability for pharmaceutical applications. (Abu Lila et al., 2017). Encapsulation within liposomes protects therapeutic agents from premature inactivation, degradation, and dilution in systemic circulation (Ulrich, 2002).

Specifically, liposomal encapsulation of dexamethasone has been shown to increase local drug concentrations at sites of inflammation while reducing systemic exposure to toxicity-sensitive organs (Anil et al., 2019). However, challenges in the pharmaceutical development of liposomal formulations remain, particularly in the areas of quality assurance and manufacturing cost (Sercombe et al., 2015).

1.2 OBJECTIVE OF THE THESIS

This dissertation is conducted within the framework of the GRK program “Tumor-Targeted Drug Delivery” and is primarily focused on the investigation of novel therapeutic strategies using MM disease models. MM remains an incurable malignancy, largely due to treatment refractoriness and frequent disease relapse. Consequently, the development and evaluation of innovative therapeutic approaches are critical to improving patient outcomes and achieving durable disease control. The aim of this work is to explore novel drug formulations and combination therapies designed to target both malignant plasma cells and TAMs, thereby disrupting their supportive role in MM progression, treatment resistance, and immune evasion.

To achieve this, the following specific objectives have been defined:

1. To evaluate the impact of liposomal encapsulation of dexamethasone, compared to its free form currently used in clinical practice, on BM targeting and niche remodelling *in vivo*.
2. To investigate the combined effects of liposomal dexamethasone and CD47 blockade on the targeting of malignant cells and the TME, with the goal of overcoming therapeutic resistance.
3. To identify and functionally characterize MM stem cell-like population and evaluate their susceptibility to the proposed therapeutic strategies.

2 MATERIALS AND METHODS

2.1 MATERIALS

Material is listed in the following tables containing information about chemicals, buffers, solutions, predesigned kits, cell culture media, cytokines, antibodies, cell lines, consumables, instruments, and software.

2.1.1 Cell culture – media, active components, and cell line

Table 2. Cell lines and culture media

	Company	Cat. #
MM1.S, gfp+/luc+	FenicsBio	CL-1622
HS-27α	ATCC	CRL-2496
MM1.S	ATCC	CRL-2974
RPMI 8226	ATCC	CCL-155
HEK 293T	ATCC	CRL-3216
Roswell Park Memorial Institute (RPMI) 1640 medium	Pan Biotech	P-04-180447
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	31885-023
Isove's Modified Dulbecco's Medium	Thermo Fischer Scientific	12440053
X-VIVO™ 15 medium	Lonza	BEBP02-061Q
Monocyte Attachment medium	PromoCell	C-28051
BIT9500 Serum Substitute	Stem Cell Technologies	09500
ELAREM™ Perform Human Platelet Lysate	PL BioScience	PE10611
Fetal Calf Serum (FCS)	PAN Biotech	P30-3306
Pancoll human	PAN Biotech	P04-601000
Penicillin-Streptomycin Gibco	Thermo Fischer Scientific	15140122
Methycellulose MethoCult H4230	Stem Cell Technologies	04230

Table 3. Cytokines for cell culture

	Company	Cat. #
rh M-CSF	Immunotools	11343113
rh GM-CSF	Immunotools	11343127
rh IL-6	Immunotools	11340066
rh TNFα	Immunotools	11343015
rh IFNγ	Immunotools	11343536
rh IL-4	Immunotools	11340045

2.1.2 Reagents and solutions

Table 4. Reagents and solutions.

	Company	Cat. #
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide	Sigma	M-2128
Ampuwa ultrapure water for injection	Fresenius-Kabi	B101211
Trypan Blue	Roth	CN76.1
Trypsin/EDTA solution 0.05%/0.02%	PAN Biotech	P10-023500
Fetal Bovine Serum Premium Forte	PAN Biotech	P30-3312
Agarose	ThermoFisher	16500-500
Cell dissociation Buffer Enzyme-Free PBS-based	ThermoFischer	13151014
Ethanol, absolute for analysis	Merck	100983
Erylyse-Puffer pH 7,2-7,4	Morphisto	15038.01000
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	8043.1
Ethidiumbromid	ThermoFisher	I5585-011
dNTPs	ThermoFisher	18427013

Alt-R S.p. HiFi Cas9 Nuclease V3	IDT	1081060
Alt-R CRISPR-Cas9tracrRNA	IDT	1072532
Dimethyl sulfoxide DMSO	Serva	20385.01
1,2-Distearoyl-<i>sn</i>-glycero-3-phosphocholine (DSPC)	Lipoid GmbH	816-94-4
1,2-distearoyl-<i>sn</i>-glycero-3-phosphoethanolamine-<i>N</i>-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000)	Lipoid GmbH	247925-28-6
1,2-distearoyl-<i>sn</i>-glycero-3-phosphoethanolamine-<i>N</i>-[amino(polyethylene glycol)-2000]-<i>N</i>-(Cyanine 7)	Avanti Polar Lipids	810892C
Cholesterol	Whatman® Anodisc	
DNA ladder	ThermoFisher	104880058
Random primer	Promega	C118A
SYBR select Master Mix	ThermoFisher	4472908
Dream Taq DNA polymerase (5U/μl)	ThermoFischer	EP0701
MgCl₂	Bio-Rad	170-8872
DTT	ThermoFisher	Y00147
RNasin Ribonuclease Inhibitor	Promega	N261B
M-MLV Reverse Transcriptase	ThermoFisher	28025013
Methylthiazolyldiphenyl-tetrazoliumbromid (MTT)	ThermoFischer	M-2128
Formalin	Carl Roth	P087.3
Forene/Isofluorane 100% (v/v)	Piramal	09714675
Isopropanol	Roth	AE73.2
Phosphate buffered saline	Gibco	10010-015

5x First Strand Buffer	ThermoFisher	Y02321
Cotrim-ratiopharm Ampoule SF 480mg/5ml	Ratiopharm	A75338A
CASY ton	OLS OMNI Life Science	5651808
Dexamethasone	Sigma Aldrich	D4902-25MG
Dexamethasone 21-phosphate disodium salt	Sigma Aldrich	D1159
Bortezomib	Millipore	5.04314.0001
Phytohemagglutinin L	PAN Biotech	P05-01010
Phosphate buffered saline – powder (PBS)	ThermoFischer	P04-36050P
β-mercaptoethanol	ThermoFischer	21985023
IVISbrite D-I Luciferin Ultra, RediJect	PerkinElmer	770505

2.1.3 Kits

Table 5. Kits

	Company	Cat. #
NucleoSpin RNA	Macherey-Nagel (MN)	740955
NucleoSpin RNA XS	MN	740902
APC Annexin V Apoptosis Detection kit with 7-AAD	BioLegend	640930
Amaya P3 Primary Cell 4D-Nucleofector X kit S	Lonza	V4XP-3032
pHrodo Cell labeling Kit for Incucyte™ Phagocytosis Assay	Sartorius	4649
Aldefluor™ kit	StemCell Technologies	01700
Human Lambda ELISA kit	Bethyl Laboratories	E88-116

2.1.4 CRISPR-Cas9 crRNA sequences

Table 6. Small guide RNAs

Target gene	Exon	Strand	Sequence 5' - 3'	On-target score ¹	Off-target score ²
CD47	2	-	CTACTGAAGTATACGTAAAG TGG	73	67
	4	-	TAAATATAGATCCGGTGGTA TGG	51	76

2.1.5 Antibodies and staining

Table 7. Antibodies and stainings

	Species	Company	Application
CD138 (MI15) PB	Human	BioLegend	FACS
CD47 (CC2C6) APC / FITC	Human	BioLegend	FACS
CD47 FITC (CC2C6)	Human	BioLegend	FACS
Anti-Calreticulin (EPR3924) PE	Human	Abcam	FACS
CD24 (ML5) BV786	Human	BD bioscience	FACS
CD90 (Thy1) BV421	Human	BioLegend	FACS
CD163 (RM3/1) PE/Cy7	Human	BioLegend	FACS
CD319 (162.1) APC	Human	BioLegend	FACS
CD206 (15-2) BV421 / APC	Human	BioLegend	FACS
CD86 (IT2.2) PE-Dazzle 594		BioLegend	FACS
Human True Stain FcX™	Human	BioLegend	FACS

¹ Indicates the predicted editing performance of the gRNA at the intended target site. A higher value is better.

² Higher value indicates lower off-target risk Score is from 0-100.

CD204 (7C9C20) PE	Human	BioLegend	FACS
CD14 (63D3) AF 700 / (M5E2) BV510	Human	BioLegend	FACS
SIRPα (15-414) APC	Human	BioLegend	FACS
CD11b (M1/70) PB	Human	BioLegend	FACS
CD73 FITC		BioLegend	FACS
CD105 PE		BioLegend	FACS
CD38 (H-B7) PE/Cy7	Human	BioLegend	FACS
CD80 (2D10) BV510	Human	BioLegend	FACS
Gr-1 (RB6-8C5) PB	Mouse	BioLegend	FACS
Ly6G (1A8) PB	Mouse	BioLegend	FACS
CD11c (N418) PB	Mouse	BioLegend	FACS
Ly6C (HK1.4) PE	Mouse	BioLegend	FACS
CD11b (M1/70) PerCP/Cy5.5	Mouse	BioLegend	FACS
F4/80 (BM8) PE/Cy7	Mouse	BioLegend	FACS
CD206 (C068C2) BV605	Mouse	BioLegend	FACS
CD163 (S15049I) APC/Cy 7	Mouse	BioLegend	FACS
SIRPα (P84) PE	Mouse	BioLegend	FACS

2.1.6 Consumables

Table 8. Consumables

Consumable	Company	Cat. #
Cell culture Flask T-75	Greiner Bio-One	660 175
Cell culture Flask T-175	Greiner Bio-One	658 170
Cell culture Flask T-25	Sarstedt	83.3910.002
Nunc Easyflask 25 Filt Nunclon delta	ThermoFischer	156367
96 well-plates	Greiner Bio-One	651 180
24 well-plates	Greiner Bio-One	662160
6 well-plates	Greiner Bio-One	10536952
12-Well Cell Culture inserts 0,4µm	cellQART	9310412
Pipette Tip 10µl	Sarstedt	70.3010
Pipette Tip 100µl	Sarstedt	70.3030
Pipette Tip 1000µl	Sarstedt	70.3050.020
Serological Pipette 5ml	Sarstedt	86.1253.001
Serological Pipette 10ml	Sarstedt	86.1254.001
Serological Pipette 25ml	Sarstedt	86.1685.001
Falcon tube 15ml	Sarstedt	62.554.100
Falcon tube 50ml	Sarstedt	62.547.254
Casy Cups	Omni Life Sciences	5651794
Eppendorf Tube 1,5ml	Sarstedt	72.706.006
PCR stripes	Sarstedt	79.982.002
MicroAmp Optical Fast 96-Well Reaction plate	ThermoFisher	4481190
Multichannel pipette 200µl	VWR	613-5816
Multichannel pipette 10µl	VWR	613-5818

2.1.7 Devices

Table 9. Devices

Device	Model	Company
Flow Cytometer	Navios/Gallios	Beckman Coulter
Flow Cytometer	Fortessa	Becton Dickinson (BD)
FACS sorter	Aria	Becton Dickinson (BD)
Microplate reader	Multiskan FC	ThermoFisher
Fluorescence microscope	Evos FL Autoimaging System	ThermoFisher
Nucleofector	4D-Nucleofector System	Lonza
Light microscope	AE2000	Motic
Cell Counter	CASY	Omni Life Sciences
RT-qPCR machine	Fast 7500 Real-Time PCR System	ThermoFisher
Spectrophotometer	Nanodrop 2000c	ThermoFisher
Plate reader	RT2100c Microplatereader	Rayto
Thermocycler	PeqStar 96X	PeqLab
Fluorescence microscope	IncuCyte 2020A	Sartorius
X-ray irradiation	MultiRad225	Precision
Imaging device	U-CT OI	MILabs B.V.

2.1.8 Software and database

Table 10. Software and database

Software	Company/Website	Version
Excel	Microsoft	MSO 365 (16.61.1)
Powerpoint	Microsoft	MSO 365 (15.56)
Incucyte Base Analysis Software	Sartorius	2020A
Fiji	National Institutes of Health, USA	1.53q
FlowJo	BD Life Sciences	10.8.1
Prism	GraphPad	10.0.0
tic@lab	a-tune Software AG	3.0
Word	Microsoft	2005
BioRender	https://biorender.com	-
Pubmed	https://pubmed.ncbi.nlm.nih.gov	-
Primerblast	https://www.ncbi.nlm.nih.gov/tools/primer-blast/	-
IDT design tool	https://eu.idtdna.com/site/order/designtool/index/CRISPR_PREDESIGN	
GSEA	Broad institute UC San Diego	4.3.3

2.2 METHODS

2.2.1 Cell biology methods

2.2.1.1 Cultivation of cell lines

Human MM1.S, RPMI8226 MM and stroma HS-27 α cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin / streptomycin (P/S), while MM1.S GFP^{pos}luc^{pos} cells were additionally supplemented with 0,05 mM 2-mercaptoethanol.

HEK-293T cells were maintained in DMEM supplemented with 10% FCS, 1% P/S, and were passaged every 20-48h. All cells were cultured at 37 °C, in a humidified atmosphere with 5% CO₂, and medium was replaced three times per week. Adherent cells were trypsinized and passaged at a ratio of 1:2 (HS-27 α) or 1:5 (HEK-293T) upon reaching full confluency. For trypsinization, the medium was first removed, and cells were washed with PBS (e.g., 10 mL for a T-75 flask). PBS was then aspirated, and 4 mL of 1 $\times$ trypsin was added, followed by incubation at 37 °C for 3–5 minutes. Trypsin was inactivated by adding three volumes of medium containing 10% FCS. The cell suspension was centrifuged at room temperature (RT) for 5 minutes at 1200 rpm. Suspension cells were directly collected and centrifuged under the same conditions. Cell pellets were resuspended in fresh medium and counted using a CASY cell counter according to the manufacturer's instructions, or manually using a Neubauer chamber. MM cells were maintained at a density of 5 $\times$ 10⁵ cells/mL.

2.2.1.1.1 Multiple myeloma patient samples

BM aspirates from MM patients, undergoing diagnostic BM biopsy were collected at RWTH University Hospital Aachen, following approval by the institutional ethics committee (EK 206/09). Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

2.2.1.1.2 Peripheral blood healthy donor samples

Leukocyte reduction system cones from healthy donor samples were kindly provided by Transfusion medicine department, RWTH University Hospital. Ethics committee

approval and informed consent were obtained from all donors according to the Declaration of Helsinki.

2.2.1.2 Cryopreservation

For long-term storage of cell lines and primary mononuclear cells (MNCs), aliquots containing $1-5 \times 10^6$ cells were pelleted and resuspended in 1ml of freezing medium, consisting of FCS supplemented with 10% DMSO, then slowly frozen to -80°C using a Mr. Frosty container, at a cooling rate of $1^{\circ}\text{C}/\text{min}$.

2.2.1.3 Separation of mononucleated cells

MNCs were isolated using Pancoll density gradient centrifugation from heparinized BM samples of MM patients or from white blood cells (WBCs) obtained via leukoreduction of healthy donor blood. Samples were diluted 1:5 in cold PBS supplemented with 2% FCS, layered over 15 mL of Pancoll, and centrifuged at 1200 rpm for 30 minutes at room temperature (RT) without brake. After centrifugation, cells separated into distinct layers: erythrocytes aggregated at the bottom, the mononuclear cell layer - buffy coat was located at the interface above the Pancoll, and diluted plasma remained on top. The MNC layer was carefully collected into a new Falcon tube, washed twice with cold PBS containing 2% FCS, passed through a $100\ \mu\text{m}$ cell strainer, and counted using a CASY cell counter.

2.2.1.4 Differentiation of peripheral blood mononuclear cells to macrophages

Human peripheral blood (PB) MNCs were seeded in Nunclon™ Delta surface culture plates and resuspended in Monocyte attachment medium at a concentration of 6-10 million cells/ml. Monocytes were isolated by incubating the MNCs for 30 min at 37°C , followed by washing and removal of non-adherent cells. The adherent monocytes were then incubated overnight (o/n) at 37°C in X-Vivo medium, supplemented with 0,5% P/S. On the following day (D1) recombinant human (rh) M-CSF was added at final concentration of 50ng/ml. The M-CSF supplemented medium was replaced three times per week until day 7–11 to generate M0 macrophages. For polarization to M2

macrophages, from day 7 onward, cells were cultured for three days in RPMI 1640 medium supplemented with 10% human platelet lysate, 0,5% P/S and 10 ng/ml IL-4. For M1 polarization, starting from day 1, the medium was supplemented with 50 ng/ml GM-CSF and from day seven, respective medium was supplemented with 100 ng/ml LPS, and 20 ng/ml IFN γ . Differentiated macrophages were characterized by flow cytometric analysis of surface marker expression of CD14, CD204, CD163, CD206, CD86.

2.2.2 Drug treatments

Dexamethasone and DMSO were purchased as stated in table 3. Daratumumab was kindly provided by university hospital pharmacy. Hu5F9-IgG2 δ and Her2-IgG2 δ antibodies were produced and generously provided by Dr. Katja Klausz and Prof. Dr. Mathias Peipp from the Division of Antibody-Based Immunotherapy, University medical center Schleswig-Holstein, Campus Kiel, Germany.

PEG-liposomal formulation containing dexamethasone was prepared by our collaboration partner, Sara Mohamed Mamdouh Mohamed Elsafy, from the Institute for Experimental Molecular Imaging, RWTH Aachen. The ethanol injection method was used. Briefly, a lipid mixture, consisting of DSPC (table 2), cholesterol, and DSPE in a molar ratio of 62:33:5 (total conc.37mM) was dissolved in 1ml of ethanol. For fluorescent labelling 0.2 mol% of DSPE PEG (2000)-N-Cy7 (Cyanine 7) was added. The lipid mixture was then injected into 100 mg/ml dexamethasone solution and incubated at 70°C, 100rpm, 30min to facilitate ethanol removal. Empty liposomes were prepared by adding the lipid mixture to water instead of the drug solution. Liposomes were extruded through Whatman® Anodisc inorganic membranes with pore sizes of 200 nm and 100 nm at 60 °C using a Liposofast LF-50 liposome extruder. The resulting liposomes were dialyzed at 4 °C against PBS using a 10 kDa MWCO dialysis membrane for 24 hours to remove residual ethanol and unencapsulated dexamethasone. The encapsulated dexamethasone content was quantified using high-performance liquid chromatography (HPLC). The mean particle size was determined via dynamic light scattering (Malvern Zetasizer Nano series, UK). Finally, the liposomal formulation was sterilized by filtration through a 0.22 μ m syringe filter prior to injection.

2.2.3 2D in vitro cell culture

MM1.S GFP^{pos}Luc^{pos} cells were co-cultured with macrophages derived from healthy donors using 0.4 µm transwell inserts, which prevent direct cell–cell contact. M0, M1, and M2 macrophages were differentiated as previously described, using 6-well or 12-well plate formats. Upon assembling the macrophages to the well bottom, transwell inserts were placed into the wells, and MM1.S cells were added to the upper chamber at a density of 5×10^5 cells/mL. Free and liposomal dexamethasone treatments, at conc. 2 µM were applied, and the co-cultures were incubated for 72 hours at 37 °C in a humidified atmosphere with 5% CO₂.

2.2.4 In vitro Phagocytosis assay for Incucyte

M0 differentiated macrophages were detached using 5ml of enzyme-free cell dissociation buffer and incubated for 20 minutes at RT, followed by gentle scraping from the flask. Cells were centrifuged at 1200 rpm for 5 minutes at RT, counted, and seeded into a 96-well plate at a density of 40,000 cells per well. Plates were incubated for 30 minutes to 1 hour at 37 °C to allow cell adherence. Human MM1.S GFP^{pos}Luc^{pos} cells were then co-cultured with healthy donor-derived macrophages at an effector-to-target (E:T) ratio of 1:1 under the following treatment conditions: DMSO vehicle control, 2 µM dexamethasone, 10 µg/mL Hu5F9-IgG2δ antibody treatment, Her2-IgG2δ isotype control, and a combination of dexamethasone with Hu5F9-IgG2δ, and daratumumab with Hu5F9-IgG2δ antibody treatment as a control. Each experimental group included three biological replicates and three technical replicates. Control wells containing macrophage or MM1.S monocultures were included to assess baseline fluorescence.

2.2.4.1 Phrodo labeling

Target cells were labeled with pHrodo Cell Labeling dye, at final concentration of 0,5 µg/ml according to the manufacturer's instructions. The labeled cells were then counted, resuspended in X-vivo medium and added to the macrophage-containing wells along with their respective treatments.

Incucyte imaging was initiated 30 minutes after co-culture assembly and continued for 24 hours. Antibody-dependent cellular phagocytosis (ADCP) was evaluated using the Incucyte Analysis Software (Basic Analyzer). In the red fluorescence channel, a detection radius of 20 μm was set to identify MM cells, with a mean intensity threshold of 9,000. Results were quantified as “count per image” in the red channel.

2.2.5 CFU assay

For the colony forming unit (CFU) assay, MM1.S and RPMI8226 cell lines were sorted into two populations: CD138^{pos} and CD138^{neg}. After a recovery period of 2-5 days in culture, cells were treated with 2 μM dexamethasone for 72 hours. Following treatment, cells were washed twice with medium to remove residual drug and then plated in methylcellulose under the following conditions: 1000 CD138^{pos} and 10000 CD138^{neg} cells/mL were seeded into incomplete methylcellulose-based medium supplemented with 10% lymphocyte-conditioned medium. Colonies were counted 14 days after plating.

2.2.5.1 Lymphocyte conditioned medium

MNCs isolated from healthy donor blood via density gradient centrifugation were seeded at a density of 1×10^6 cells/mL in RPMI 1640 medium supplemented with 10% FCS, 1% P/S, and 10 $\mu\text{g/mL}$ phytohemagglutinin-L (PHA-L) and incubated for 72 hours at 37 °C with 5% CO₂. The resulting supernatant was collected, centrifuged, and filtered through a 0.2 μm Millex® filter to remove cells and debris. The medium was stored at –20 °C for future use.

2.3 MOLECULAR BIOLOGY METHODS

2.3.1 RNA extraction

Bulk RNA sequencing was performed on sorted GFP^{pos} MM cells and GFP^{neg}Ly6G^{neg}CD11c^{neg}Ly6C^{pos}CD11b^{pos}F4/80^{pos} macrophages. Following Fluorescence-Activated Cell Sorting (FACS), cells were pelleted and resuspended in recommended volume of RA1 buffer supplemented with TCEP or β -mercaptoethanol.

RNA extraction was carried out according to the manufacturer's instructions. For samples with fewer than 1×10^5 cells, an additional step was included by adding carrier RNA working solution prior to lysate filtration. To adjust RNA binding conditions, a 1:1 volume of 70% ethanol:RA1 buffer was added to the homogenized lysate and mixed thoroughly by pipetting. Depending on the sample size, either the NucleoSpin RNA XS column (for small samples) or the standard RNA column was used. Columns were placed into fresh collection tubes and the lysates were applied to the columns. After centrifugation at $11,000 \times g$ for 30 s, the flow-through was discarded, and the columns were transferred to new collection tubes. To enhance membrane desalting and improve rDNase digestion efficiency, an equal volume (1:1) of membrane desalting buffer (MDB) to RA1 was added to the columns, followed by centrifugation at $11,000 \times g$ for 30 s. For samples processed using the RNA XS kit, 25 μ L of rDNase reaction mix was applied to the membrane and incubated for 15 min at RT with the lid closed. After DNase digestion, the column was washed with 100 μ L of RA2 buffer, incubated for 2 min at RT, and centrifuged for 30 s at $11,000 \times g$. This was followed by two washing steps using 400 μ L and 200 μ L of RA3 buffer, centrifuged for 30 s and 2 min, respectively. RNA was eluted in a 1.5 mL collection tube using 15 μ L of RNase-free water, followed by centrifugation at $11,000 \times g$ for 30 s. For samples processed with the standard NucleoSpin RNA kit, 95 μ L of DNase reaction mix was applied, followed by a 15 min incubation at RT. The columns were then washed with 200 μ L of RAW2 buffer and centrifuged for 30 s at $11,000 \times g$. Two additional washes were performed using 600 μ L and 250 μ L of RA3 buffer, followed by centrifugation for 30 s and 2 min, respectively. After drying the membranes, RNA was eluted into a 1.5 mL collection tube using 40–60 μ L of RNase-free water, depending on the initial cell number. RNA concentration and purity were assessed using a NanoDrop spectrophotometer. All samples were stored at -80°C until further processing.

2.3.2 Determination of nucleic acid concentration

The quality and quantity of the isolated RNA were verified by using a NanoDrop spectrometer by measuring the optical density (OD) of 1 μ L of the sample. Purity was assessed based on the $A_{260/280}$ and $A_{260/230}$ ratios, with expected values around 2.0, for pure RNA samples.

2.3.3 CRISPR-Cas9 gene editing

For CRISPR-Cas9 genome editing, transient transfection of ribonucleoprotein (RNP) complexes was used. Guide RNAs (gRNAs) targeting exon 2 and exon 4 of the CD47 gene (table 6) were designed using the Integrated DNA Technologies (IDT) online tool. The protocol described here is based on the 4D Amaxa Cas9 protein nucleofection system. The first step involves annealing the CRISPR (cr) RNA with the tracrRNA to form a functional guide RNA. This is done by mixing 2.5 μ l of crRNA and tracrRNA, each at a final concentration of 100 μ M, followed by incubation at 95 °C for 5 minutes and cooling at RT for 15–30 minutes. The crRNA:tracrRNA duplex can be stored at –20 °C for up to three months. The next step is the formation of the RNP complex by mixing 1 μ l of Cas9 protein with an equimolar amount of the crRNA:tracrRNA duplex prepared separately for each crRNA. This mixture is incubated for 20–60 minutes at RT and stored on ice until transfection. RNP complexes are stable for up to six months at 4 °C and for more than one year when stored at –80 °C. To prepare the cells for nucleofection, 2×10^6 MM1.S GFP^{pos}luc^{pos} cells were resuspended in nucleofector solution, that comprises of 16.4 μ l SF Cell Line Solution and 3.6 μ l Supplement 1. Then, 1 μ l of the RNP complex and 1 μ l of electroporation enhancer were added to the cell suspension. Nucleofection was performed in a microcuvette strip using the electroporation program DN100 on the 4D Nucleofector device, as described by Fedele et al., 2018. Immediately after the electroporation pulse, the cells were transferred to a 6-well plate containing 5 ml of fresh medium. Electroporation efficiency was assessed 24- and 48-hours post-transfection via flow cytometry. Subsequently, three distinct populations: CD47^{hi}, CD47^{dim} and CD47^{neg} were sorted with FACS.

2.3.4 Library preparation and RNA sequencing

RNAseq was conducted in collaboration with Dr. Julia Franzen from the Genomics facility at the Interdisciplinary center for clinical research (IZKF), RWTH University of Aachen. Library preparation was carried out using QuantSeq 3'mRNA-Seq library preparation kit FWD for Illumina. Sequencing was performed on an Illumina NextSeq 500 platform using single end reads. An average of 130 million reads/sample were attained. The *ex vivo* experiments were performed on bulk-sorted GFP^{pos} MM cells and GFP^{neg}CD11c^{neg}Gr-1^{low}Ly6C^{pos}CD11b^{pos}F4/80^{pos} macrophages. Each treatment group was subjected to RNAseq in triplicates, at least.

2.4 FLOW CYTOMETRY METHODS

Fluorescently conjugated antibodies against cell surface antigens, commonly referred to as cluster of differentiation (CD) markers, used in following experiments are listed in table 5 in Materials section.

2.4.1 Surface marker staining

For the characterization of *in vitro* differentiated macrophages, surface staining with CD14, CD204, CD163, CD206, CD86 and SIRPα was performed. For the characterization of *in vitro* differentiated macrophages, surface staining with CD14, CD204, CD163, CD206, CD86, and SIRPα was performed. To assess surface marker expression on MM cells, CD47, CALR, CD138, CD38, and CD24 were used. Anti-murine antibodies employed for sorting and characterizing mouse macrophages included Ly6G, CD11c, Ly6C, CD11b, F4/80, CD163, and CD206. For staining, the cells of interest were first washed by centrifugation in PBS supplemented with 2% FCS. Subsequently, cells were incubated with 1 µl of Fc block or anti-CD16/32, for human and murine samples respectively for 15 minutes at room temperature in the dark. After washing, antibody mixtures were added at a 1:100 dilution and incubated for 20 minutes at 4°C in the dark. Following incubation, cells were centrifuged and resuspended in cold PBS + 2% FCS for flow cytometry analysis. Unstained controls

and corresponding isotype (IgG) controls were included for each cell type and antibody conjugate.

2.4.2 Evaluation of apoptosis

To distinguish between early and late apoptosis in different experimental setups APC/ or PB stained Annexin V (Ann V) Apoptosis Detection Kit with 7-amino-actinomycin D (7-AAD) was used for flow cytometry. The principle of the assay is based on the externalization of phosphatidylserine (PS) during early apoptosis, while the cell membrane remains intact. This population is identified as AnnV^{pos}7-AAD^{neg}. In contrast, late apoptotic or necrotic cells exhibit compromised membrane integrity, allowing both Annexin V and 7-AAD binding, identifying as AnnV^{pos} 7AAD^{pos}.

Cells were stained according to the manufacturer's instructions. Briefly, cells were pelleted by centrifugation and resuspended in Annexin V binding buffer, followed by incubation for 15 minutes at room temperature in the dark. After staining, additional Annexin V binding buffer was added, and samples were analyzed by flow cytometry. A heat-killed control cells, incubated at 65°C for 10–15 minutes were included as a positive control for cell death.

2.5 EVALUATION OF PHAGOCYTOSIS

Phagocytosis following dexamethasone treatment was evaluated using flow cytometry and fluorescence microscopy. M0 and M2 differentiated macrophages were serum-starved for 3 hours, while MM1.S GFP^{pos}luc^{pos} cells were treated with 2 µM dexamethasone for 72 hours. After macrophage starvation, a direct co-culture was assembled and incubated for 2 hours at 37 °C. The cell suspension was collected, and the adherent cells were washed three times with PBS supplemented with 2% FCS. For flow cytometry, adherent cells were detached using 1× trypsin for 5 minutes at 37 °C, washed, and combined with the respective supernatant. Cells were stained with an anti-human CD14 antibody, filtered through a 25 µm cell strainer, and analyzed via flow cytometry. Doublets were excluded based on FSC and SSC parameters, and CD14^{pos}GFP^{pos} events were considered indicative of phagocytosis. For fluorescence microscopy, macrophages were pre-labeled with LysoTracker™ Red DND-99, a cell-

permeable dye that stains acidic compartments such as lysosomes, according to the manufacturer's protocol. Briefly, macrophages were stained with 75 nM LysoTracker Red DND-99 for 1 hour at 37 °C and subsequently washed three times with PBS + 2% FCS. Co-cultures of macrophages and MM1.S GFP⁺/Luc⁺ cells were established at a 1:1 ratio and incubated for 2 hours at 37 °C. The supernatant was then removed, adherent cells were washed with PBS + 2% FCS, fresh medium was added, and cells were visualized using a fluorescence microscope.

Stained macrophage and MM1.S monocultures were used as negative controls to determine baseline fluorescence. A minimum of 500 objects were counted across three different fields. The phagocytic rate was calculated as the percentage of LysoTracker DND-99⁺/GFP⁺ cells relative to the total number of cells.

2.6 ALDEFLUOR ASSAY

To identify MM stem-cell like cells Aldefluor™ assay kit was used. High-expressing human ALDH cells are characterized as viable stem, progenitor, and cancer precursor cells that can be detected by flow cytometry. Cells with high ALDH activity are characterized as viable stem, progenitor, and cancer precursor cells, and can be detected by flow cytometry. Cells were processed according to the manufacturer's instructions. Detection was performed using the FITC channel of the flow cytometer, with the test sample compared to a control, inhibitor-treated sample to determine basal fluorescence levels. It is essential to perform all stainings—including any combined with antibody labeling—in Aldefluor assay buffer, and to keep samples on ice throughout the procedure to prevent efflux of the Aldefluor reaction product.

2.7 MOUSE EXPERIMENTS

Animal experiments were approved by the local authorities of North Rhine-Westphalia, Germany (LANUV AZ: 81-02.04. 2021.A276). Animals were housed at the Institute of Laboratory Animal Science, RWTH University Hospital, Aachen, in facilities designated by supervisory authorities and maintained according to internationally recognized standards and guidelines established by the Federation of European Laboratory Animal Science Associations (FELASA) and the Society for Laboratory Animal Science

(GV-SOLAS). Room temperature was maintained at 20–24°C, with relative humidity between 45–65%, and a 12-hour light/dark cycle. Food and water were provided *ad libitum* as part of routine care by trained animal caretakers.

Female NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, NOD-scid IL2Rgamma^{null}, NOD-scid IL2Rg^{null}) were obtained from Charles River Laboratories. Mice arrived at 6–8 weeks of age and were allowed to acclimate to the housing conditions for at least one week. Due to their severe immunodeficiency, NSG mice were maintained under specific pathogen-free (SPF) conditions.

For xenograft MM studies, mice underwent total body irradiation using a 0.3 mm copper filter, 226 kV, 10 mA, at a final dose of 240 cGy. This was followed by intravenous tail vein injection of a cell mixture containing 1×10^6 MM1.S GFP^{pos}luc^{pos} cells and 5×10^5 HS-27 α human stromal cells. To prevent infections, 100 μ g/ml cotrimoxazole was added to the drinking water for two weeks post-irradiation. *In vivo* expansion of MM cells was evaluated via bioluminescence tomography combined with computed tomography (μ CT-BLT). Luciferin, the substrate for MM1.S GFP^{pos}luc^{pos} cells was administered intraperitoneally (i.p.) at concentration of 30 mg/ml, and imaging was performed after a 10-minute incubation period.

In studies evaluating the effect of liposomally encapsulated dexamethasone versus free dexamethasone, intravenous (i.v.) treatment began two weeks after transplantation. Doses of 1 mg/kg and 2.5 mg/kg of both free and liposomally encapsulated dexamethasone were administered twice weekly. Respective control groups received 0.9% sodium chloride or empty liposome vehicles. After assessing the impact of free and liposomally encapsulated dexamethasone on MM disease progression, a subsequent experiment was conducted to evaluate the effect of the Hu5F9-IgG2 δ anti-CD47 antibody, either alone or in combination with 1 mg/kg liposomal dexamethasone. In this experiment, 2 mg/kg of Hu5F9-IgG2 δ and the corresponding isotype control, Her2-IgG2 δ were administered i.p. three days post-transplantation. Liposomal dexamethasone treatment commenced in the second week after transplantation and was administered i.v. according to fig.1.

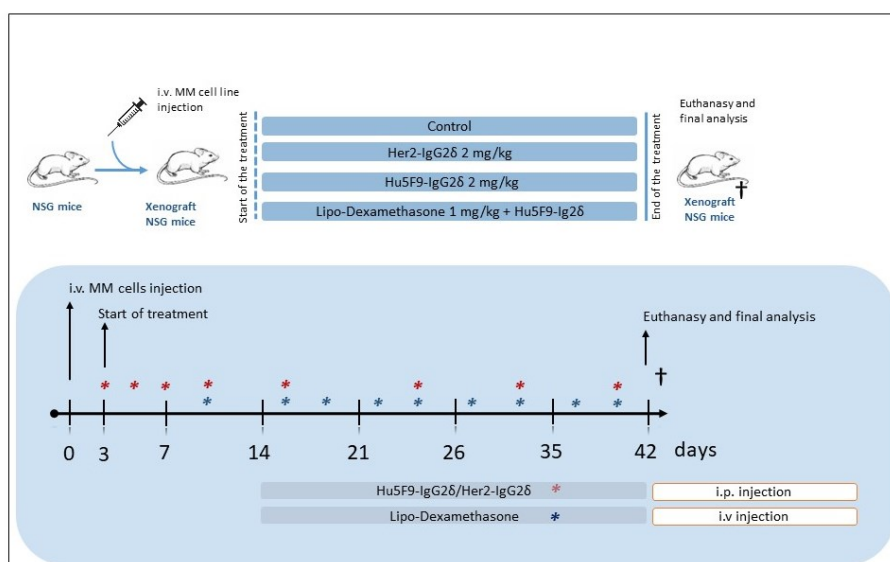


Figure 6. Schematic representation of *in vivo* experimental set-up. *In vivo* testing of Hu5F9-IgG2δ anti-CD47 antibody, alone or in combination with 1 mg/kg liposomal dexamethasone.

The animals were sacrificed and analyzed 4-5 weeks after transplantation to ensure proper comparison of results.

2.7.1 Imaging protocol

The imaging was conducted in a collaboration with Dr. Alexandros Sofias from the department of Experimental Molecular Imaging, Institute for Experimental Molecular Imaging, RWTH Aachen University. For *in vivo* biodistribution studies, animals were placed on a chlorophyll-free diet seven days prior to imaging. Images were acquired by using a hybrid micro-computed tomography and fluorescence tomography (FLT/ μ CT) device (U-CT OI, MILabs B.V., Utrecht, Netherlands). Phantom imaging was performed in advance to validate the acquisition settings before proceeding to *in vivo* imaging. Depilated and anesthetized mice were subjected to a pre-scan and positioned in the designated holder to acquire FLT images, followed by a CT scan to anatomically localize the regions of interest (ROI). Background signal scans were obtained for all animals prior to injection. Following the pre-scan, animals were injected intravenously with Cy7-labeled liposomal dexamethasone using a 30G cannula connected to a sterile polypropylene tube. Imaging was performed 6 hours after administration of the Cy7-labeled liposomes. After the scans, the shaved animals were housed on TEK-fresh

litter to provide better insulation. Hybrid bioluminescent tomography and computed tomography (BLT/CT) imaging was performed following i.p. injection of 150mg/kg luciferin, a substrate for enzyme luciferase, with a 10-minute incubation to monitor MM cell engraftment in the different treatment groups. For all acquisitions, animals were anesthetized with 3% isoflurane and maintained at 1.7% in oxygen-enriched air. The eyes were protected with eye ointment.

2.8 ELISA

Detection of human Ig λ , secreted by MM1.S cells transplanted in our in vivo studies was performed on mouse serum samples according to the manufacturer's protocol. The kit is based on a sandwich ELISA. Briefly, samples were diluted 1:1000 and loaded, along with standards, onto the provided plate pre-coated with anti-human λ antibody. The plate was incubated for 1 hour at RT and then washed. Biotinylated detection antibody was added, incubated for 1 hour at RT, and washed. Next, streptavidin-conjugated horseradish peroxidase (SA-HRP) was added and incubated for 30 minutes at RT to catalyze the colorimetric reaction. After washing unbound HRP from the wells, the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added and incubated for 30 minutes at RT in the dark. The colorimetric reaction produces a blue color, which turns yellow after adding the sulfuric acid stop solution. Absorbance was measured at 450 nm, with background subtraction at 550 nm. Measurements were taken in duplicates, and sample values were calculated from a standard curve generated from adjacent standard dilutions.

2.9 BIOINFORMATICS ANALYSIS

Bioinformatics analysis was performed in collaboration with partners from the Genomic and Proteomic facilities at the Interdisciplinary Center for Clinical Research (IZKF), RWTH University of Aachen. Basic analysis was conducted by Dr. Chao-Chung Kuo, while advanced bioinformatics analysis was carried out by Dr. Mirna Barsoum. Gene set enrichment analysis (GSEA) analysis was performed using GSEA software with the settings described in table 11.

Table 11. GSEA settings applied in MM dataset.

Expression dataset	Upload file of interest: DGEA_xlsx
Gene set database	Human/mouse database of interest
Number of permutation	1000
Phenotype labels	Treatment vs Control
Collapse/Remap to gene symbols	No_collapse
Permutation type	Gene_set
Chip platform	ILLUMINA_Array_MSigDB.v2024.1.Hs.chip

2.10 STATISTICAL ANALYSIS

was performed using GraphPad Prism v.5. For comparisons between two conditions, a t-test was applied. For multiple comparisons, one-way or two-way ANOVA was used, followed by Tukey's or Bonferroni's post-hoc tests for comparisons of three or more conditions. Probability values were defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Data are presented as mean $\pm$ standard deviation (SD), unless stated otherwise.

3 RESULTS

3.1 PATIENT DERIVED MM CD47 EXPRESSION AND DEXAMETHASONE TREATMENT

Increased expression of CD47 on the cell surface has been reported to occur in multiple cancers and results in the escape of phagocytic clearance by macrophages (Huang J et al, 2022). This is considered a hallmark of drug resistance, recurrence, and metastasis in hematological malignancies (Majeti, 2009; Chao MP, 2010; Galli S, 2015). In MM, significant CD47 upregulation was observed on MM plasma cells compared to non-myeloma (Kim D et al., 2012) and MGUS plasma cells (Sun et al., 2020; Rendtlew Danielsen, 2007).

To provide proof of concept, the expression of CD47 on patient-derived myeloma and non-myeloma cells was evaluated. MNCs were isolated from patient BM and PB samples and stained with CD38, CD138, CD319 and CD47 antibodies for subsequent flow cytometric analysis. Myeloma cells were identified as CD38^{pos}CD138^{pos} or CD38^{pos}CD319^{pos} cells and CD47 expression was quantified by mean fluorescence intensity (MFI) (fig. 7A). To evaluate CD47 and calreticulin (CALR) surface expression within CD38^{pos}CD138^{pos} or CD38^{pos}CD319^{pos} MM compartment, patient derived MNCs were subjected to dexamethasone treatment. For this purpose, three MM BM patient samples at first diagnosis were analyzed. MNCs were isolated via density gradient centrifugation, treated with dexamethasone and surface marker expression was determined by flow cytometric analysis (fig 7B+C). Briefly, after selecting viable single cells, we gated on CD38^{pos}CD138^{pos} or CD38^{pos}CD319^{pos} MM cells, and evaluated the expression of CD47^{hi} and CALR. While dexamethasone treatment of two different patient samples did not result in a significant change in malignant cell number (fig. 7D), nor differential surface expression of CD47, (fig. 7E) and CALR (fig. 7F), a trend toward increased CD47 (fig. 7H) and CALR expression (fig. 7I) was observed in one patient sample that showed reduced MM cell numbers upon dexamethasone treatment (fig. 7G).

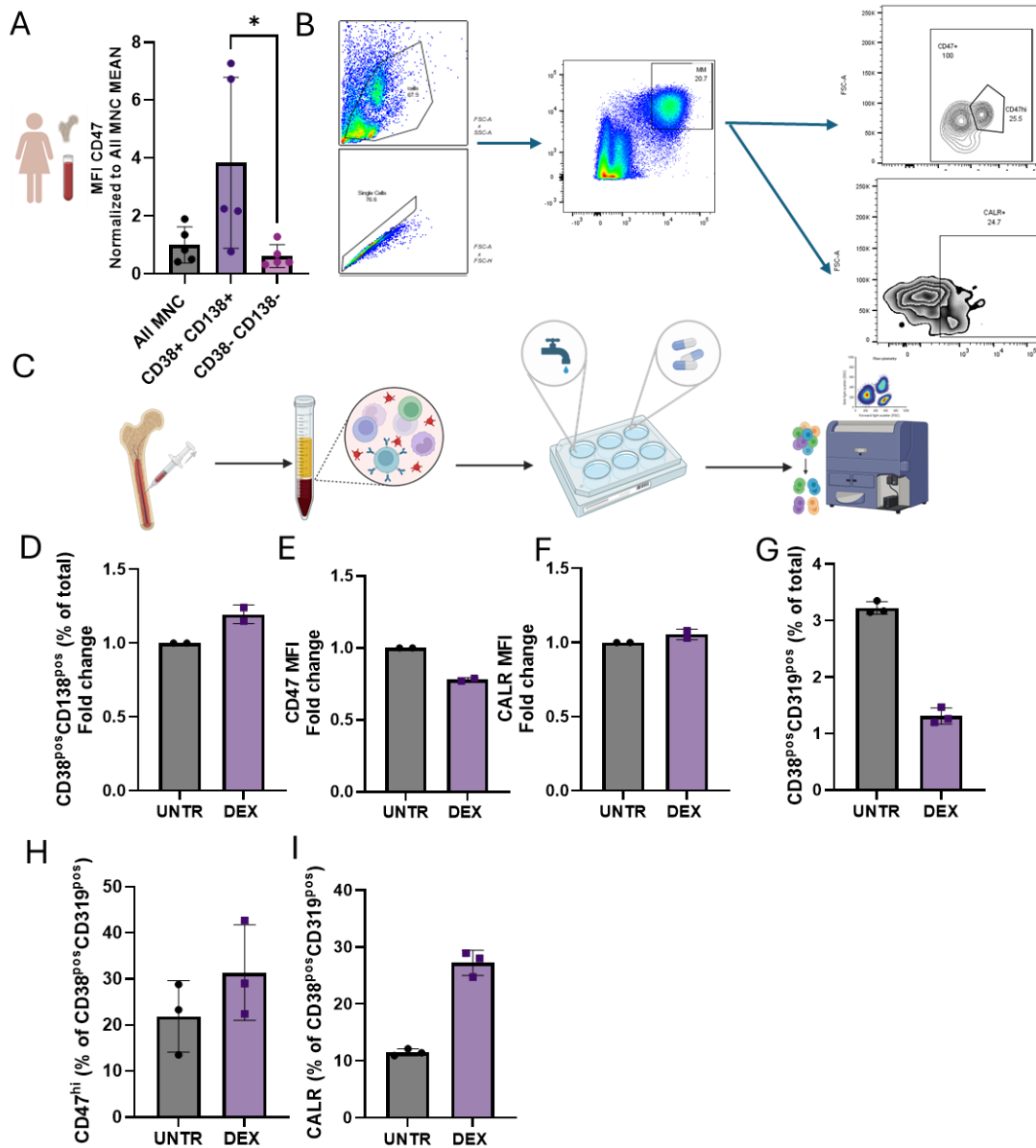


Figure 7. CD47 and CALR expression in MM patient samples. Flow cytometric analysis was performed on patient derived MNCs. Analyses showed that CD47 expression was significantly higher in malignant cell population, compared to non-malignant BM cell compartment (A). Further flow cytometric analyses of patient derived cells that had been treated using 2 μ M dexamethasone were performed as depicted (B) and according to the experimental scheme (C). While no change in MM cell abundance (D), CD47 (E) and CALR (F) surface expression upon treating two patient samples was observed, in the third patient that responded to dexamethasone therapy (G) we observed a trend towards increased CD47 (H) and CALR (I) surface expression. Shown is the mean value of $n=5$ normalized to bulk MNCs expression $\pm$ SD. Dexamethasone treatments were performed at the range of conc. 2-5 μ M. P-value was calculated using one-way Anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Taken together, these data indicate elevated surface expression of CD47 on MM cells and suggest that the upregulation of CD47 and CALR on the cell surface following dexamethasone treatment may contribute to mechanisms underlying dexamethasone-mediated effects and represent a potential therapeutic target.

3.2 DEXAMETHASONE TREATMENT DIFFERENTIALLY REGULATES CD47 EXPRESSION AND SPARES STEM CELL-LIKE CELLS DEFINED BY IMMUNE PHENOTYPE *IN VITRO*

Multiple studies have shown a correlation between the CD47 expression profile and the abundance of stem cell-like cells in MM, with drug resistance and immune evasion (Rastgoo et al., 2020; Yue Y et al., 2022; Gunes EG et al., 2024). The RPMI 8226 and NCI-H929 cell line derived MM stem cell-like cells are described in the literature as a CD138^{neg} population (Matsui W et al., 2004, Hoosen N et al., 2012, Guo W et al., 2021), which makes them easier to assess, compared to patient material. We aimed to monitor the change in CD138, CD47 and CALR positivity upon dexamethasone treatment using two *in vitro* experimental setups. First, MM1.S cells were cultured and treated with 2µM dexamethasone for 72h. After washing, the cells were stained with CD138, CD47 and CALR antibodies and analyzed by flow cytometry. Visual inspection of live and dead cell ratios in histogram plots revealed an increased presence of cell debris at the expense of live cells in dexamethasone-treated versus untreated samples, shown as changes in the gray and yellow population ratios (fig. 8A). To quantify this effect, we applied a live gating strategy (fig. 8B) and observed a significant decrease in live cells following dexamethasone treatment (fig. 8C). Dexamethasone treatment increased the percentages of stem cell-like, CD138^{neg} cells (fig. 8D). The enrichment of CD138^{neg}, stem cell-like population upon treatment was visualized on the histogram plot, by the emergence of a second population, depicted in blue (fig. 8E right). To demonstrate that this enrichment reflects an actual increase in the stem-cell like population, rather than merely a shift in the ratio between CD138^{pos} and CD138^{neg} cells, we calculated the fold change and observed a 10.6-fold increase in cell numbers (fig. 8E left).

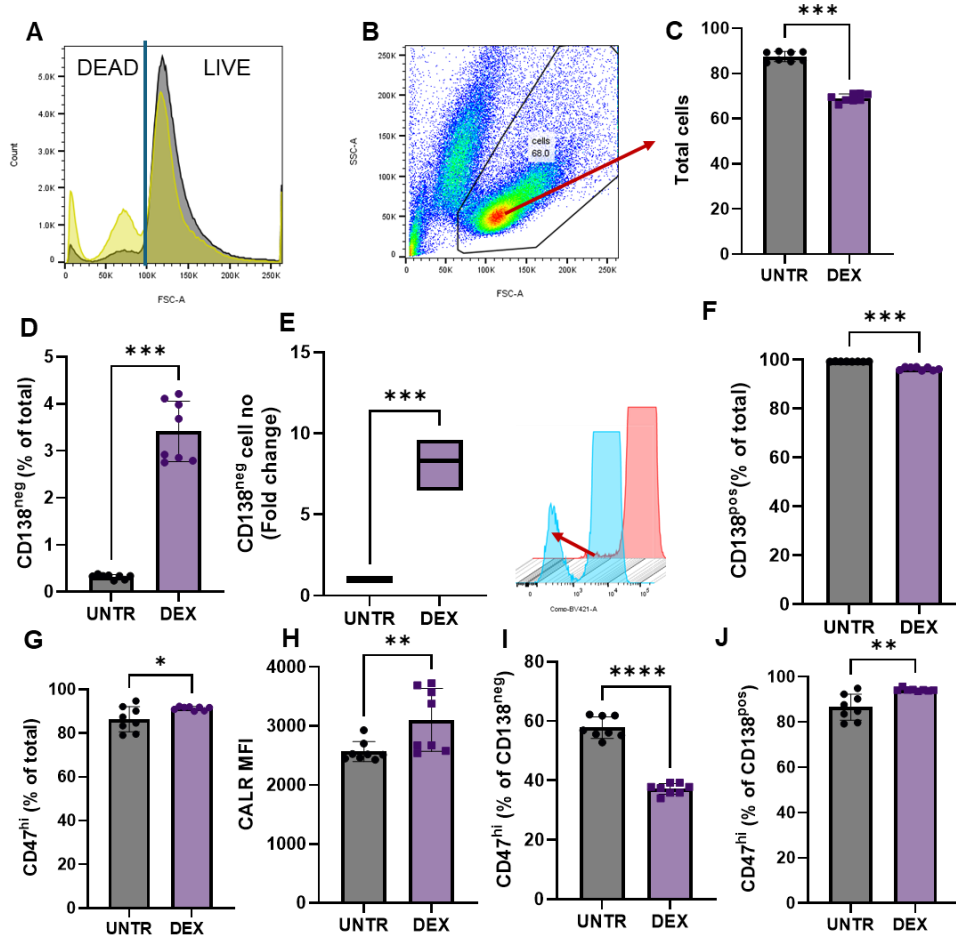


Figure 8. Effect of dexamethasone treatment on MM monoculture. For surface marker expression, SSC-A/FSC-A analysis showed increased accumulation of cell debris upon dexamethasone treatment (A) and this population was excluded from further analysis (B). Hampered cell viability was observed by evaluating total cell numbers upon dexamethasone treatment (C), along with enrichment of CD138^{neg} cell percentage (D) and number (E) while slightly decreasing CD138^{pos} mature MM1.S cell population (F). CD47 (G) and CALR (MFI=median fluorescence intensity) (H) surface expression was higher upon dexamethasone treatment in bulk MM1.S cells. Differential surface expression analyses showed that while in CD138^{neg} compartment CD47 expression decreases upon dexamethasone treatment (I), CD138^{pos} population follows the same trend towards increase CD47 surface expression, as seen in bulk cells (J). Dexamethasone treatments were performed at final conc. 2 μ M. n=8. Shown are the mean values of $\pm$ SD. P-values were calculated using two-tailed student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The CD138^{pos} fraction, that reflects more mature cell population (Guo W et al., 2021) was assessed, as well. Here we observed a subtle effect of dexamethasone treatment on CD138^{pos} cells (fig. 8F). CD47 expression was evaluated by stringent gating of highly expressing CD47 cells, referred to as CD47^{hi}, while CALR expression was assessed based on changes in MFI. Upon dexamethasone treatment, an increase in both CD47^{hi} (fig. 8G) and CALR expression (fig. 8H) was observed when analyzing

bulk MM1.S cells. Interestingly, the expression of CD47^{hi} was significantly decreased within CD138^{neg} cell compartment following treatment (fig. 8I). In contrast, CD138^{pos} cells showed the same trend toward increased CD47^{hi} expression as seen in bulk cells (fig. 8J). Overall, dexamethasone treatment affected MM1.S cells by slightly reducing the total number of viable cells. It enriched the CD138^{neg}, stem cell-like population, both in terms of percentage and absolute cell numbers, while showing only a very subtle, but opposite effect on more mature MM compartment. The observed increase in CD47 and CALR expression in bulk cells following treatment aligns with studies suggesting that malignant cells may protect themselves and develop drug resistance through upregulation of the “don’t eat me” signal CD47, along with the “eat me” signal CALR (Hassan EM et al., 2023; Rastgoo N et al., 2019). Interestingly, CD47 expression was differentially regulated across cell types: it was prominently decreased in CD138^{neg} stem cell-like cells, while it was upregulated in CD138^{pos} mature cells, potentially reflecting differences in treatment response between these subpopulations. While the surviving mature cells potentially protect themselves with increased CD47 surface expression, CD138^{neg} may become more susceptible to immune mediated clearance by CD47 reduction.

To investigate these phenomena in a more physiologically relevant context, we established a co-culture experiment. M0 macrophages were differentiated from healthy donor blood samples, following the protocol outlined in subsequent sections. These cells were seeded onto the lower chamber of a 6-well plate, and an insert with 0.4 μ m pore size was carefully placed on top. MM1.S cells were then added to the upper chamber at a 1:1 ratio relative to macrophages. This setup enabled paracrine communication through the exchange of soluble factors, while preventing direct cell-cell contact (fig. 9A). The co-cultures were treated with 2 μ M dexamethasone or an equivalent concentration of DMSO as a vehicle control for 72h. Quantification of MM1.S cells was performed following the same procedure used for monoculture experiments described previously.

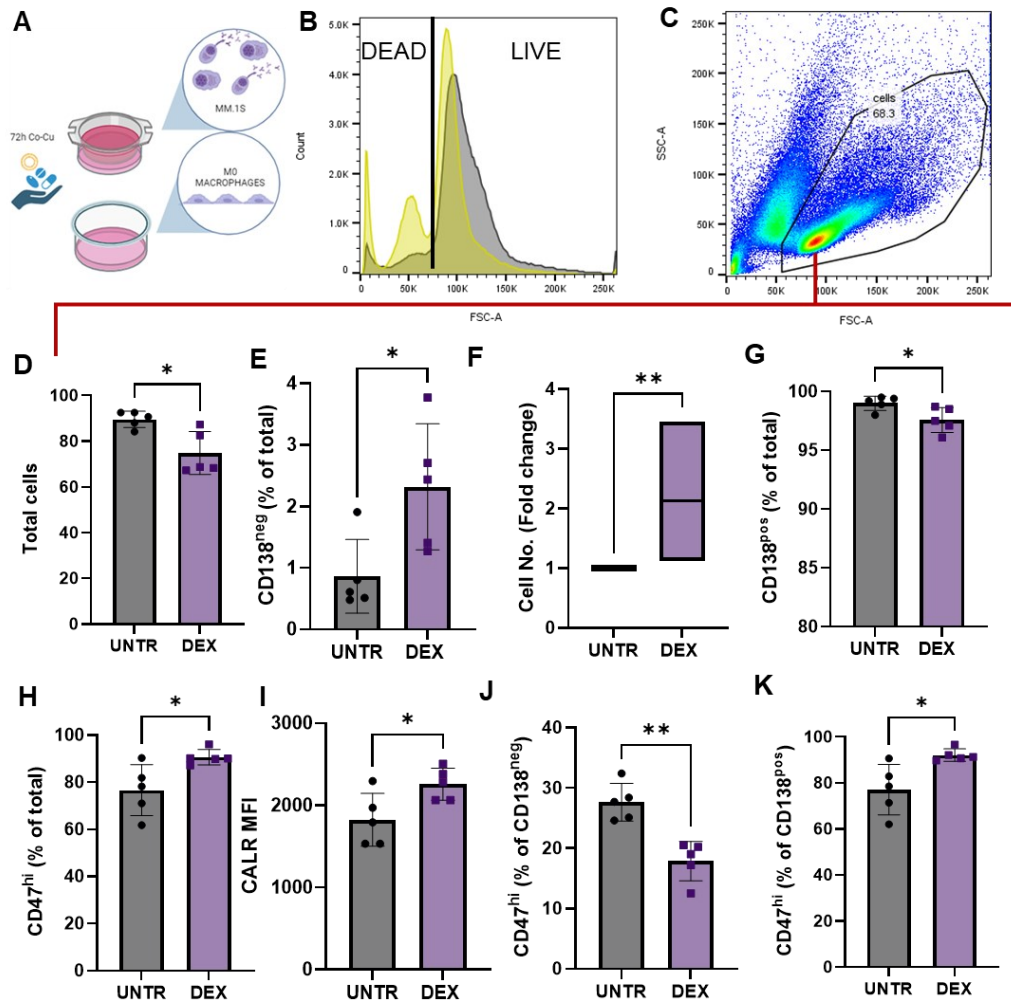


Figure 9. Effect of dexamethasone treatment on MM cells in co-culture. MM1.S cells were seeded on top of 0.4 μ m pore transwell system, with M0 macrophages previously differentiated and seeded in the lower chamber, with or without 2 μ M dexamethasone treatment, for 72h (A). In this manner, cell-cell communication is enabled only via secretion cell factors. The increase of dead cells, upon dexamethasone treatment was observed in MM1.S cells (B). Upon gating on the live cell population (C), a decrease in total cell number was detected (D). Enrichment of CD138^{neg} cells, percentage (E) and number (F) wise, as well as a slight decrease in CD138^{pos} cells (G) was observed. CD47 (H) and CALR (MFI=median fluorescence intensity) (I) expression were upregulated upon dexamethasone treatment within the bulk cells. Differential analysis showed that CD47 expression was downregulated in the CD138^{neg} compartment upon treatment (J), while CD138^{pos} cells followed the same increase (K), comparable to bulk cells. n=5. Shown are mean values $\pm$ SD. P-values were calculated using two-tailed student's t-test. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

Dexamethasone treatment exerted a cytotoxic effect on MM cells, as evidenced by an increased proportion of dead cells (fig. 9B). Following live cell gating (fig. 9C) a reduction in the number of the live cells was observed in response to dexamethasone treatment (fig. 9D). The enrichment of the CD138^{neg} population was evident, with a 2.7-fold increase in percentage (fig. 9E) and a 2.1-fold increase in the absolute cell number (fig. 9F), though this effect was less prominent compared to monoculture (fig. 9D+E).

A modest reduction in CD138^{pos} population upon dexamethasone treatment was also observed in the co-culture setting (fig. 9G). Furthermore, the expression of CD47^{hi} (fig. 9H) and CALR (fig. 9I) was upregulated; however, the differential effects of the treatment varied between CD138^{neg} and CD138^{pos} subpopulations. Specifically, CD138^{neg} cells showed decreased CD47^{hi} expression following dexamethasone treatment (fig. 9J), whereas CD138^{pos} cells upregulated the expression of this molecule (fig. 9K).

Overall, we observed similar effects in both monoculture and co-culture experiments, although the enrichment of CD138^{neg} cells in co-culture was less pronounced compared to monoculture (2.7-fold vs. 10.6-fold). Dexamethasone treatment reduced the overall number of malignant cells and primarily targeted CD138^{pos} cells, which represent more mature compartment, thereby enriching for CD138^{neg} cells. An overall increase in CD47^{hi} and CALR expression was observed in the bulk and CD138^{pos} cell populations, while the opposite trend was seen in CD138^{neg} cells. These findings suggest the potential use of dexamethasone in combination with anti-CD47 therapy to overcome the immune evasion strategies. In particular, targeting CD47 overexpressing malignant cells, previously reported to create a protective environment for CD47 low-expressing cancer stem cells, may render the latter vulnerable to immune-mediated elimination and enhance therapeutic efficacy (Chang CL et al., 2020).

3.3 DEXAMETHASONE TREATMENT SPARES STEM CELL-LIKE CELLS IN CFU ASSAYS

The colony-forming capacity of MM cell lines identifies a progenitor cell population with stem cell-like properties *in vitro*. Although specific MM cell lines exhibit colony-forming capacity associated with progenitor cells, functional heterogeneity is evident both within and between cell lines (Matsui W et al., 2008; Ziel M et al., 2007). To assess the impact of dexamethasone treatment on the clonogenic potential of MM cell lines, CFU assays were performed. For this purpose, the basic human H4230 MethoCult medium supplemented with 10% lymphocyte conditioned medium was used as a source of growth factors. The lymphocyte conditioned medium was included to support optimal CFU medium composition.

MM1.S and RPMI 8226 MM cell lines were plated at a density of 1000 cells/mL and colonies consisting of more than 40 cells were scored at day 14. While RPMI 8226 cells demonstrated the colony-forming capacity (fig. 10A upper panel), this was not observed in case of MM1.S cells (supp. fig. 1). RPMI 8226 cells were subsequently FACS-sorted into CD138^{pos} and CD138^{neg} population (fig. 10A lower panel). Cells were seeded at conc. of 350000 cells/mL and treated with 3 μ M dexamethasone, or corresponding concentration of DMSO for 72h (fig. 10B). Following treatment, CD138^{pos} cells were seeded at 1000 cells/mL, and CD138^{neg} cells at 10000 cells/mL in CFU medium. Different seeding densities were selected due to the previously observed sensitivity of CD138^{neg} cells to FACS sorting, which affected their viability. To avoid underestimation of clonogenic capacity in this population, the undiluted CD138^{neg} fraction was used in CFU assay. Colonies were counted 14 days upon CFU assembly.

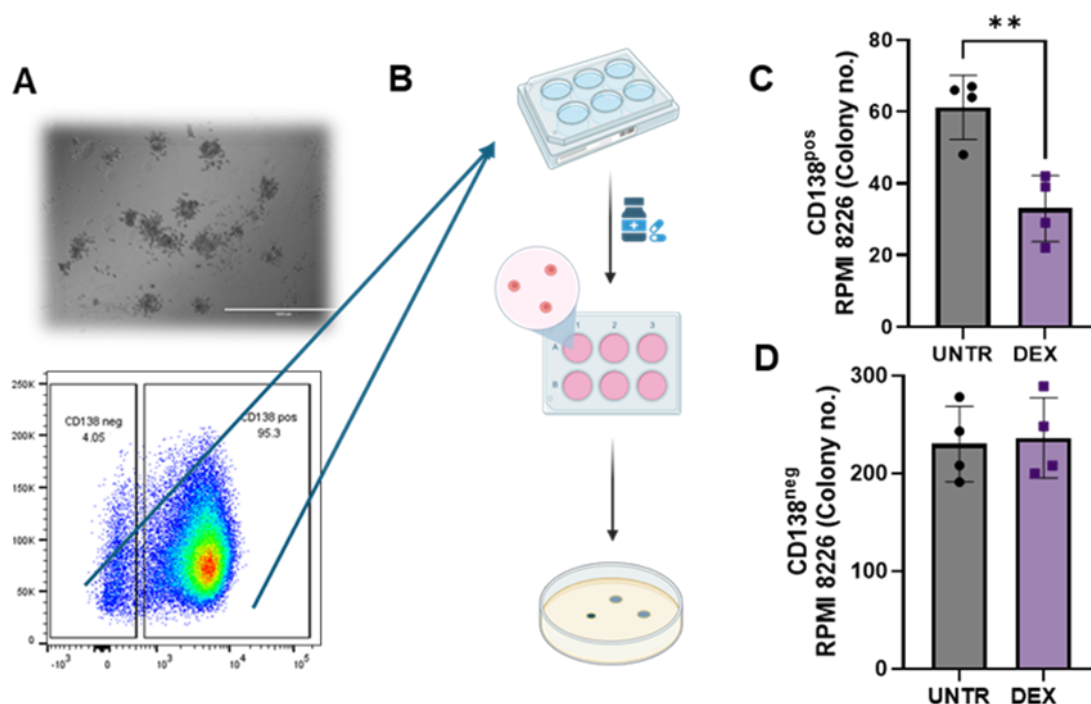


Figure 10. Effect of dexamethasone treatment on CFU potential in CD138^{neg} and CD138^{pos} RPMI8226 MM cells. For CFU assay RPMI 8226 MM cell line capable of forming colonies in methylcellulose were used (A). CD138^{pos} and CD138^{neg} cells were sorted and treated with 3 μ M dexamethasone. After 72h 1000 cells/ml, and 10000 cells/ml of CD138^{pos} and CD138^{neg} cells, respectively, were seeded, along with appropriate controls in methylcellulose medium supplemented with lymphocyte conditioned medium (B). The colonies were counted 14 days after seeding and the effect of dexamethasone was evaluated in both, CD138^{pos} and CD138^{neg} RPMI 8226 cells. While significant reduction of colony formation was observed in dexamethasone treated CD138^{pos} cells (C), this effect was absent in CD138^{neg} cells (D). Mean values of $n=3 \pm SD$ are shown, and p -values were calculated using two-tailed student's t -test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

The CFU assay indicated that dexamethasone treatment didn't affect the colony forming potential of CD138^{neg} RPMI 8226 cells (fig. 10C), while it had a significant inhibitory effect on CD138^{pos} cells (fig. 10D). These results are consistent with previous experiments, suggesting that dexamethasone preferentially targets the more mature MM cell population, while sparing the stem cell-like CD138^{neg} cells. However, the observed effects may be attributable to differences in cell density in the CFU assays, potentially influencing colony counts. Therefore, these findings should be re-evaluated under standardized conditions.

3.4 DEXAMETHASONE TREATMENT PROMOTES MACROPHAGE M2

POLARIZATION *IN VITRO*

To investigate the treatment effects on macrophage polarization, MNCs were isolated from healthy donor blood samples using density gradient centrifugation and subsequently subjected to a macrophage differentiation protocol (fig. 11A). MNCs were seeded on day 0 (D0) in macrophage medium (MACM) at a density of $12-15 \times 10^6$ PBMC per well in 6-well plate format and left O/N to allow adherence. From D1 MACM was supplemented with 100 ng/mL recombinant human (rh) M-CSF and was replenished every 2-3 days. Cells were cultured until D6, or D7, at which point they were considered fully differentiated but non-polarized M0 macrophages. From D7 to D10, further polarization towards anti-inflammatory M2 macrophages was introduced by supplementing MACM with 10 ng/mL rh IL-4. For TAM polarization, conditioned medium collected from 24h cultures of U266 and MM1.S cells, at density 1 million/mL was used and mixed in 1:1 ratio and added to macrophage cultures. To differentiate M1 pro-inflammatory macrophages, cells were cultured from D1 to D7 in MACM supplemented with 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), 100 ng/ml LPS and 20 ng/ml IFN- γ and continued until D10.

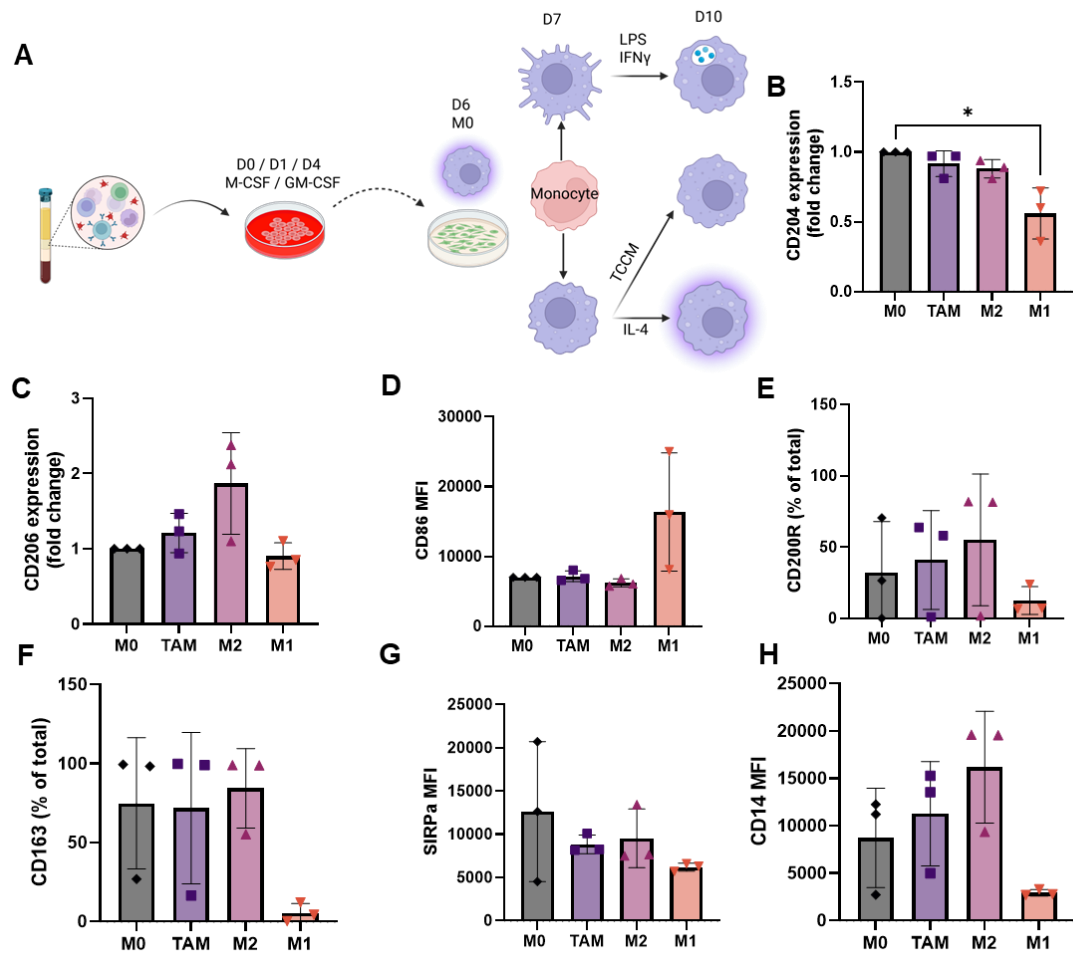


Figure 11. Macrophage polarization and characterization. Macrophages were differentiated and polarized from healthy donor PBMNCs towards M0, TAM, M2, and M1, according to the described protocol (A). Surface expression of markers associated with anti-inflammatory macrophage phenotype: CD204 (B), CD206 (C), CD200R (E), and CD163 (F), as well as the marker associated with pro-inflammatory phenotype, CD86 (D), SIRP α (G) and CD14 (H), was assessed to evaluate the efficiency of polarization protocol and its impact on cell phagocytosis. TCCM: tumor cell-conditioned medium.

Surface expression of CD204, CD206, CD86, CD200R, CD163, SIRP α and CD14 was assessed across different groups via flow cytometry. Upregulation of markers associated with the anti-inflammatory macrophage phenotype: CD204 (fig. 11B), CD206 (fig. 11C), CD200R (fig. 11E) and CD163 (fig. 11F) was observed in M2-polarized macrophages. In contrast, M1 polarization resulted in upregulation of CD86, a marker characteristically expressed by M1 pro-inflammatory macrophages (fig. 11D). SIRP α and CD14, associated with phagocytic regulation and immune evasion signaling, were evaluated as well. SIRP α expression remained constantly high across macrophage subtypes (fig. 11G), whereas CD14 expression was reduced in the M1 population (fig. 11H), likely due to interference by LPS which binds to the CD14 surface

receptor. Overall, these results demonstrate characteristic dynamic changes in surface marker expression during the differentiation and polarization of distinct macrophage subtypes.

Subsequently, the effects of free and liposomal dexamethasone on macrophage polarization were evaluated using a transwell co-culture system. M0 differentiated macrophages were seeded at the bottom of the wells, while MM1.S cells were placed in the upper compartment using a 0.4 μm pore size-insert to prevent direct cell-cell contact (fig. 12A).

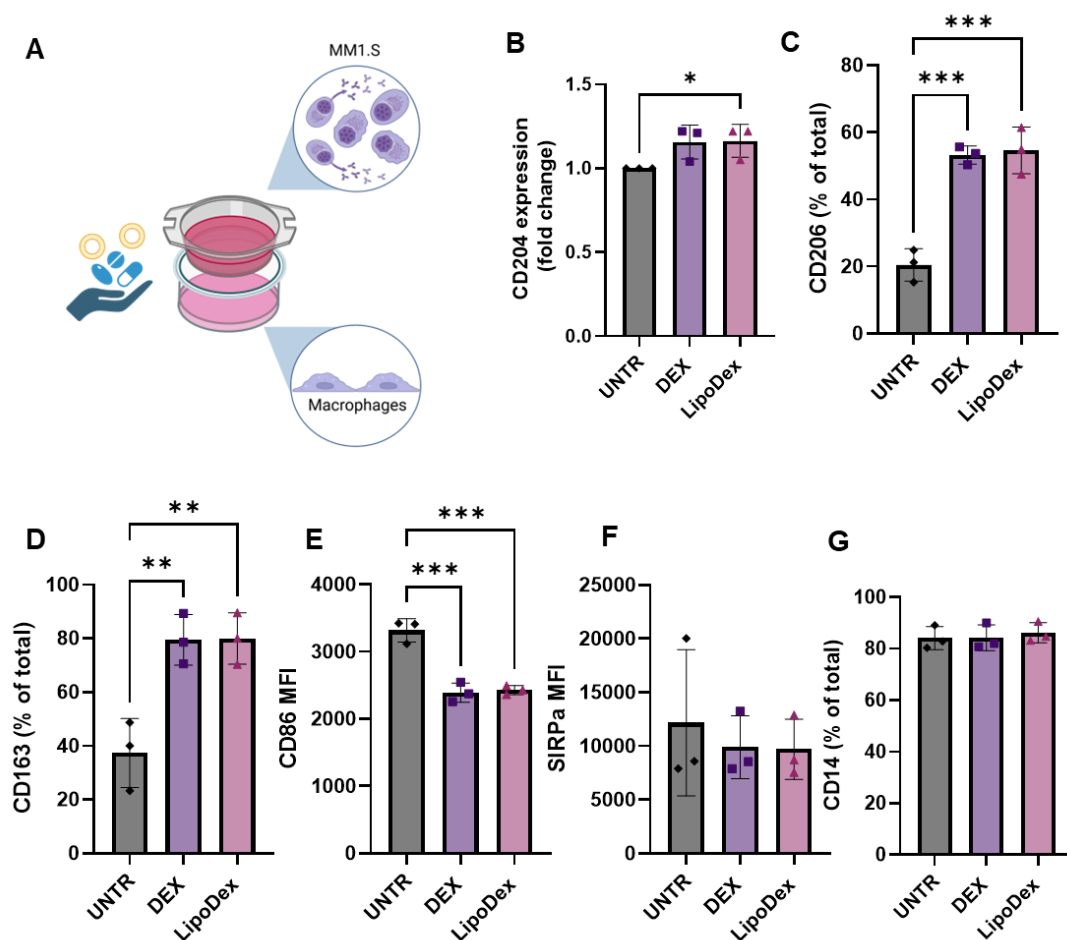


Figure 12. Effects of free and liposome-encapsulated dexamethasone on macrophage polarization. A transwell co-culture system, which prevents direct cell-cell contact was used to evaluate the impact of MM1.S secretory profile on M0 macrophage polarization (A). Upon both, free and liposomal dexamethasone treatments, at conc. 2 μM , significant upregulation of macrophage anti-inflammatory marker expression signature, e.g. CD204 (B), CD206 (C) and CD163 (D) was detected. Conversely, the surface expression of proinflammatory associated marker, CD86 was significantly downregulated (E). Expression levels of SIRP α (F) and CD14 (G) were not affected by the treatments. Mean values of $n=3 \pm \text{SD}$ are shown. P-values were calculated using one-way Anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

After treatment of co-cultures with 2 μ M dexamethasone or liposomal dexamethasone for 72h, the transwell inserts containing MM1.S cells were removed, and macrophages were detached by trypsinization. The cells were subsequently prepared and stained for flow cytometric analysis of CD204, CD206, CD163, CD86, SIRP α and CD14 surface expression. Markers characteristically expressed by anti-inflammatory macrophages: CD204 (fig. 12B), CD206 (fig. 12C) and CD163 (fig. 12D) were similarly upregulated following treatment with both, free and liposomal dexamethasone. Downregulation of the pro-inflammatory response was evidenced by decreased CD86 expression (fig. 12E) in both treatment groups. Expression of SIRP α (fig. 12F) and CD14 (fig. 12G) remained unaffected by either treatment (fig. 12F, G). Overall, both dexamethasone formulations induced a comparable shift in macrophage polarization toward an anti-inflammatory, M2-like phenotype.

3.5 DEXAMETHASONE SLIGHTLY INCREASES PHAGOCYTOSIS OF MM CELLS

After confirming an anti-inflammatory effect of dexamethasone on macrophage polarization, we evaluated the phagocytic capacity of these cells. To investigate the impact of dexamethasone treatment on MM cell phagocytosis, M0 or M2 macrophages were differentiated as previously described and subsequently cultured in a direct contact with dexamethasone treated MM1.S GFP^{pos} luciferase (luc)^{pos} cells. Phagocytosis was evaluated using two independent approaches: fluorescent microscopy and flow cytometry. For fluorescence microscopy, macrophages were serum-starved and subsequently labeled with the cell permeable red LysoTracker dye according to manufacturer's instructions. Briefly, the dye accumulates in highly acidic compartments, e.g. lysosomes, due to the low pH level, emitting the fluorescence detectable in the red fluorescent protein (RFP) channel. The MM1.S were visualized by GFP expression. Phagocytic activity was evaluated after 2h of co-incubation, by quantifying the overlap of LysoTracker - red and GFP - green signals in merged fluorescence images. Monocultures of stained macrophages and MM1.S cells served as negative controls to determine the baseline fluorescence levels. Phagocytosis was identified by the presence of yellow-orange signal within the macrophage-sized regions, as shown in figure 13A. An increased trend in phagocytic activity was observed in both M0 (fig. 13B) and M2 (fig. 13C) macrophages following dexamethasone treatment of MM cells, under this experimental setting.

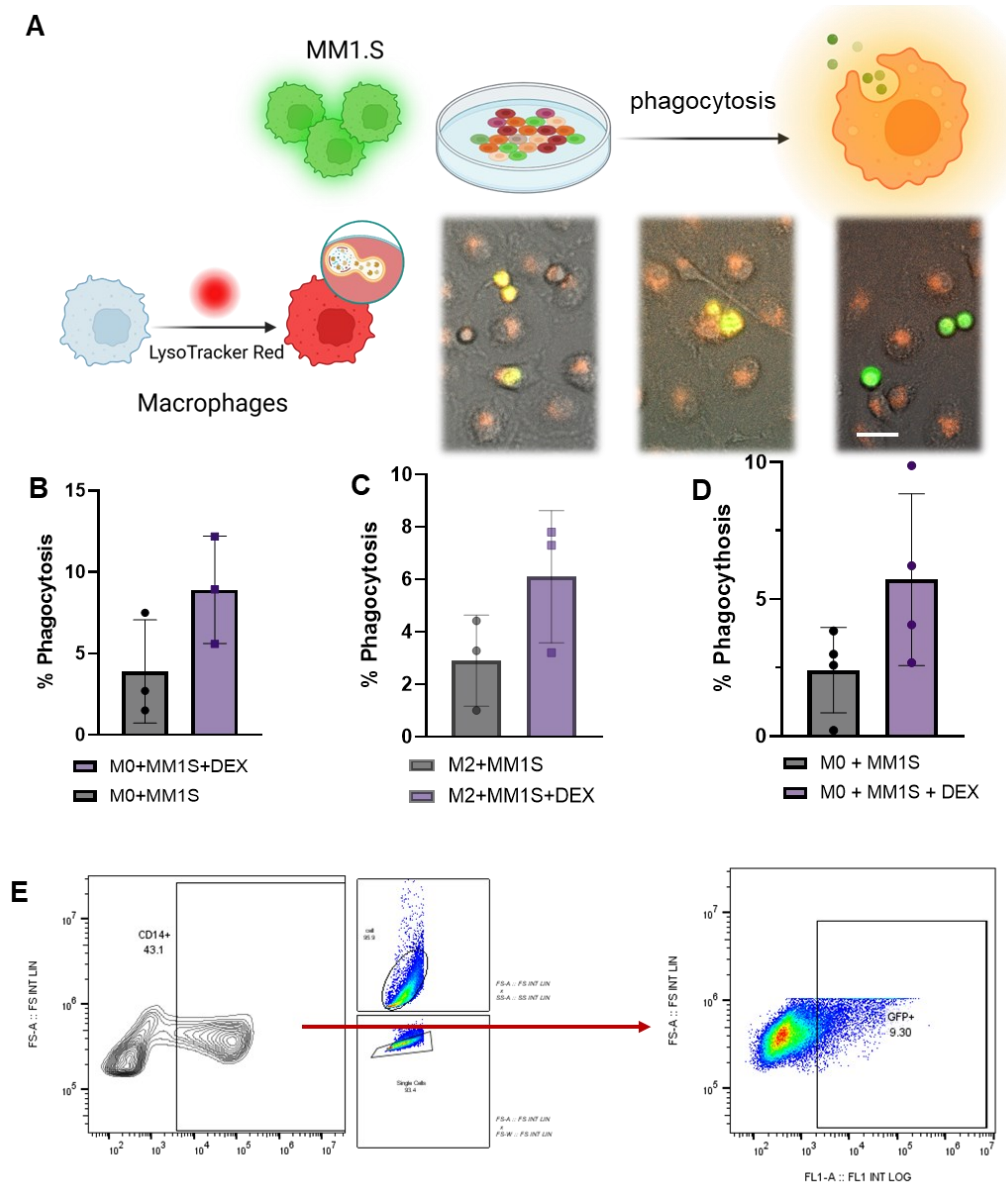


Figure 13. Effect of dexamethasone on phagocytosis. For the purposes of accessing dexamethasone effect on macrophage phagocytosis with fluorescence microscopy, dexamethasone treated GFP^{pos} MM1.S cells were co-cultured with LysoTracker red DND99 labelled macrophages and incubated for 2h. The phagocytosis was visualized as the presence of intense yellow signal within the macrophage cytoplasm. The size bar corresponds to 30 μ m (A). The percentage of phagocytosis was calculated as number of yellow-positive (double positive) cells of total counted cells. Trend towards increased phagocytic activity was observed in both, M0, $p=0.2$ (B) and M2, $p=0.4$ (C) dexamethasone treated co-cultures. This phenomenon was also assessed in flow cytometric analysis, by following double positive CD14, GFP M0 cells, upon exclusion of doublets, $p=0.108$ (E). Same trend of increased phagocytosis upon dexamethasone treatment was observed in flow cytometric analysis (D). Dexamethasone, at conc. 2 μ M was used in all experimental setups. Mean values $n \geq 3 \pm SD$ are shown. P -values were calculated using two-tailed student's t -test.

For flow cytometric analysis, serum starved M0 macrophages were co-incubated with GFP^{pos}luc^{pos} MM1.S cells, as previously described. The phagocytic activity was quantified as percentage of MM GFP^{pos} cells within macrophage CD14^{pos} cell population (fig. 13E). An increase in the phagocytic activity of M0 macrophages

following dexamethasone treatment was also observed by flow cytometry (fig. 13D). The enhanced phagocytosis observed by both microscopy- and flow cytometry-based assays is likely attributable to the pro-apoptotic effect of dexamethasone on MM cells. This represents controlled clearance of apoptotic cells by macrophages, a process known as efferocytosis which contributes to the resolution of inflammation associated with cell death.

3.6 LIPOSOMAL DEXAMETHASONE SHOWS FAVORABLE BIODISTRIBUTION IN BONE MARROW

To monitor the expansion of MM cells and evaluate the MM-targeting efficacy of liposomal dexamethasone, bioluminescent imaging and biodistribution studies were performed. To evaluate the biodistribution of liposomally encapsulated dexamethasone and its colocalization with MM cells, Cy7-labeled liposomes were administered i.v. two weeks after transplantation of MM1.S GFP^{pos}Luc^{pos} cells, coinciding with the first treatment timepoint. Six hours post-injection μ CT/FLT was performed to visualize the distribution of fluorescent signal (fig. 14A). Simultaneously, μ CT/BLT imaging was performed, to determine the localization of malignant cells at matched timepoint. This was achieved by i.p. injection of luciferin, a substrate for enzyme luciferase, expressed by GFP^{pos}Luc^{pos} MM1.S cells administered 10 minutes prior to imaging, to enable detection of bioluminescent signal. As expected, the highest accumulation of liposomes was observed in organs involved in liposomal excretion and clearance, predominantly liver, followed by kidneys, lungs, and spleen (fig. 14B). A significant portion of liposomes remained in circulation, 6h post-injection, as indicated by signal detected in the heart (fig. 14B). The BM uptake was estimated to be approximately 5% of initial injected dose (fig. 14B). Next, BLT/ μ CT scans were performed at week three post-MM transplantation, concurrently with μ CT/FLT imaging, as well as at weeks four and five after to monitor the MM cell engraftment over time, and to potentially assess differences between treatment groups (fig. 14C, showing a representative μ CT/FLT image). Taken together, an increase in bioluminescence signal, normalized to total body weight was observed over time and was consistent with the expected progression of disease (fig 14D-E).

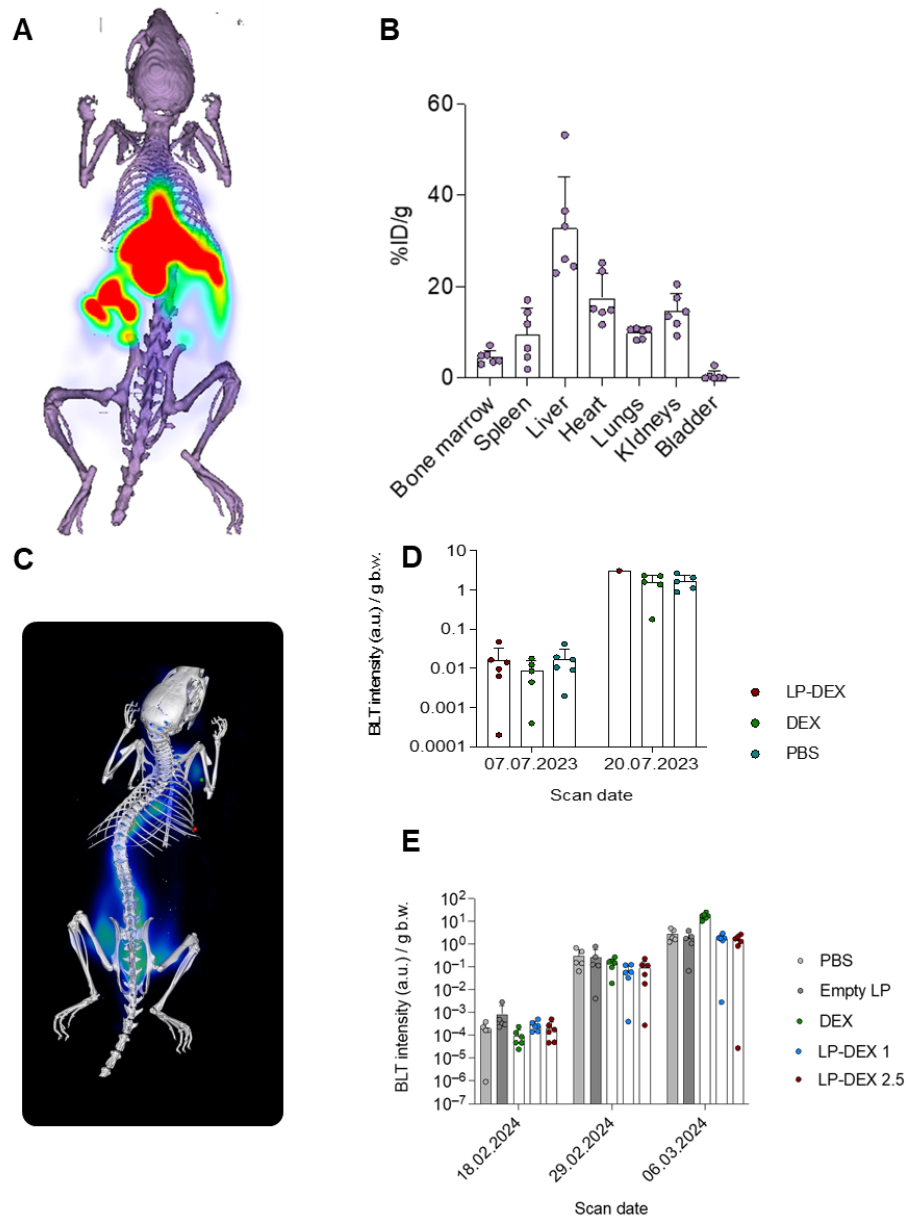


Figure 14. Biodistribution study in vivo. To follow-up the biodistribution of Cy-7 labeled liposomal dexamethasone, the treatment was administered i.v. and the animals were subjected to FLT/ μ CT scans at two time points (15 min and 6 h after injection) (A). The Cy-7 organ uptake was evaluated and presented as percentage of initial dose per gram. While expected accumulation of Cy-7 liposomes was detected in following organs: liver, heart, kidneys, lungs, and spleen, a 5% uptake was detected in the BM (B). After i.p. administration and of luciferase substrate, to visualize luc+ MM1.S cells, BLT/ μ CT imaging was performed, and detected signal coincided with spine localization (C). While, the increase of BLT signal was observed during the disease progression, no differences between the treatment groups could be detected (D+E).

However, quantitative analysis of bioluminescent signal intensity revealed no significant differences between treatment groups receiving 1 mg/kg dexamethasone, 2.5 mg/kg dexamethasone, 1 mg/kg liposomal dexamethasone and 2.5 mg/kg liposomal dexamethasone treatment (fig. 14 D+E). Although bioluminescence imaging is well-suited for longitudinal monitoring of tumor dynamics within individual animals, it

is not considered reliable for direct quantitative comparisons between different treatment groups. For robust inter-group comparisons, it is typically used in combination with other methods such as flow cytometry or histology (Chu T et al., 2025). Localization of malignant cells corresponded with areas of BM, as shown in fig. 14B, and this distribution aligned with the ~5% BM uptake observed for Cy7-labeled liposomes.

3.7 MULTIPLE MYELOMA CELL BIOLOGY IS AFFECTED BY LIPOSOMAL DEXAMETHASONE, BUT NOT BY FREE DEXAMETHASONE AT LOWER CONCENTRATIONS *IN VIVO*.

To investigate the effect of different dexamethasone concentrations and formulations, previously established murine xenograft model of MM, which recapitulates BM engraftment, was employed. Briefly, the human MM1.S GFP^{pos}luc^{pos} cell line, known for its sensitivity to dexamethasone was co-injected with the human fibroblast cell line HS27 α in a ratio 1:0.5 via the tail vein within 24h post-irradiation. Treatment began two weeks following transplantation. The experimental approach consisted of two consecutive studies. In the first experiment, 1 mg/kg free dexamethasone was compared to 0.9% sodium chloride (NaCl) control. In the second experiment, 1mg/kg and 2.5 mg/kg liposomal dexamethasone, as well as 2.5 mg/kg free dexamethasone were evaluated in comparison to their respective controls: 0.9% NaCl and empty liposomal particles (EL). Initially, the experimental design intended to compare both free and liposomal dexamethasone at a concentration of 1 mg/kg, with 0.9% NaCl and EL as vehicle controls. However, due to drug contamination in the process of the production pipeline, animals treated with 1 mg/kg liposomal dexamethasone exhibited adverse reactions and were withdrawn at an early stage. Consequently, only the effect of 1 mg/kg free dexamethasone could be assessed in this study. To address this limitation a follow-up experiment was performed to evaluate the effects of both formulations, free and liposomal dexamethasone, at concentrations of 1 mg/kg and 2.5 mg/kg on MM progression (fig. 15A).

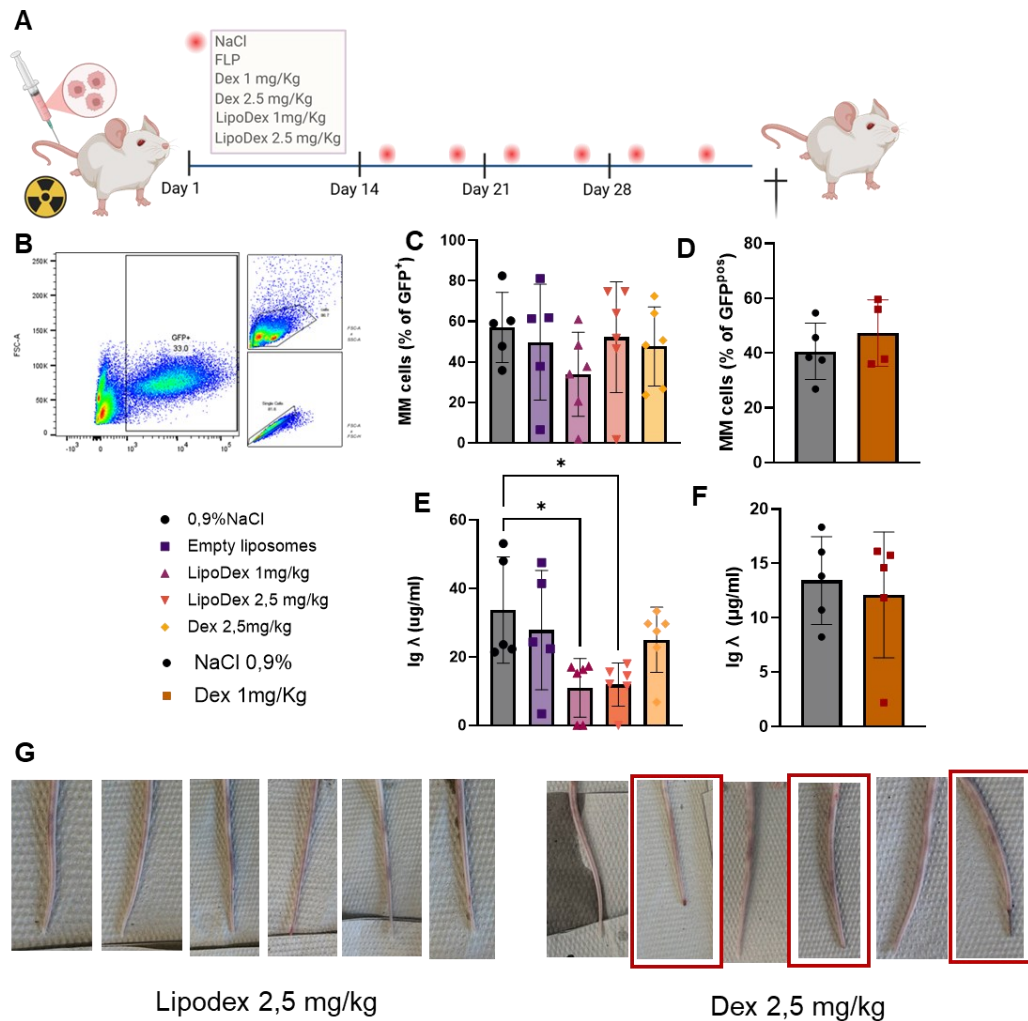


Figure 15. The effect of dexamethasone treatment on malignant cell persistence and secretory profile in a xenograft MM model. Two weeks after establishment of the xenograft model via tail vein transplantation of MM1.S cells in combination with HS-27 α fibroblasts into severely immunocompromised NSG mice, treatment with free or liposomal dexamethasone, at concentrations 1 mg/kg and 2.5 mg/kg was initiated via i.v. injection, administered twice weekly (A). Final assessment of the malignant cell infiltration was performed by flow cytometric analysis of GFP^{pos} cells in the BM (B). No statistically significant differences were observed between treatment groups; however, treatment with 1 mg/kg liposomal dexamethasone showed a trend toward reduced abundance of GFP^{pos} cells in BM samples, whereas free dexamethasone did not show an effect (C-D). Serum analyses revealed a significant reduction in IgA levels in mice treated with liposomal dexamethasone at both 1 mg/kg and 2.5 mg/kg compared to controls (E-F). In terms of tolerability, tail necrosis was observed in 3 out of 6 mice receiving 2.5 mg/kg free dexamethasone following repeated intravenous injections, whereas no such adverse effect was noted in the liposomal dexamethasone group. (G). $n \geq 4$ per group. Data are presented as mean $\pm$ SD. Significances were calculated using one-way Anova. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To evaluate the impact of different dexamethasone formulations on MM progression, total BM cells were harvested from femurs and tibias and analysed by flow cytometry to quantify GFP^{pos} MM1.S cells (fig. 15B). This approach enabled the assessment of malignant cell expansion within BM across the respective treatment groups. While malignant cell expansion reached 80% in some samples, intra-group variability was

observed (fig. 15C+D). Notably, a reduction in the frequency of malignant cells within the BM was observed in the group treated with 1 mg/kg liposomal dexamethasone, although this trend did not reach statistical significance (fig. 15C). Given the established utility of serum free light chain (sFLC) measurements, specifically κ and λ chains, as biomarkers for MM disease burden (Tosi et al., 2013), serum samples were collected via retro-orbital bleeding and analyzed by ELISA to quantify levels of Ig λ , the predominant light chain secreted by the MM1.S cell line (ATCC, Manassas, VA). No significant differences in Ig λ levels were observed in animals treated with free dexamethasone at either 1 mg/kg or 2.5 mg/kg when compared to the respective vehicle controls. In contrast, treatment with liposomal dexamethasone at both concentrations resulted in a significant reduction in circulating Ig λ levels (fig. 15E–F), indicating a more pronounced suppression of tumor burden. In addition to therapeutic efficacy, treatment-associated toxicity was also observed. Notably, tail necrosis was observed in 3 of 6 animals receiving free dexamethasone, an adverse effect that was absent in the liposomal dexamethasone-treated cohorts. Collectively, these findings suggest that liposomal dexamethasone may offer enhanced therapeutic efficacy and improved tolerability relative to the free dexamethasone formulation in this xenograft model of MM.

Next, MM cells were isolated by FACS, based on GFP^{pos} expression, and the resulting cell populations were subjected to RNA extraction and subsequent 3'mRNA bulk sequencing analysis was performed (fig. 16A). All experimental procedures and bioinformatic analyses were conducted in-house.

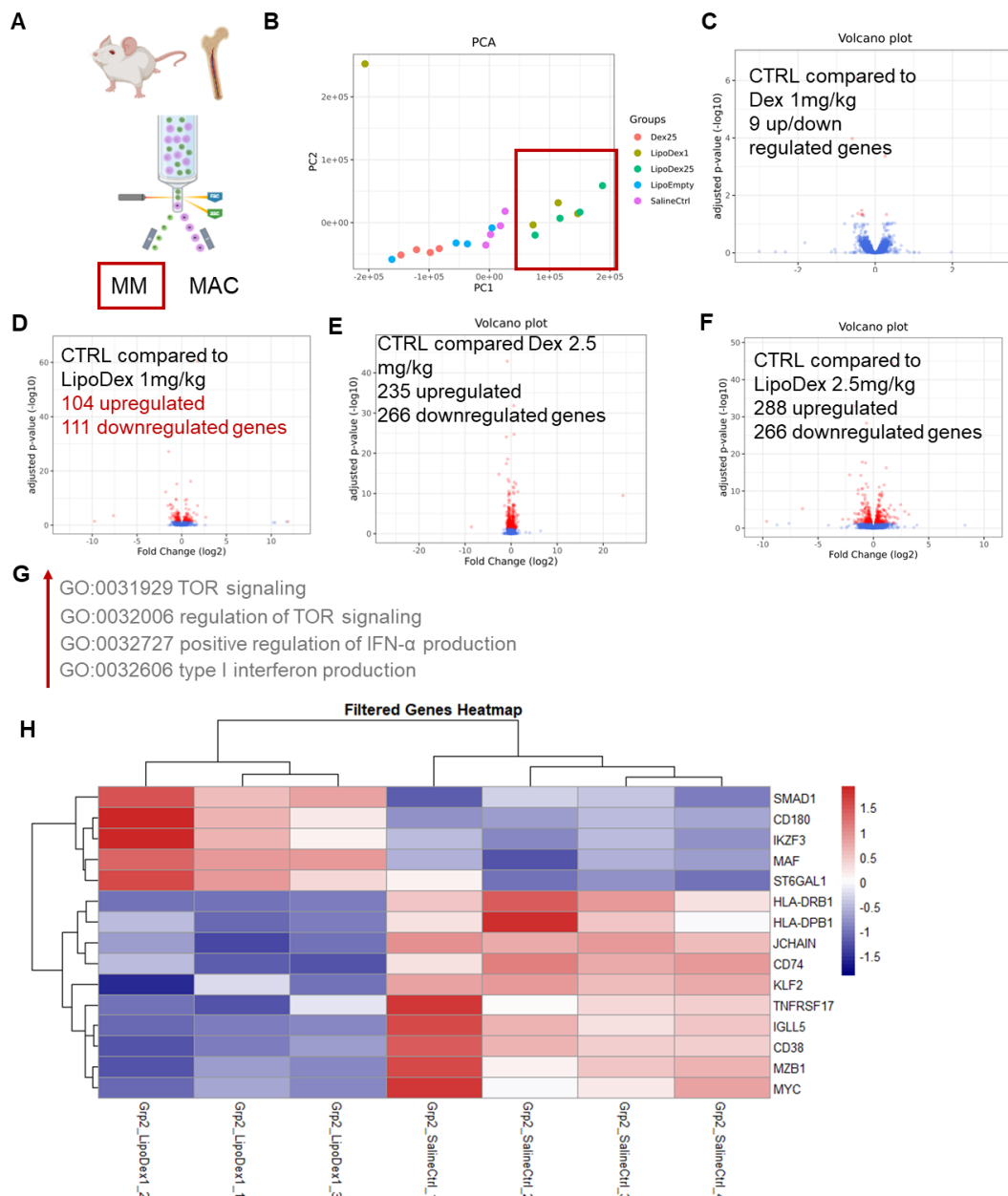


Figure 16. RNA sequencing analysis of MM cells isolated from murine BM. MM cells were isolated based on GFP expression using FACS (A). RNA was extracted, subjected to quality control and analysed via 3'RNA sequencing. PCA demonstrated that liposomal dexamethasone at both 1 mg/kg and 2.5 mg/kg clustered separately from 0.9% NaCl, EL and the 2.5 mg/kg free dexamethasone group, indicating distinct transcriptional profiles (B). Volcano plots illustrate significantly up- and downregulated genes, highlighted in red in treatment groups compared to their respective controls: 1 mg/kg dexamethasone (C) and 1 mg/kg liposomal dexamethasone (D). A substantially greater transcriptional response was observed in the 1 mg/kg liposomal dexamethasone group, with 215 significantly regulated genes compared to the free dexamethasone group, at the same concentration, with 9 significantly regulated genes. At the higher 2.5 mg/kg dose both dexamethasone formulations induced a comparable number of significantly regulated genes (E+F). GO analysis revealed significant upregulation of the TOR signaling pathway and IFN- α production (G). A heat map of genes significantly regulated by the 1 mg/kg liposomal dexamethasone treatment highlights key genes involved in MM cell proliferation, invasiveness, and drug resistance (H). $n \geq 3$. Raw sequencing reads were aligned to reference genome using standard alignment tools. Differential gene expression analysis was performed using DESeq2 package in R. P-values were adjusted for multiple testing using the Benjamini-Hochberg procedure. Genes with an adjusted p value < 0.05 and a log2 fold change > 1 were considered significantly differentially expressed.

Following differential expression analysis, gene ontology (GO) enrichment analysis was carried out to evaluate biological processes (BP) and molecular function (MF). In addition, Kyoto encyclopaedia of genes and genomes (KEGG) pathway analysis was performed. GSEA was also conducted to identify biological processes that were significantly activated or suppressed under specific treatment conditions. Principal component analysis (PCA) revealed distinct clustering of samples treated with liposomal dexamethasone at both 1 mg/kg and 2.5 mg/kg separated from those treated with 2.5 mg/kg free dexamethasone and untreated controls (fig. 16B). This indicates that liposomal dexamethasone induces a transcriptional profile that is distinct from both free dexamethasone and vehicle controls, and that both liposomal treatment concentrations exert similar directional shifts in gene expression. Differential gene expression analysis, visualized as volcano plots, further underscored these distinctions. Notably, the 1 mg/kg free dexamethasone group exhibited minimal transcriptional changes, with only 9 genes reaching statistical significance (fig. 16C). In contrast, the 1 mg/kg liposomal dexamethasone treatment induced significant regulation of 215 genes: 104 up- and 111 downregulated (fig. 16D). At the higher 2.5 mg/kg dose, both formulations induced more extensive transcriptional alterations, with approximately 500 genes significantly up- or downregulated in each group (fig. 16E+F). GO analysis revealed enrichment in pathways relevant to MM pathophysiology. Notably, upregulation of the TOR signaling pathway, previously described as a hallmark of MM stem cell-like, SP cells (Liu B et al., 2017) was observed. In addition, increased expression of IFN- α production pathways was detected, which has been implicated in remodeling of the TME and may act synergistically with CD47 blockade (Pogue S et al., 2017) (fig. 16G). A heat map analysis of selected genes associated with MM progression and drug resistance is shown in fig. 16H. Among genes significantly upregulated in the 1 mg/kg liposomal dexamethasone group were SMAD1, CD180, IKZF3, MAF and ST6GAL1, all of which are associated with pro-tumorigenic functions, including promotion of proliferation, migration, survival, and therapeutic resistance. Conversely, genes significantly downregulated by treatment included HLA-DR1, HLA-DR2, TNFSF and CD38 whose reduced expression has been linked to immune evasion and disease dissemination in MM (Wu J et al., 2021, Kikuchi J et al., 2018, Krönke J et al., 2014, Jiang Q et al., 2022, Notarfranchi L et al., 2024). Additional downregulated genes JCHAN, KLF2, IGLL5, MZB1 and MYC are known to impair immunoglobulin secretion, inhibit cell proliferation and survival, and contribute to

enhanced therapeutic sensitivity when suppressed (Zavidij O et al., 2021, Nobari ST et al., 2022; Chanukuppa, V et al., 2020; White BS et al., 2018; Ohguchi, H et al., 2016, Castro CD et al., 2014, Holien T et al., 2012). Overall, the RNA-seq data demonstrate activation of tumor-promoting pathways associated with MM stemness, consistent with prior *in vitro* findings indicating an enrichment of stem cell-like populations following dexamethasone treatment. Simultaneously, transcriptional signatures indicative of impaired MM cell survival and immunoglobulin secretion suggest a therapeutic sensitization effect mediated by liposomal dexamethasone, aligning with previously observed reductions in Igλ secretion.

3.8 EXPRESSION PROFILING OF DEXAMETHASONE TREATED MICE REVEALS M2-LIKE ANTI-INFLAMMATORY SIGNATURE IN MACROPHAGES

To validate the anti-inflammatory effect of dexamethasone on murine macrophage polarization *in vivo*, BM cells were harvested and subsequently stained for FACS (fig. 17A). A macrophage-enriched population was isolated based on the following surface marker profile: GFP^{neg}CD11C^{neg}Ly6G/C^{low}CD11b^{pos}F4/80^{pos} (fig. 17B). To further characterize the polarization state, additional staining was performed to assess the expression of M2-associated markers CD206, CD163 and SIRPα (fig. 17B). Surface marker analysis revealed a moderate increase in the expression of M2-associated markers CD163 (fig.17C) and CD206 (fig.17D) along with stable expression of SIRPα (fig. 17E) in response to dexamethasone treatment. These findings were further supported by gene expression profiling (fig. 17F), which demonstrated significant upregulation of oxidative phosphorylation, accompanied by downregulation of key pro-inflammatory pathways, including IL-6/JAK/STAT3 signaling, inflammatory response, IFN-γ response, TNF-α signaling, complement activation, and IL-17 signaling. Additionally, pathways associated with immune activation, such as myeloid leukocyte migration, leukocyte activation involved in immune response, and phagocytosis were significantly downregulated. Together, these transcriptional changes support the polarization of macrophages toward an M2-like anti-inflammatory phenotype in response to dexamethasone treatment.

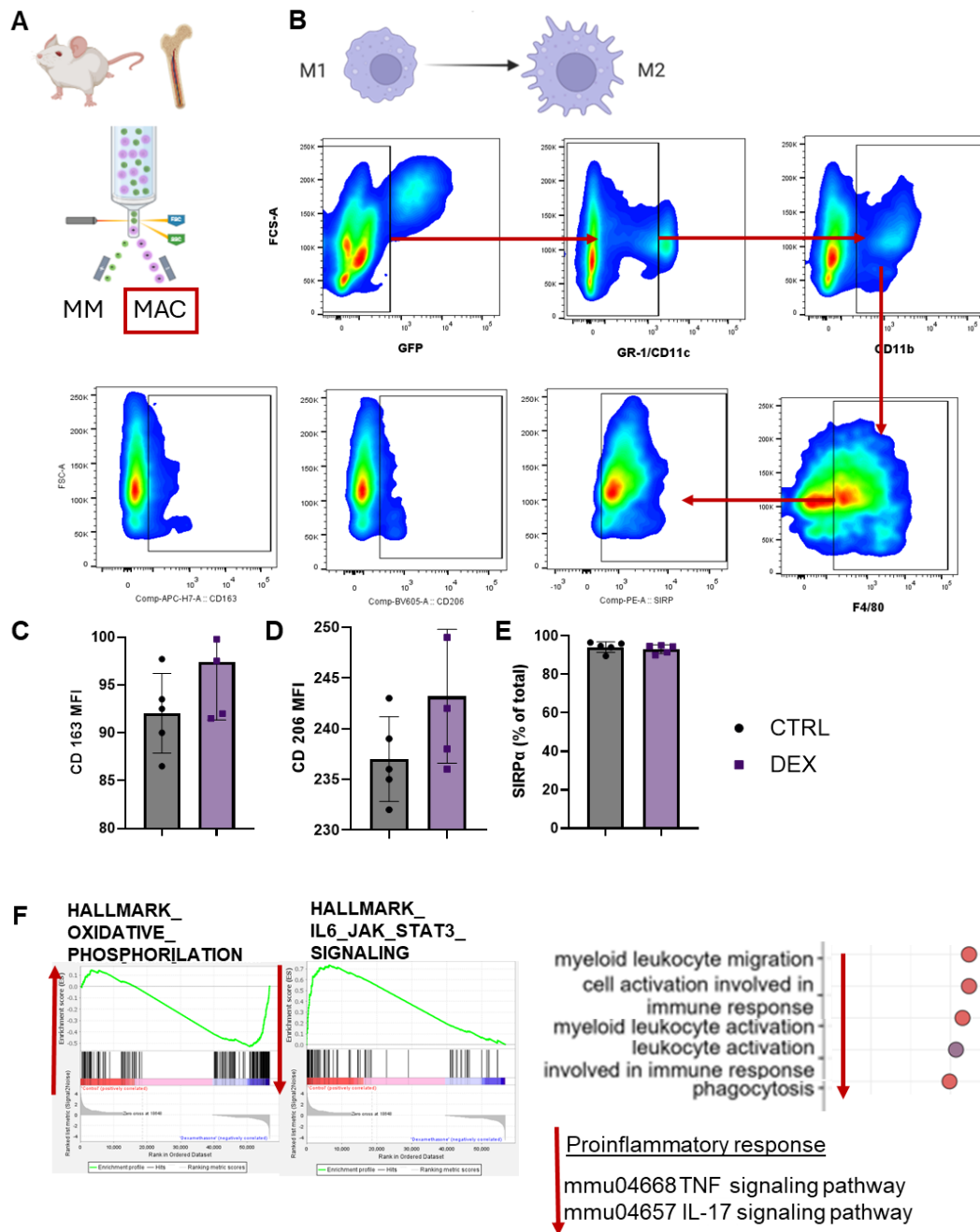


Figure 17. Expression analysis of BM derived murine macrophages upon dexamethasone treatment. Macrophages were isolated from murine bone marrow and sorted by flow cytometry based on the following surface marker profile: GFP^{neg}CD11C^{neg}Ly6G/C^{low}CD11b^{pos}F4/80^{pos} cells (A). The gating strategy is shown in panel (B), along with additional characterisation of CD163, CD206 and SIRPα surface expression within the macrophage population (B). A moderate increase in M2-associated markers CD163 (C) and CD206 (D) and stable expression of SIRPα (E) was observed following treatment with 1 mg/kg dexamethasone. Subsequent 3'RNA sequencing was performed on the sorted macrophages with reads aligned to the murine reference genome using the same pipeline as for MM cell analysis. Transcriptomic profiling revealed upregulation of oxidative phosphorylation and downregulation of IL-6/JAK/STAT3, TNF, and IL-17 signaling pathways, as well as suppression of genes involved in myeloid cell activation, immune response, and phagocytosis collectively indicating an anti-inflammatory, M2-like polarization state induced by dexamethasone treatment (F).

3.9 DEXAMETHASONE ENHANCES ANTI-CD47 MEDIATED PHAGOCYTOSIS OF MM1.S CELLS *IN VITRO*

The CD47 gene encodes a transmembrane protein, that functions as a critical immune checkpoint by delivering “don’t eat me” signal, upon binding to SIRP α , a receptor expressed on phagocytic cells (Kim D et al., 2012). To evaluate the functional consequences of CD47 disruption *in vitro*, a CRISPR/Cas9-mediated knockout (KO) was generated in the MM1.S GFP^{pos} luc^{pos} cell line (fig. 18A).

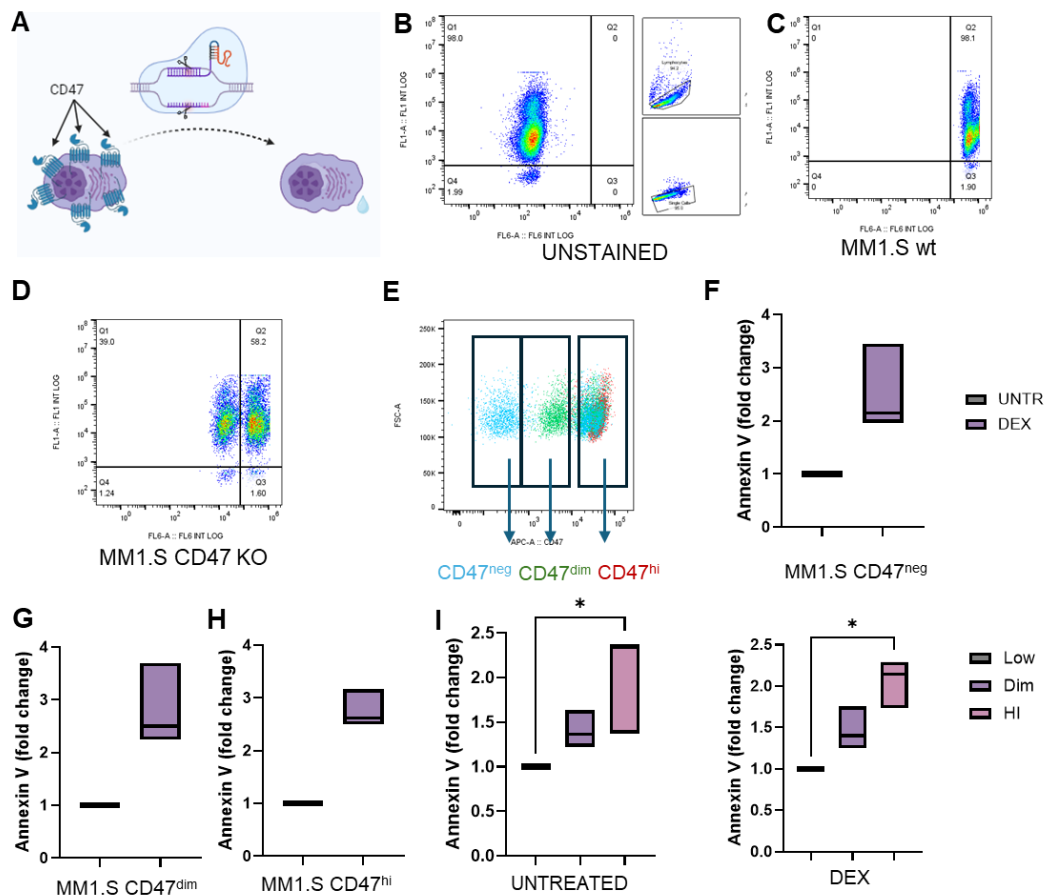


Figure 18. MM1.S CD47 KO evaluation. CD47 KO was introduced by CRISPR/Cas9 nucleofection (A). Two days after nucleofection MM1.S WT and KO cells were validated for CD47 expression by flow cytometry. Compared to unstained control (B) MM1.S WT cells exhibited the expected high CD47 expression (C). In contrast, MM1.S KO cells showed the emergence of a second population, with reduced CD47 expression (D). CD47^{neg}, CD47^{dim} and CD47^{hi} expressing cells were FACS sorted, as depicted in (E). Annexin V and 7-AAD staining was used to validate the effect of 2 μ M dexamethasone treatment in different cell population sorted. CD47^{neg} (F), CD47^{dim} (G) and CD47^{hi} (H) showed the same trend towards dexamethasone sensitivity ($p=0.10$) while CD47^{neg} showed highest viability when compared to untreated ($p=0.74$; $p=0.15$) and dexamethasone treated ($p>0.99$; $p=0.46$) CD47^{dim} and CD47^{hi} cells, respectively (I). $n=3$.

Following nucleofection, MM1.S WT and KO cells were assessed for CD47 surface expression. Compared to the unstained sample (Fig. 18B), MM1.S WT cells showed

high CD47 expression, as expected (Fig. 18C). Two days after nucleofection, MM1.S cells subjected to CRISPR/Cas9 exhibited two distinct populations. (fig. 18D). By day seven post-nucleofection, three distinct populations had emerged within the nucleofected bulk culture: CD47^{neg}, CD47^{dim}, and CD47^{hi}, which were subsequently isolated by FACS (fig. 18E). Along with phenotypic assessment of CD47 expression, an additional functional assay was performed to determine whether the KO affects cell survival. Therefore, sorted cells were subjected to apoptosis assays. All subpopulations exhibited sensitivity to dexamethasone treatment, showing comparable levels of response (fig. 18F-H). Notably, the CD47^{neg} subset demonstrated superior viability relative to CD47^{dim} and CD47^{hi} cells under both basal and dexamethasone-treated conditions (fig. 18I). This enhanced resistance of CD47 KO cells may be attributed to impaired caspase-independent cell death pathways, potentially due to reduced cytochrome c release, associated with CD47 deficiency, as previously reported (Lamy L et al., 2003). To evaluate the effect of CD47 blockade, by using an antibody in combination with dexamethasone treatment on the induction of macrophage-mediated phagocytosis, a co-culture system was established using M0-differentiated macrophages and either MM1.S WT, or MM1.S CD47 KO cells in direct contact (fig. 19A). The experimental treatment groups were designed as follows: untreated, Her2-IgG δ isotype control, Hu5F9-IgG δ CD47-blocking antibody, dexamethasone, dexamethasone combined with CD47-blocking antibody, and daratumumab in combination with CD47-blocking antibody, as a positive control. MM1.S WT and CD47 KO cells were pre-treated with 2 μ M dexamethasone for 72 hours prior to co-culture assembly and subsequently labeled with pHrodo dye, as previously described. Labeled MM1.S cells were seeded with M0-differentiated macrophages in 96-well flat-bottom plates, and the respective antibody treatments were added directly to the co-culture system. Image acquisition using the Incucyte live-cell imaging system began 30 minutes after co-culture initiation and was performed every 2 hours for a total duration of 24 hours.

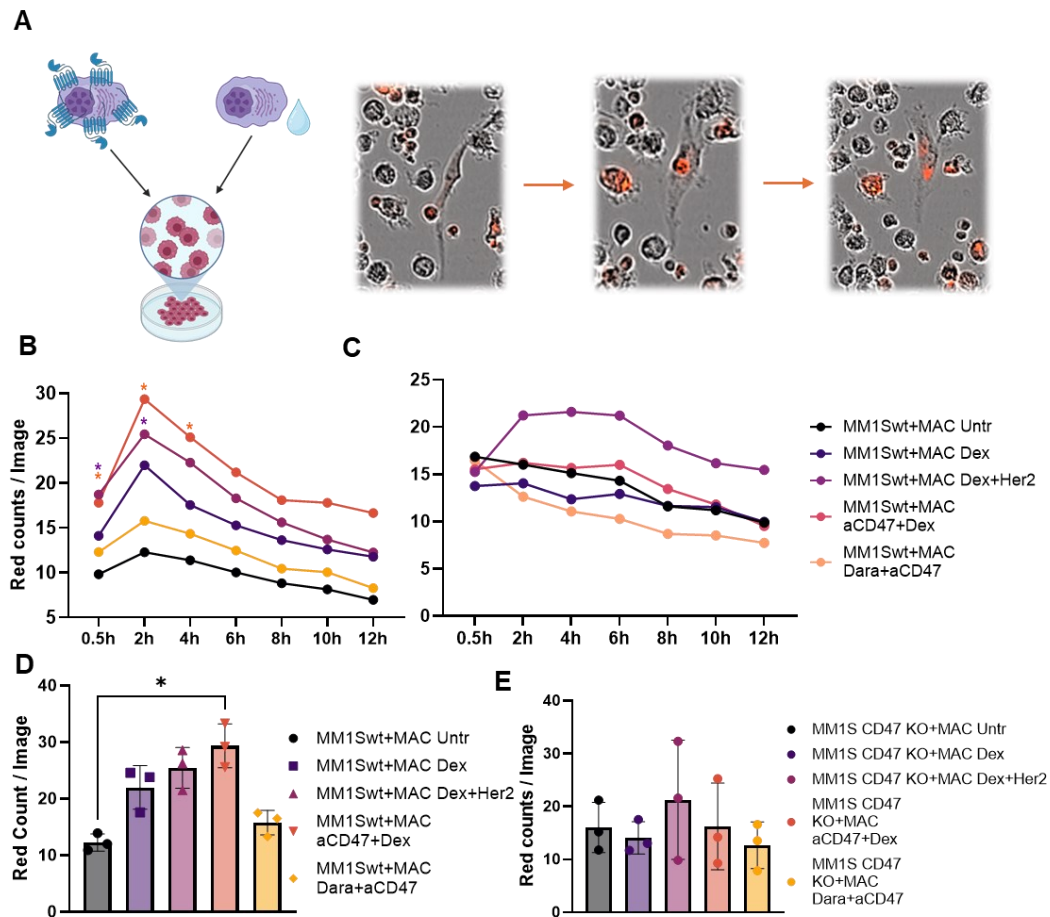


Figure 19. Phagocytosis assay evaluating the effect of anti-CD47 and dexamethasone treatment on macrophage-mediated clearance of MM1.S cells. MM1.S WT or MM1.S CD47 KO were cultured with pHrodo-labelled macrophage to evaluate the effect of various treatments, on macrophage-mediated phagocytosis (A). The treatment groups included: untreated (Untr), 2 μ M dexamethasone (Dex), 2 μ M dexamethasone combined with 10 μ g/mL Her2-IgG2 σ isotype control (Dex + Her2), 10 μ g/mL Hu5F9-IgG2 σ anti-CD47 antibody (anti-CD47), 2 μ M dexamethasone combined with 10 μ g/mL aCD47 (Dex + aCD47), and 10 μ g/mL daratumumab (Dara) combined with 10 μ g/mL anti-CD47. Phagocytic activity was quantified by measuring the increase in red fluorescence intensity per image using Incucyte analysis software. In MM1.S WT co-cultures, significant increase in phagocytosis was observed in the Dex + anti-CD47 treatment group from 0.5 to 4 hours post co-culture initiation, whereas a transient increase was observed in the Dex + Her2 control group between 0.5 and 2 hours (B). $n=3$. Mean $\pm$ SD. Significance was calculated by using two-way Anova. In MM1.S CD47 KO co-cultures, the same treatment strategy was applied, but no significant increase in phagocytosis was detected under any condition (C). Analysis at the 2-hour time point showed a statistically significant increase in phagocytosis only in the Dex + anti-CD47 treatment group in MM1.S WT cells (D), an effect not observed in the CD47 KO setting (E). $n=3$. Mean $\pm$ SD. Significance was calculated by using one-way Anova. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. MAC: macrophages.

The increase in red fluorescence signal, originating from the acidic lysosomal compartments within macrophages following MM1.S cell phagocytosis, was quantified over time. Phagocytic activity was further visualized through time-lapse imaging and video acquisition during the incubation period (fig. 19B). Phagocytosis assays revealed a transient, time-dependent increase in the engulfment of MM1.S WT cells, with a

marked elevation observed within the first 0.5 to 4 hours of co-culture incubation. Quantitative analysis revealed enhanced phagocytosis of MM1.S WT cells treated with dexamethasone in combination with anti-CD47 antibody, evident between 0.5 and 4 hours of incubation. A transient increase in phagocytic activity was also observed in cells treated with dexamethasone and the Her2-IgG δ isotype control; however, this effect diminished after 2 hours (fig. 19B). In contrast, no significant upregulation of phagocytosis was observed in any treatment group involving MM1.S CD47 KO cells (fig. 19C). Given that the peak of phagocytic activity occurred approximately 2 hours post-incubation across most treatment conditions, this time point was selected for focused comparative analysis of MM1.S WT and CD47 KO co-cultures. A significant increase in phagocytic uptake was detected exclusively in the group treated with dexamethasone combined with CD47-blocking antibody, involving MM1.S WT cells co-cultured with macrophages (fig. 19D). This effect was absent in the CD47 KO co-culture system (fig. 19E).

These findings support the hypothesis that blockade of CD47, in conjunction with dexamethasone treatment, enhances the phagocytosis of MM cells *in vitro*.

3.10 ANTI-CD47 THERAPY HAS AN EFFECT ON MM BM INFILTRATION WHILE THE COMBINATORY TREATMENT USING ANTI-CD47 AND LIPOSOMAL DEXAMETHASONE TARGET STEM CELL-LIKE CELLS

To assess the effects of anti-CD47 monotherapy and its combination with liposomal dexamethasone, the previously established murine model was employed. Experimental groups included saline control, Her2-IgG δ isotype control antibody, Hu5F9-IgG δ (anti-CD47) antibody monotherapy, and the combination of Hu5F9-IgG δ with liposomal dexamethasone. Antibody treatments were administered via i.p. injections, at a dose of 2 mg/kg, while liposomal dexamethasone was delivered intravenously at 1 mg/kg. Anti-CD47 or control antibody administration commenced three days post-transplantation, whereas liposomal dexamethasone treatment was initiated during the second week post-transplant, as outlined in figure 20A.

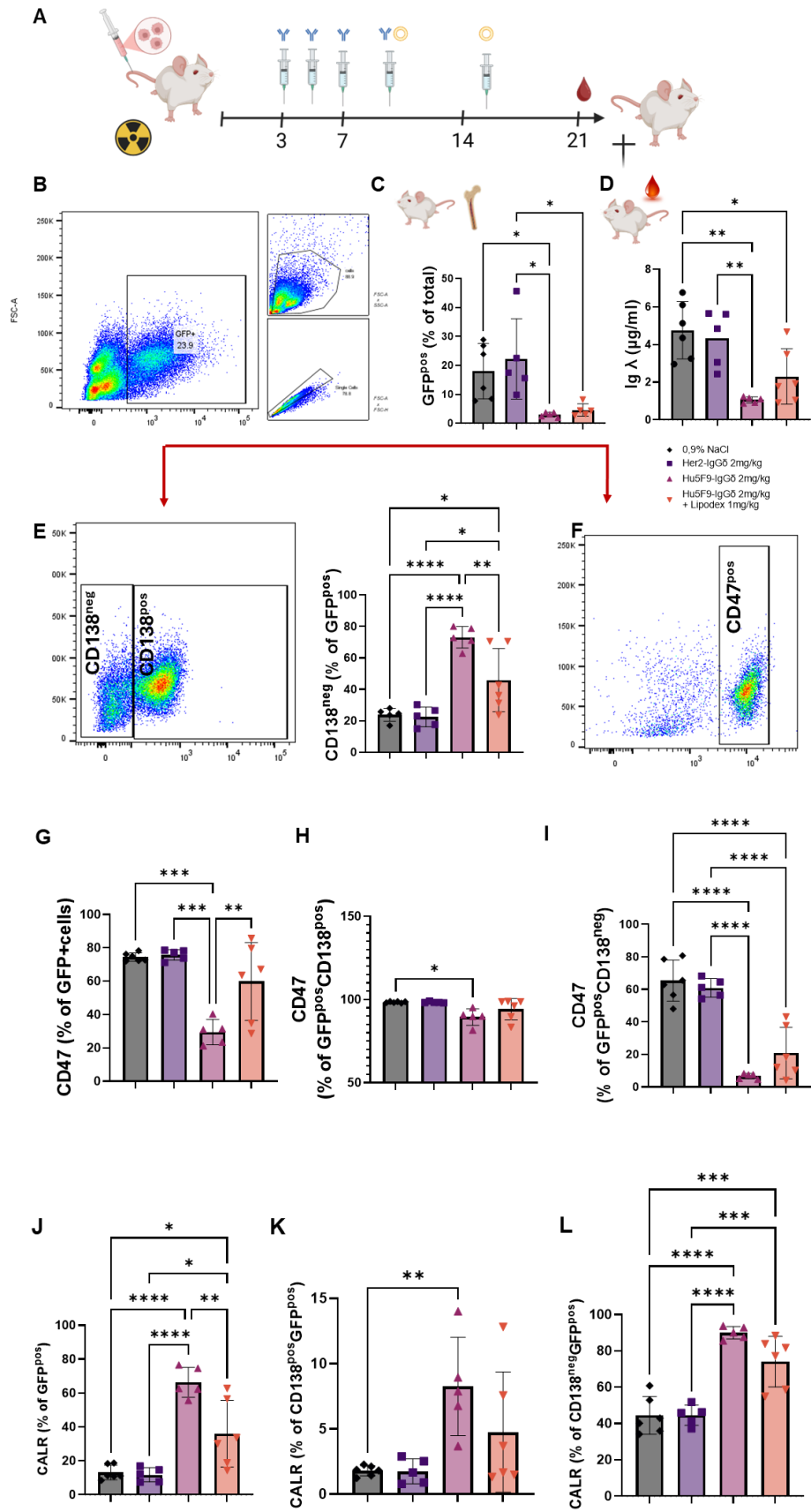


Figure 20. Effect of anti-CD47 monotreatment and combination of anti-CD47 blocking with liposomal dexamethasone on malignant cell populations. Evaluation of anti-CD47 therapy, alone or in combination with liposomal dexamethasone was investigated in previously described xenograft mouse model. 2 mg/mkg Hu5F9-IgG2 σ , or Her2-IgG2 σ antibody was given i.p. three days after MM1.S and HS-27 α transplantation. Liposomal dexamethasone was introduced from the second week after transplantation and was given i.v. at a conc. of 1 mg/kg. Treatment groups were distributed as follows: saline control, 2mg/kg Her2-IgG2 σ , 2 mg/kg Hu5F9-IgG2 σ , and 2 mg/kg Hu5F9-IgG2 σ + 1 mg/kg liposomal dexamethasone (A). Characterization of MM1.S GFP^{pos}Luc^{pos} cells was done by flow cytometric analysis. Gating strategy is shown (B). Significant treatment effect of anti-CD47 therapy on MM cell abundance was observed within the BM, while isotype control antibody didn't show any effect compared to the saline control. Liposomal dexamethasone, given along with anti-CD47 therapy didn't show any additional effect on malignant cell abundance (C). Same effect was observed upon analysis of blood Ig λ levels in diseased animals (D). Gating strategy for CD138^{pos} and CD138^{neg} quantification is represented in figure (E left). Analysis of CD138^{neg} cell compartment, within bulk GFP^{pos} cells showed enrichment of this population upon anti-CD47 monotreatment, while the addition of liposomal dexamethasone significantly decreased the abundance of these cells within this group (E right). Gating strategy for CD47 expression was performed as represented in figure (F). CD47 surface expression was assessed within bulk malignant cells (G), CD138^{pos} (H) and CD138^{neg} compartment (I). CALR expression was analysed using the same approach in bulk GFP^{pos} cells (J), CD138^{pos} (K) and the CD138^{neg} compartment (L). $n \geq 5$. Mean $\pm$ SD. Significances were calculated using one-way Anova. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

At the study endpoint, BM cells were harvested from femurs and tibias and subjected to flow cytometry, FACS and 3'RNA sequencing analyses. Engraftment of MM1.S cells within the BM was quantified based on GFP expression (fig. 20B). Additionally, samples were stained with antibodies against human CD138, CD47, and CALR for phenotypic characterization. Macrophages and MM cells were sorted, as previously described, and subjected to 3'RNA sequencing for gene expression profiling. A significant reduction in MM cell burden was observed in both the anti-CD47 monotherapy and combination treatment groups (fig. 20C). Correspondingly, serum Ig λ levels were decreased in these groups (fig 20D).

While liposomal dexamethasone did not further reduce the overall malignant cell burden (fig. 20C), its effect was evident within the stem cell-like compartment (fig. 20E). To show this, the GFP^{pos}CD138^{neg} malignant cell population was investigated. Although anti-CD47 monotherapy led to a significant enrichment of this stem cell-like population, the addition of liposomal dexamethasone selectively reduced this compartment, indicating its potential targeted effect on stem-like malignant cells (fig. 20E). CD47 and CALR expression levels were assessed across defined malignant cell subsets, including total GFP^{pos} cells, GFP^{pos}CD138^{pos} cells, and the stem cell-like GFP^{pos}CD138^{neg} population. The addition of liposomal dexamethasone to anti-CD47 therapy resulted in elevated CD47 expression within the total malignant cell population, (fig. 20G) as well as a moderate increase in the GFP^{pos}CD138^{pos} subset (fig. 20H), compared to anti-CD47 monotherapy. In contrast, this trend was not observed in the

stem cell-like GFP^{pos}CD138^{neg} compartment (fig. 20I), consistent with previously obtained *in vitro* data. Notably, CALR expression was upregulated across all malignant cell populations; within the bulk (fig. 20J), CD138^{pos} (fig. 20K) and CD138^{pos} (fig. 20L) MM cells.

Sorted MM cells were subjected to transcriptomic expression analysis (fig. 21A). In response to anti-CD47 monotherapy, there was an upregulation of gene signatures associated with NF-κB pathway activation, oxidative phosphorylation, IL-4-mediated responses (fig. 21B) and KRAS oncogenic signalling (fig. 21C). These pathways are commonly linked to enhanced cell survival and are considered hallmarks of stem cell-like properties. In contrast, combined treatment with anti-CD47 and liposomal dexamethasone resulted in the downregulation of JAK2 (fig. 21D) and Hox9 (fig. 21E) signalling pathways, both of which have been associated with cancer stemness. In sorted macrophages (fig. 21A) anti-CD47 treatment induced the upregulation of genes involved in heme metabolism compared to the untreated control (fig. 21F), consistent with the known role of CD47 blockade in promoting erythrocyte clearance and the central role of macrophages in iron recycling. Notably, the combination therapy promoted gene expression signatures related to allograft rejection and phagocytosis, suggesting enhanced macrophage-mediated antitumor activity (fig. 21G).

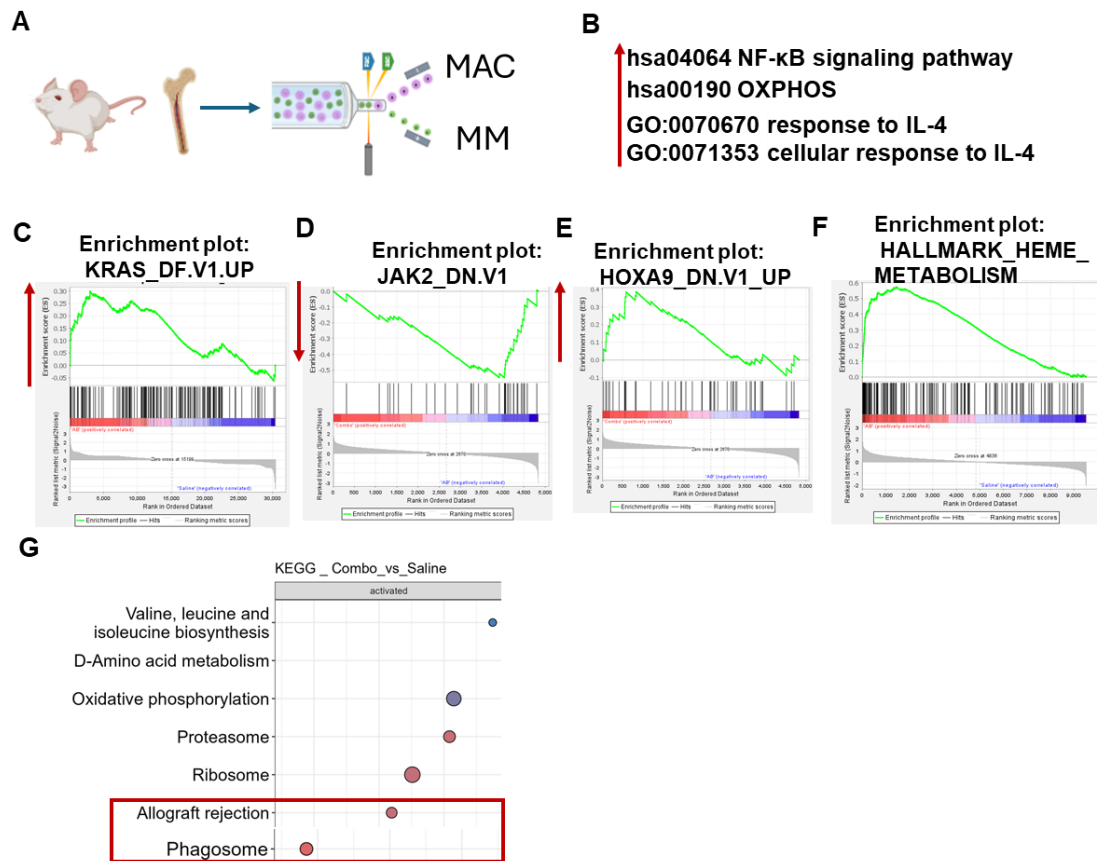


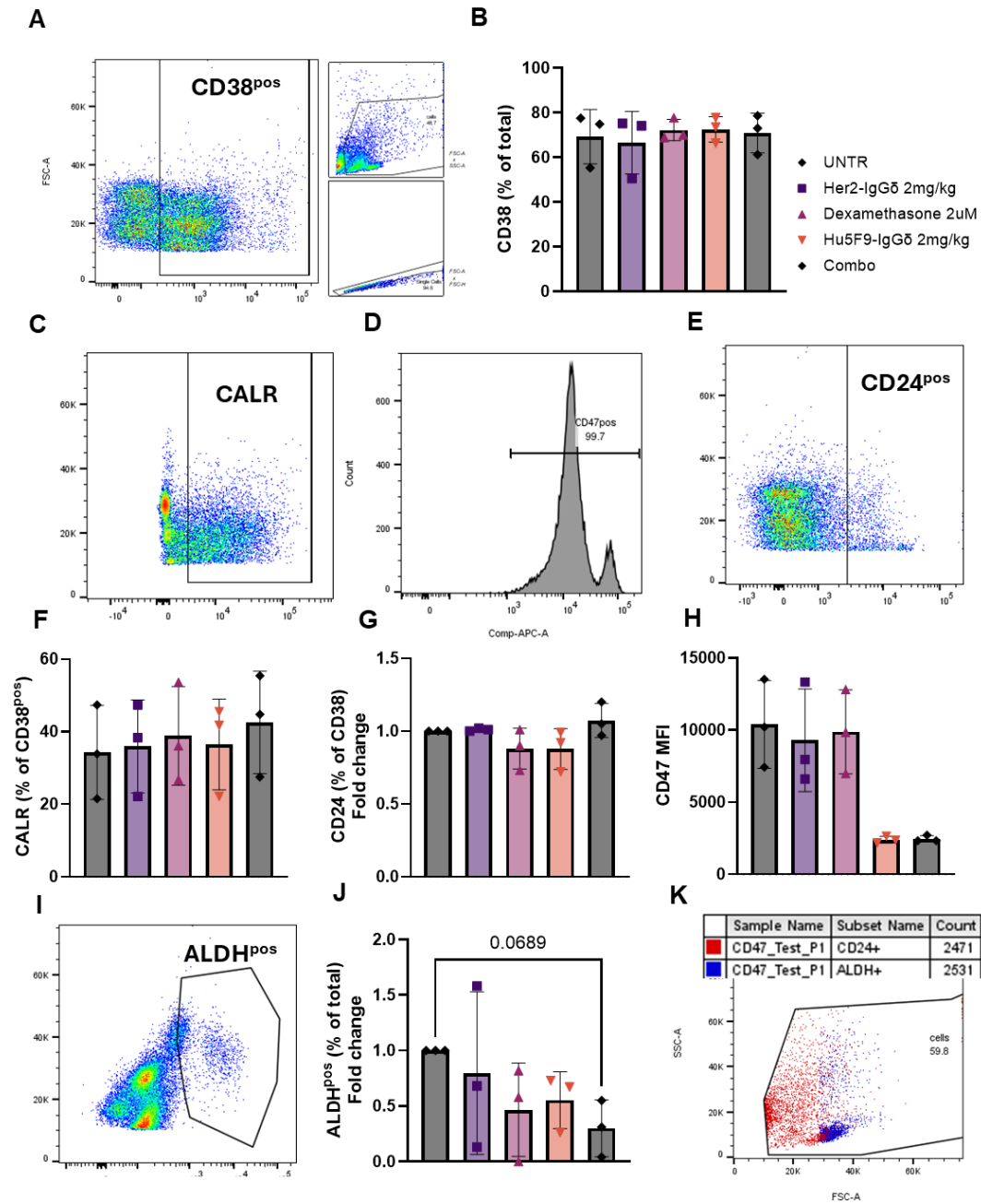
Figure 21. Transcriptomic analysis of MM cells and macrophages following anti-CD47 monotherapy or combined treatment with liposomal dexamethasone. *GFP*^{pos} MM1.S and cells and *GFP*^{neg}CD11C^{neg}Ly6G/C^{low}CD11b^{pos}F4/80^{pos} macrophages were subjected to FACS sorting followed by RNA extraction and 3'RNA expression analysis was performed, as described (A). Anti-CD47 monotherapy induced significant upregulation of gene signatures associated with NF-κB signaling, oxidative phosphorylation, IL-4 response (B), and KRAS pathways (C). In contrast, combination therapy with anti-CD47 and liposomal dexamethasone resulted in downregulation of JAK2 (D) and HOXA9 (E) signaling pathways, consistent with the stem cell-like cell targeting, observed in flow cytometric analysis. Anti-CD47 monotherapy of murine macrophages showed upregulation of genes involved in heme metabolism (F), whereas the addition of liposomal dexamethasone enhanced expression of pathways related to immune response, including allograft rejection and phagocytosis (G). *n*=3. Raw sequencing reads were aligned with reference genome using the alignment tool. Differential gene expression analysis was performed using DESeq2. *P*-values were adjusted for multiple testing using the Benjamini-Hochberg procedure. Genes with adjusted *p* value < 0.05 and a log2 fold change > 1 were considered significantly differentially expressed.

Consistent with *in vitro* findings, CD47 and CALR surface expression increased predominantly in the more differentiated malignant cell populations upon liposomal dexamethasone treatment. Overall, anti-CD47 monotherapy significantly reduced the MM cell burden, whereas the addition of liposomal dexamethasone did not further decrease the overall tumor load. However, liposomal dexamethasone selectively depleted the stem cell-like compartment, which was enriched following anti-CD47 monotherapy, and enhanced macrophage-mediated antitumor activity, as evidenced by KEGG pathway analysis.

3.11 CHARACTERIZATION OF PATIENT DERIVED STEM CELL-LIKE CELLS

Characterizing stem cell-like populations in patient-derived samples presents significant challenges compared to MM cell lines. In the literature, this subpopulation remains poorly characterized; however, functional markers such as ALDH enzymatic activity, side population analysis, surface expression of CD24 and CD138 negativity are considered key hallmarks of stem cell-like cells in MM (Guo W et al., 2021). Previously cryopreserved BMMNCs from MM patients were treated for 72 hours with the following conditions: untreated control, Her2 isotype control, dexamethasone, anti-CD47 antibody, and a combination of dexamethasone with anti-CD47 antibody. Follow-up of MM CD38^{pos} MM cells were not feasible, as CD138 surface expression was diminished - a phenomenon previously reported under various conditions, including sample handling, hypoxia, and processing techniques (Frigyesi I et al, 2014). Consequently, MM cells were identified based solely on CD38 positivity (fig. 22A+B). Within this population, surface expression of CALR (fig. 22C), CD47 (fig. 22D) and CD24 (fig. 22E) was assessed, to observe any potential changes in the expression analysis upon different treatment approaches. No significant modulation of CALR (fig. 22F), and CD24 (fig. 22G) expression was detected across treatment groups within the CD38^{pos} population. In contrast, a CD47 surface expression was diminished following anti-CD47 therapy, consistent with effective target engagement (fig. 22H). To functionally assess the stem-like cell compartment, an Aldefluor assay was included to identify ALDH^{high} cells (fig. 22I). Although differences between treatment groups did not reach statistical significance, a trend towards reduced ALDH activity was noted in the combination treatment group (fig. 22J). To further investigate the relationship between CD24 and ALDH positivity, co-expression patterns were visualized via back-gating on SSC-A and FSC-A plots. A partial overlap between CD24⁺ and ALDH⁺ subsets was observed (Fig. 22L); however, ALDH^{pos} cells demonstrated more stringent cell clustering compared to CD24^{pos} cells.

While these preliminary observations provide insight into treatment-associated modulation of stem cell-like markers in patient-derived multiple myeloma cells, additional studies will be required to comprehensively define the impact of combination therapy on this compartment.



4 DISCUSSION

Multiple myeloma is regarded as incurable disease, as nearly all patients eventually experience disease relapse (Bruno AS et al., 2020). Current standard-of-care therapies include PIs, corticosteroids, IMiDs, monoclonal antibodies, and ASCT. Management of patients who relapse or develop treatment-refractory disease remains a significant clinical challenge. Recent studies support the use of multi-agent therapeutic regimens, incorporating novel agents with potential synergistic activity alongside existing treatments. This strategy aims to enhance initial CR rates and may ultimately lead to improvements in PFS and OS (American cancer society, 2021).

Dexamethasone has been a cornerstone of MM therapy for decades, due to its pro-apoptotic, anti-inflammatory, and immunosuppressive properties. It is included at all stages of MM treatment, typically as an adjunct to novel agents. Despite its proven clinical benefit, all patients eventually relapse. Dexamethasone's therapeutic potential is limited by its suboptimal pharmacokinetics, including rapid systemic clearance, low target-site accumulation, and a high volume of distribution, factors that primarily contribute to systemic toxicity rather than efficacy (Metselaar et al., 2023). Consequently, higher and/or more frequent dosing is required, which increases the risk of adverse systemic effects such as immunosuppression, hyperglycemia, and osteoporosis. Liposomal encapsulation of dexamethasone has been shown to improve therapeutic outcomes in models of inflammation and solid tumors. This formulation enhances local drug concentrations at sites of inflammation while simultaneously reducing off-target toxicity (Deshantri et al., 2019; Metselaar et al., 2023). In addition, multiple studies have reported that MM cells overexpress CD47, an immune checkpoint molecule that enables tumor cells to evade macrophage-mediated phagocytosis via interaction with SIRP α , a mechanism commonly referred to as the “don't eat me” signal (Kim et al., 2012). Preclinical data indicate that anti-CD47 therapies effectively block this antiphagocytic signal *in vitro* (Sun et al., 2020), and *in vivo* studies have further validated CD47 as a promising therapeutic target within the MM tumor microenvironment (Kim et al., 2012). Therapeutic targeting of CD47 may potentiate the efficacy of existing treatments and contribute to improved clinical outcomes in MM. This study explored the therapeutic potential of liposomally

encapsulated dexamethasone and CD47 blockade in the treatment of MM. Specifically, the antitumor efficacy and improved targetability of liposomal dexamethasone were evaluated *in vivo*, with a focus on its capacity to enhance drug delivery to MM cells while potentially reducing systemic toxicity compared to free dexamethasone. The effects of CD47 inhibition were assessed both *in vitro* and *in vivo*, highlighting its role in promoting macrophage-mediated clearance of malignant cells. Furthermore, the study investigated the combinatorial impact of anti-CD47 therapy with liposomal dexamethasone as an adjunct to standard-of-care regimens, aiming to determine potential synergistic effects.

This chapter presents a critical evaluation of the main findings, discusses their relevance to current therapeutic strategies, and outlines potential clinical applications. In addition, the limitations of the present work are addressed, along with suggestions for future research directions within the scope of tumor-targeted therapy in MM.

4.1 LIPOSOMAL DEXAMETHASONE TARGETS MM CELLS WITHIN BM MICROENVIRONMENT

Dexamethasone is included in nearly all MM treatment regimens due to its ability to induce apoptosis in malignant plasma cells, reduce inflammation, and enhance the efficacy of other myeloma-directed therapies (Rosenberg, 2023). Glucocorticoids (GCs) have been successfully applied in cancer therapy across various animal models, with liposomal formulations often demonstrating superior clinical efficacy compared to their free-drug formulations (Schiffelers RM et al., 2005; Avnir Y et al., 2011; Kroon J et al., 2015). However, limited progress has been made in translating the preclinical findings on liposomal GC delivery into clinical practice. Although liposomal drug carriers have been shown to mitigate GC-associated systemic toxicity, concerns remain regarding carrier-related adverse effects, which must be thoroughly evaluated. In particular, some delivery vehicles may provoke immune responses upon repeated administration, potentially leading to accelerated clearance of the particles from circulation (Lühder, 2017).

This study supports the rationale for employing liposomal dexamethasone formulations in preclinical models of MM as part of a multidrug therapeutic strategy. *In vivo* data demonstrated that liposomal dexamethasone exhibited superior BM targeting

compared to the free drug, highlighting its potential to enhance therapeutic efficacy while minimizing systemic toxicity. The biodistribution study using Cy7-labeled liposomal dexamethasone revealed preferential accumulation in clearance and excretion organs, namely the liver, kidneys, spleen, and lungs, at six hours post-injection. Notably, a significant proportion of the liposomes remained in circulation, as evidenced by signal retention in the heart, and approximately 5% of the injected dose localized within the BM. This degree of BM accumulation represents a marked improvement over free dexamethasone, which typically shows <1% localization to this compartment (Spoorenberg et al., 2013). These findings are consistent with the well-documented tendency of liposomes to passively accumulate in tissues with enhanced vascular permeability, a hallmark of the MM BM niche. Unlike free dexamethasone, which is rapidly cleared and non-specifically distributed, the liposomal formulation benefits from prolonged circulation and enhanced permeability and retention (EPR) effects. The extended circulation time of liposomal dexamethasone allows for more sustained drug release, potentially reducing the need for repeated dosing and decreasing off-target exposure (Metselaar et al., 2023). Due to a weak bioluminescent signal at the time of Cy7-liposome administration, definitive colocalization of liposomal dexamethasone with malignant plasma cells could not be confirmed. Nonetheless, 3D reconstruction of BLT/ μ CT imaging demonstrated the expected exponential increase in GFP^{pos}Luc^{pos} cells over time. These observations support the hypothesis that liposomal drug delivery can achieve therapeutically relevant concentrations within the BM niche.

Together, these findings underline the therapeutic potential of liposomal dexamethasone in MM treatment, especially when considering its potential as part of a combinatorial regimen. However, further studies are warranted to more precisely quantify drug-tumor colocalization and to assess therapeutic outcomes in longitudinal models.

To assess the impact of treatment on disease progression, we followed established clinical practices by measuring serum immunoglobulin λ levels, as the overproduction of monoclonal immunoglobulins is a hallmark of MM pathology. MM.1S cells are known to secrete IgA λ monoclonal proteins (Fenics Bio, 2025), making this a suitable biomarker for monitoring therapeutic response (Schieferdecker et al., 2020). Our data demonstrated a superior reduction in plasma Ig λ levels following treatment with

liposomal dexamethasone compared to free dexamethasone, indicating enhanced therapeutic efficacy of the liposomal formulation. We also evaluated the effects of treatment on MM cell numbers within the bone marrow. However, no statistically significant differences were observed between the treatment groups. Notably, there was considerable variability in MM cell abundance across treatment groups, which may have limited our ability to detect more subtle treatment effects. Additionally, the drug concentrations used in this study were optimized for combinatorial therapeutic approaches and may have been insufficient to exert a strong cytotoxic effect as monotherapy (Deshantri et al., 2019).

Importantly, animals treated with 2.5 mg/kg of free dexamethasone developed tail necrosis, a side effect not observed in the group receiving an equivalent dose of liposomal dexamethasone. This finding supports the improved safety profile of the liposomal formulation. While necrotic effects of dexamethasone are not widely reported in the literature, higher concentrations have been associated with tissue damage and cytotoxicity resulting from extravasation injury, which aligns with our empirical observations (Okuda et al., 2017).

Gene expression analysis of sorted MM cells revealed a distinct transcriptional profile in the groups treated with 1mg/kg and 2.5 mg/kg liposomal dexamethasone compared to untreated controls and 2.5 mg/kg free dexamethasone-treated samples, as determined by PCA. Due to experimental limitations, the 1 mg/kg free dexamethasone group could not be analysed within the same PCA plot alongside the other treatment groups. However, this group was evaluated independently in comparison with its corresponding control. In addition to PCA, volcano plot analysis demonstrated a markedly greater number of differentially expressed genes in the group treated with 1 mg/kg liposomal dexamethasone compared to the free dexamethasone group, which exhibited regulation of only nine genes vs 215 genes in the liposomal group. In contrast, the 2.5 mg/kg treatment groups exhibited comparable transcriptional changes, with 501, and 554 regulated genes, in the free and liposomal dexamethasone groups, respectively. These findings suggest a more pronounced transcriptional response induced by the liposomal formulation. Lower doses of liposomal dexamethasone are known to offer improved tolerability and enhanced quality of life, supporting their potential for long-term use in MM treatment regimens (Harding S et al., 2025). Contemporary treatment strategies aim to incorporate dexamethasone as

an adjunct to monoclonal antibodies (mAbs), IMiDs, and PIs in order to amplify therapeutic efficacy. Studies have demonstrated that even at reduced doses, dexamethasone can contribute synergistically while minimizing systemic toxicity (NIH, 2025; Delforge M et al., 2015; Ruedas A et al., 2009). Based on these considerations, we selected 1 mg/kg liposomal dexamethasone for evaluation as part of a combination treatment strategy. Interestingly, we observed an upregulation of stem cell–associated gene signatures in vivo following treatment with 1 mg/kg liposomal dexamethasone, consistent with similar effects seen in vitro with the free formulation. This response was accompanied by activation of the mTOR signaling pathway, which has been implicated in the maintenance, survival, and self-renewal of CSCs, thereby contributing to therapy resistance and disease progression (Son B et al., 2024; Du J et al., 2015). Additionally, treatment with liposomal dexamethasone induced upregulation of the interferon-alpha (IFN α) signaling pathway. Historically, IFN α activation has been associated with the development of adaptive responses that promote survival and resistance to dexamethasone (Ferlin-Bezombes M et al., 1998; Liu P et al., 1999). However, former studies have demonstrated that IFN α may exert potent anti-MM activity, partly by enhancing M1 macrophage infiltration and promoting antibody-dependent cellular phagocytosis. Notably, these effects can be fully restored when combined with CD47-targeted therapy (Pogue S et al., 2017).

Gene expression analysis revealed upregulation of SMAD1, CD180, IKZF3, and MAF, along with downregulation of CD38, collectively suggesting a transcriptional profile associated with therapy resistance and enhanced myeloma stemness. Upregulation of SMAD1 facilitates unchecked cell proliferation and promotes cell survival by inhibiting caspase-8–mediated apoptosis (Wu J et al., 2021). CD180, a downstream target of IKZF1, has been implicated in maintaining cellular dormancy and mediating drug resistance in MM (Kikuchi J et al., 2018). Increased expression of IKZF3 further promotes autonomous MM cell growth and survival (Krönke J et al., 2014). MAF upregulation is a well-established marker of poor prognosis and has been associated with resistance to treatment (Jiang Q et al., 2022). Conversely, downregulation of CD38 has been linked to increased disease aggressiveness and extramedullary dissemination (Notarfranchi L et al., 2024). This finding is particularly relevant for evaluating anti-CD38–directed therapies, such as daratumumab, in combination with dexamethasone. Furthermore, downregulation of HLA-DRB1, HLA-DPB1, and CD74 was observed following treatment with 1 mg/kg liposomal dexamethasone, indicating

a potential immune evasion mechanism via reduced presentation of tumor-associated antigens through MHC class II molecules (Zavidij O et al., 2021). Also notable was the downregulation of TNFRSF17 (encoding BCMA), MZB1, IGLL5, KLF2, and MYC—all genes crucial for MM cell survival (Nobari ST et al., 2022; Chanukuppa V et al., 2020; White BS et al., 2018; Ohguchi H et al., 2016; Holien T et al., 2012). These findings highlight potential dexamethasone-sensitive molecular targets. Of particular interest is the observed downregulation of BCMA, which may limit the efficacy of BCMA-targeted therapies when used in combination with dexamethasone. However, further validation is necessary to substantiate this hypothesis. Additionally, we observed upregulation of ST6GAL1, a gene involved in immunosuppressive mechanisms through inhibition of granulopoiesis (Gc S et al., 2022), and downregulation of JCHAIN, which is essential for the formation of polymeric IgA and IgM (Castro CD et al., 2014). This is consistent with the reduced plasma Ig λ levels observed in mice treated with liposomal dexamethasone, further supporting the immunosuppressive effects of glucocorticoid therapy.

Importantly, treatment with empty liposomes did not result in observable adverse effects, such as weight loss or injection-associated tail necrosis. Although some gene expression changes were noted in this group relative to untreated controls, PCA indicated no significant differences along the primary axis of variation, PC1. However, differences were detected along the second principal component, PC2. This effect may be attributable to a single outlier within the empty liposome group, which could artificially inflate variance along PC2 and exaggerate intergroup differences (supp. fig. 3) (Chen X et al., 2020).

4.2 DEXAMETHASONE TREATMENT DIFFERENTIALLY REGULATES CD47 EXPRESSION AND SELECTIVELY SPARES STEM CELL-LIKE CELLS

CD47 is a multifunctional surface protein that is overexpressed in various malignancies, including MM (Zhan F et al., 2002). Its overexpression underscores a critical role in immune evasion and the acquisition of drug resistance. The most well-characterized immune checkpoint interaction contributing to evasion of phagocytosis is the binding of CD47 on MM cells to SIRP α on macrophages. This interaction delivers a "don't eat me" signal that inhibits macrophage-mediated clearance (Kim D et al., 2012). In addition to these inhibitory signals, cancer cells can also express pro-

phagocytic or "eat me" signals, which influence the balance between immune evasion and immune clearance (Gardai SJ et al., 2005). One of the most studied "eat me" signals is the surface exposure of CALR, which is part of the immunogenic cell death (ICD) pathway. CALR exposure typically occurs following treatment with apoptotic inducers and promotes phagocytosis through binding to the low-density lipoprotein receptor-related protein (LRP) on macrophages (Orr AV et al., 2003). In MM, aberrant CALR expression has been associated with poor clinical outcomes (Serrano Del Valle A et al., 2022).

In this study, we evaluated the impact of dexamethasone treatment on CD47 and CALR surface expression in MM1.S cells. The analysis was stratified by subpopulations characterized as stem cell-like, CD138^{neg} and mature CD138^{pos} cells, in accordance with previous definitions of MM1.S cellular hierarchy (Reghunathan R et al., 2013). CD47 expression was assessed independently within each compartment to elucidate potential mechanisms underlying dexamethasone resistance and to explore the rationale for combining dexamethasone with anti-CD47 antibody therapy. We observed moderate upregulation of both CD47 and CALR in bulk MM1.S cells following dexamethasone treatment. This is consistent with previous studies reporting CD47 upregulation as a resistance mechanism (Rastgoo N et al., 2020). Notably, CD47 surface expression has been shown to correlate with CALR surface exposure, suggesting that increased CD47 expression may serve as a protective mechanism against CALR-mediated immunogenic apoptosis (Chao MP et al., 2010). Dexamethasone treatment appeared to selectively target the CD138^{pos} compartment while enriching the stem cell-like CD138^{neg} population. Consistently, the CFU capacity was significantly affected in the CD138^{pos} cells, whereas the clonogenic potential of the CD138^{neg} population remained largely unaffected by the treatment. These findings suggest that the stem cell-like subpopulation exhibits inherent resistance to dexamethasone and may contribute to disease persistence and relapse, which is in agreement with previously published data (Gong L et al., 2021). Interestingly, and contrary to what has been reported for drug-resistant cancer cell populations, we observed downregulation of CD47 expression specifically within the stem cell-like CD138^{neg} compartment following dexamethasone treatment. This effect was consistently seen in both monoculture and transwell co-culture systems with macrophages. In contrast, CD47 expression remained higher in the CD138^{pos} population and in bulk cells. These findings diverge from prior reports describing CD47

upregulation as a hallmark of CSC drug resistance and aggressiveness (Rastgoo N et al., 2020). To our knowledge, this is the first study to specifically assess CD47 expression in MM stem cell-like populations in response to dexamethasone. Although CD47 is widely recognized as an immune checkpoint molecule, its expression appears to vary across tumor types and differentiation states. For example, in bladder cancer, tumor-initiating cells exhibited higher CD47 levels than their mature counterparts (Chan KS et al., 2009). Conversely, in myelofibrosis, progenitor cells showed decreased CD47 expression compared to controls (Nonino A et al., 2018). Similarly, ALDH^{low} ovarian cancer cells expressed CD47 at approximately half the level of ALDH^{high} cells (Sharro AC et al., 2016). A study on primary murine endothelial cells also demonstrated an inverse relationship between CD47 expression and self-renewal capacity, with low-CD47 cells displaying higher expression of stemness-associated transcription factors such as c-Myc, Klf4, Oct4, and Sox2 (Kaur S et al., 2013).

MM tumor-initiating stem cell-like cells reside in a complex BM niche shaped by immunosuppressive cues from the TME. In this context, mature MM cells, characterized by high CD47 expression, may contribute to niche remodeling and immune evasion, thereby protecting the more quiescent and treatment-resistant stem cell-like population. However, these findings have primarily been derived from MM cell line studies and require validation in a more physiologically relevant TME setting. Nonetheless, both expression profiling and functional analyses support the stem-cell like characteristics of these cells.

4.3 DEXAMETHASONE TREATMENT INDUCES M2 ANTI-INFLAMMATORY MACROPHAGE PHENOTYPE

For studies assessing the impact of macrophages and their polarization in MM we established in house a differentiation and polarization protocol based on published literature (Orrechioni M et al., 2020). Morphological assessment (supp. fig. 2) and surface marker analysis—specifically CD204, CD206, CD86, CD200R, and CD163—confirmed the expected phenotypes for M0, M1, M2, and TAMs (Hourani T et al., 2023; Kaku Y et al., 2014; Koning N et al., 2010; Yunna C et al., 2020). On D6, M0 and M1 macrophages displayed a rounded morphology, while M2 macrophages exhibited a more elongated, spindle-like shape (supp. fig 1). M2-polarized macrophages expressed higher levels of CD204, CD206, CD200R, and CD163, whereas M1

macrophages showed elevated surface expression of CD86. SIRP α and CD14 were also analyzed, given their relevance in macrophage-mediated phagocytosis (Andrejeva G et al., 2021; Schlegel RA et al., 1999). CD14 was underrepresented in the M1 population, likely due to LPS-induced masking of the CD14 receptor, as previously documented (Schlegel RA et al., 1999). Treatment with both free and liposomal dexamethasone promoted polarization toward an anti-inflammatory M2 phenotype in co-culture with MM1.S cells, consistent with the known immunomodulatory effects of glucocorticoids (Carrati G et al., 2023; Bartneck M et al., 2014).

We further examined the effects of 1 mg/kg dexamethasone on murine macrophages *in vivo*. For these purposes, we designed FACS panel to sort and further characterize the murine macrophage populations according to CD206, CD163 and SIRP α expression (Güç E et al., 2020; Cassetta L et al., 2016; Wang C et al., 2013; Zhang X et al., 2008). Due to the use of NSG mice, which lack functional NK, T, and B cells (Shultz LD et al., 2012), lineage exclusion markers for these populations were omitted. Following dexamethasone treatment, macrophages showed a moderate increase in CD163 and CD206 while SIRP α levels remained stable, consistent with *in vitro* findings. Sorted macrophages were then analyzed via 3' RNA sequencing. Transcriptomic analysis indicated a shift toward an M2-like state, evidenced by upregulated oxidative phosphorylation and downregulated glycolysis, hallmarks of anti-inflammatory macrophage metabolism (Viola A et al., 2019). Additionally, dexamethasone treatment led to suppression of pro-inflammatory gene expression, including downregulation of IFN γ signaling, IL-6/JAK/STAT3, TNF α signaling, complement expression, and IL-17 pathway activity (Xia T et al., 2023; Viola A et al., 2019; Ruan CC et al., 2015; Zhang Q et al., 2013). Reduced myeloid cell activation and migration were also observed, in line with previous findings (Kim BY et al., 2017; Bartneck M et al., 2014). A decrease in macrophage phagocytic activity was noted, supporting earlier reports of dexamethasone-mediated inhibition of phagocytosis (Becker J et al., 1985), which has been linked to granulocyte maturity and glucocorticoid exposure (Shezen E et al., 1985).

4.4 COMBINED DEXAMETHASONE AND ANTI-CD47 THERAPY ENHANCES MACROPHAGE-MEDIATED PHAGOCYTOSIS *IN VITRO*

Macrophages, associated with MM exhibit a predominantly immunosuppressive, tumor supportive M2 phenotype, commonly referred to as TAMs (Sun J et al., 2022). An increasing body of evidence highlights their critical role in MM pathogenesis and association with poor clinical outcomes (Beider K et al., 2014; De Beule N et al., 2017; Chen H et al., 2017; Suyani E et al., 2013). As key components of the innate immune system, macrophages possess the capacity to engulf malignant cells and present tumor antigens to adaptive immune cells, positioning macrophage-mediated phagocytosis as a promising target in cancer immunotherapy (Feng M et al., 2019). To investigate this further, we first evaluated the effect of dexamethasone-treated MM cells on the phagocytic activity of M0 and M2 macrophages in a co-culture system. Although an increased trend in phagocytosis of dexamethasone-exposed MM cells was observed, the effect did not reach statistical significance. This process of apoptotic cell clearance, known as efferocytosis, plays a critical role in tissue homeostasis, resolution of inflammation, immune tolerance, and cancer progression (Lin D et al., 2020). Several studies have demonstrated that efferocytosis can facilitate tumor immune evasion (Poon IK et al., 2014) and promote disease progression through rapid clearance of apoptotic cells (Zhou X et al., 2021).

In contrast, the induction of anti-tumor immunity through enhanced phagocytic clearance of malignant cells has emerged as a promising therapeutic strategy aimed to disrupting CD47-SIRP α immune checkpoint (Lin F et al., 2020) thereby promoting a more favorable immune response in MM patients. SIRP α functions as a key inhibitory receptor on phagocytes, acting as a negative regulator that counterbalances activating signals from Fc receptors, complement receptors, and lipoprotein-related proteins (Logtenberg ME et al., 2020). CD47, often overexpressed on tumor cells, represents an evolutionary adaptation to evade immune surveillance by delivering a "don't eat me" signal, thus rendering it an attractive target for mAb-mediated blockade to reverse immune suppression and stimulate macrophage-mediated phagocytosis of MM cells (Zhang W et al., 2020). Current therapeutic strategies increasingly focus on targeting the CD47–SIRP α axis using anti-CD47 monoclonal antibodies and SIRP α -Fc fusion proteins (Sun J et al., 2022). Clinical trials investigating agents such as magrolimab,

TTI-622, AO-176, and ALX148 have shown encouraging results (Zhang W et al., 2020; Maute R et al., 2022). In parallel, alternative approaches such as RNA interference to suppress CD47 expression via siRNA-mediated knockdown have also demonstrated enhanced tumor cell phagocytosis (Zhao X et al., 2013; Wang Y et al., 2013). In this study, we investigated the effect of CD47 blockade using Hu5F9-IgG2 α —a CD47-targeting monoclonal antibody—with and without dexamethasone, to assess potential additive effects on macrophage phagocytic capacity *in vitro*. The Hu5F9-IgG2 α antibody used in our experiments is an Fc-inactive variant, engineered with six mutations in the Fc region to eliminate Fc receptor binding and subsequent Fc-mediated effector functions (Vafa O et al., 2014). This design enabled us to isolate the direct effect of CD47–SIRP α axis inhibition without confounding Fc γ R engagement. To further validate the Fc-null nature of this antibody, we employed Her2-IgG2 σ as an isotype control, consistent with previous experimental protocols (Schewe DM et al., 2024; Kim D et al., 2012).

Our experimental setup included treatment groups of untreated control, dexamethasone monotherapy, dexamethasone combined with Her2 control antibody, and dexamethasone combined with anti-CD47 antibody. Additionally, we tested a combination of daratumumab with anti-CD47 as a potential strategy to further enhance macrophage-mediated phagocytosis, based on previously reported synergistic effects (Klausz K et al., 2021). All treatment groups were assessed in co-culture systems of macrophages with either WT or CD47 KO MM1.S GFP^{pos}luc^{pos} cells to directly assess the functional contribution of CD47 in phagocytic evasion. We observed the strongest induction of phagocytosis in WT MM1.S cells following combined treatment with dexamethasone and anti-CD47 antibody, within the 0.5–4-hour time window. Due to logistical constraints, the 0-hour time point could not be recorded, as co-culture assembly was performed in our laboratory prior to imaging at a collaborating institute. Focusing on the 2-hour time point, at which the highest phagocytic activity was recorded, we observed a statistically significant increase in phagocytosis exclusively in the WT cells treated with the combination of dexamethasone and anti-CD47 antibody. No significant induction was detected in other WT treatment conditions, nor was any increase observed in CD47 KO cells across all treatment groups. The absence of effect in CD47 KO cells was partially expected, particularly in the context of anti-CD47 therapy, confirming the specificity of the observed response. The absence of

increased phagocytosis in untreated control and dexamethasone monotherapy was somewhat counterintuitive, given that loss of the “don’t eat me” signal, mediated by CD47, typically renders cells more susceptible to phagocytic clearance (Tang Z et al., 2023). However, considering the multifunctional role of CD47 in cellular processes (Polara R et al., 2024), including its involvement in stress responses (Soto-Pantoja DR et al., 2015) and caspase-3-independent apoptosis (Lamy L et al., 2003), the improved viability of CD47 KO cells under our experimental conditions may reflect an adaptive advantage. This notion was further supported by annexin V-based apoptosis assay, where we observed that apoptosis was, in part, CD47-dependent in both untreated and dexamethasone-treated conditions. CD47 KO cells consistently exhibited the lowest levels of apoptosis, suggesting enhanced survival and potentially altered susceptibility to phagocytic elimination. In our experiments, we did not observe an increase in phagocytosis following combined treatment with daratumumab and anti-CD47 antibody. This finding contrasts with previously published data demonstrating that daratumumab, as an IgG1 monoclonal antibody, effectively promotes ADCP by engaging activating Fcγ receptors (Gao Y et al., 2022; Overdijk MB et al., 2015). However, ADCP is known to be highly dependent on the balance between activating, CD64, CD16, and inhibitory, CD32 Fcγ receptors (Junker N et al., 2020; Herter S et al., 2014). The expression of these receptors is often limited in M0, non-activated, resting macrophages, such as those used in our experimental model, potentially explaining the lack of observed phagocytic enhancement (Mendoza-Coronel E et al., 2017). However, the observed induction of macrophage phagocytic activity following dexamethasone and anti-CD47 treatment of MM1.S WT cells may be attributed to the direct modulation of the surface expression of “don’t eat me” signal CD47 and the “eat me” signal CALR signals. Notably, we previously confirmed that dexamethasone treatment increases CALR surface expression, suggesting a shift in the balance of pro- and anti-phagocytic signals toward enhanced macrophage-mediated phagocytosis.

4.5 CD47 BLOCKADE IS EFFECTIVE *IN VIVO*, AND ITS COMBINATION WITH DEXAMETHASONE FURTHER REDUCES STEM CELL-LIKE CELLS

Based on the encouraging *in vitro* results demonstrating the superior effect of combined dexamethasone and anti-CD47 treatment, we proceeded to validate these findings in a preclinical *in vivo* setting. Using a MM xenograft mouse model, we investigated the following treatment strategies: saline control, Her2-IgG2 σ (isotype control), Hu5F9-IgG2 σ (anti-CD47), and the combination of anti-CD47 with dexamethasone (combo). A significant reduction in MM cell burden within the BM, as well as decreased serum Ig λ levels, was observed following anti-CD47 monotherapy. This finding aligns with a previous study that assessed anti-CD47-based combination therapies in B-cell precursor acute lymphoblastic leukemia (Schewe DM et al., 2024). The underlying mechanism for this effect has been explored in prior research, which suggests that differential responsiveness between murine and human macrophages to CD47 blockade may contribute to the observed outcomes. A study investigating the effect of CD47 blockade in combination with rituximab in non-Hodgkin lymphoma (NHL) demonstrated that CD47 inhibition alone significantly reduced lymphoma burden and prolonged overall survival in mice, while the addition of rituximab further enhanced these effects (Chao MP et al., 2010). Another study exploring the mechanism underlying phagocytic clearance following disruption of the CD47–SIRP α axis reported that this effect is dependent on Fc–Fc γ receptor interactions. Specifically, phagocytosis was observed in co-culture systems involving human tumor cells and murine macrophages in the presence of CD47 blockade. However, the same effect was absent in *in vitro* systems comprising only human-derived cells, leading the authors to conclude that CD47 blockade alone is insufficient to trigger optimal macrophage-mediated phagocytosis without Fc receptor engagement (Jain S et al., 2019). In contrast, a study published in *Nature Immunology* identified expression of the fully human β 2-microglobulin (β 2M) subunit of MHC class I on cancer cells as a mechanism for protection against phagocytosis. This occurs via interaction with the inhibitory LILRB1 receptor on macrophages. The findings also help explain anemia frequently observed during anti-CD47 therapy, as erythrocytes, which lose MHC I expression during maturation, become susceptible to phagocytic clearance. The authors proposed that low or absent MHC I expression on cancer cells could serve as a predictive biomarker for responsiveness to anti-CD47 or anti-SIRP α therapies, particularly in

tumors with impaired neoantigen presentation (Barkal AA et al., 2018). Additionally, the NSG mouse strain is considered ideal for xenograft studies due to its expression of a specific SIRP α isoform with approximately 65-fold higher affinity for human CD47 compared to other murine SIRP α variants. This high-affinity interaction closely mimics human CD47–SIRP α binding and facilitates efficient engraftment of human cells (Kwong LS et al., 2014). This enhanced binding affinity may also help overcome some of the limitations of cross-species interactions in the xenograft model. (Kwong LS et al., 2014). In our study, we observed increased surface expression of CALR following anti-CD47 treatment. CALR is a well-characterized “eat me” signal, and its upregulation in the presence of CD47 blockade may contribute to the observed clearance of malignant cells, consistent with previous findings that identified CALR as a potent driver of phagocytosis when CD47 signaling is inhibited (Chao MP et al., 2010).

In the combination treatment, we did not observe any additional effect of liposomal dexamethasone on anti-CD47 therapy in terms of reducing malignant cells within the BM or lowering serum Ig λ levels. However, the contribution of liposomal dexamethasone was evident in the stem cell-like compartment, where it induced a reduction in the stem cell-like population that remained unaffected by anti-CD47 monotherapy. This effect was further supported by gene expression analysis, which revealed upregulation of CSC-associated signatures following anti-CD47 monotherapy. These included KRAS, which is involved in PI3K/AKT pathway activation (Zhang Y et al., 2020; Guo W et al., 2021), as well as other pro-survival and anti-apoptotic pathways, such as NF- κ B signaling (Ang DA et al., 2024), oxidative phosphorylation (Yu Xiang et al., 2019), cellular response to IL-4 (Mirlekar B et al., 2022), and TGF- β signaling (Gunes EG et al., 2024). Comparative expression analysis of the combination treatment versus anti-CD47 monotherapy showed downregulation of stemness-related pathways, including HOXA9 and JAK2, suggesting that liposomal dexamethasone may counteract CSC-associated transcriptional programs when used alongside anti-CD47 therapy (Filipek-Gorzala J et al., 2024; Paul B et al., 2020).

We evaluated the impact of dexamethasone, anti-CD47 monotherapy, and their combination on frozen BMMNCs derived from MM patients. Initially, we assessed infiltration of CD138^{pos}CD38^{pos} cells; however, this marker combination proved suboptimal, as CD138 expression is known to decline rapidly after sampling (Chen D

et al., 2021). Therefore, we focused on the expression of CD24, CD47, and CALR within CD38^{pos} cells, as well as ALDH activity, to evaluate treatment effects on MM stem cell-like populations along with CALR and CD47 expression in patient-derived samples. CD24 expression and ALDH positivity are established markers of stem-like cells (Gunes EG et al., 2024; Guo W et al., 2021). Although no significant changes in CD24 expression were observed, a trend toward ALDH downregulation was detected following combined dexamethasone and anti-CD47 treatment. Using a back-gating strategy to identify CD38^{pos}CD24^{pos} and ALDH^{pos} populations, we observed a distinct SSC/FSC profile exclusively in the ALDH^{pos} subset, supporting the utility of ALDH activity as a marker for identifying patient-derived stem cell-like MM cells. However, this assay requires further validation through improved MM cell detection using more stable markers such as CD319^{pos}CD38^{pos}, as well as testing with a greater number of fresh patient samples. Finally, CD47 surface expression was diminished following anti-CD47 antibody treatment, confirming the targetability of this molecule with the antibody used in our experimental system. CD47 expression following treatment was also evaluated *in vivo*. We observed reduced CD47 expression in the anti-CD47 monotherapy group, likely due to receptor occupancy by the antibody. However, CD47 expression was upregulated upon administration of liposomal dexamethasone in combination with the anti-CD47 antibody, consistent with our previously described *in vitro* findings. Notably, the upregulation of CD47 was restricted to CD138^{pos} cells, while CD138^{neg} cells showed no such increase, again aligning with our *in vitro* observations, which demonstrated a differential effect of dexamethasone on CD138^{pos} versus CD138^{neg} cell populations. The impact of anti-CD47 monotherapy and its combination with liposomal dexamethasone was evaluated in macrophages through expression analysis of 3' RNA-sequencing data, as described previously. Anti-CD47 treatment resulted in the upregulation of heme metabolism pathways in macrophages, consistent with existing literature suggesting this reflects hemoglobin catabolism and iron recycling following erythrocyte clearance (Vallelian F et al., 2022; Burger P et al., 2012). When combined with anti-CD47, liposomal dexamethasone further promoted an M2-like metabolic signature, marked by increased oxidative phosphorylation and fatty acid metabolism, compared to anti-CD47 monotherapy (Viola A et al., 2019). Importantly, the combined treatment induced the upregulation of pathways associated with allograft rejection and phagocytic activity in macrophages, relative to the untreated

control group. This indicates a potential activation of anti-tumor macrophage functions (Wang H et al., 2010; Rastgoo N et al., 2020). Combined with the observed superior targeting of stem cell-like MM cells in our mouse model, these findings provide a strong rationale for further preclinical evaluation of the anti-CD47 and dexamethasone combination strategy.

4.6 CONCLUSIONS AND PERSPECTIVES

In this study, we demonstrate the superiority of liposomal encapsulated dexamethasone over the free formulation in an *in vivo* MM model. Liposomal dexamethasone exhibited favourable biodistribution with a BM-targeting pattern and effectively reduced Ig λ levels, regardless of the concentration tested. At lower concentrations, it also induced a substantially greater change in the expression profile, compared to the free formulation. This suggests prolonged circulation time and sustained drug release, which may allow for extended dosing intervals and reduced off-target effects associated with free dexamethasone toxicity.

We evaluated the impact of dexamethasone treatment on surface CD47 expression and MM stemness to identify a suitable combination treatment strategy. Dexamethasone induced upregulation of both "don't eat me" CD47 and "eat me" CALR, on MM cells, suggesting that increased CD47 expression may serve as a mechanism for survival and evasion of phagocytosis. In parallel, we observed an enrichment of the stem cell-like population following dexamethasone treatment, alongside the depletion of mature cells, indicating a higher resistance of the stem-like compartment to therapy. To our knowledge, this is the first study to investigate the differential effects of dexamethasone on CD47 expression in both mature and stem cell-like MM cell populations. Notably, dexamethasone treatment led to an upregulation of CD47 expression in both bulk and CD138^{pos} cells. In contrast, within the stem cell-like compartment, dexamethasone induced a downregulation, though not a complete loss, of CD47 expression. This differential response may represent a potential resistance mechanism, where mature MM cells may upregulate CD47 to evade immune clearance, while stem cell-like cells, with reduced CD47 expression may remain more susceptible to immune-mediated elimination. *In vivo*, a similar trend was observed: the addition of dexamethasone to anti-CD47 therapy led to modest CD47 upregulation in the bulk and mature MM populations, an effect not seen in untreated cells.

The effect of CD47 blockade, alone or in combination with liposomal dexamethasone, was evaluated in an MM xenograft model *in vivo*. The impact of anti-CD47 monotherapy on MM progression was assessed by measuring the reduction of malignant cell numbers in the BM and Ig λ levels in the serum. Anti-CD47 treatment

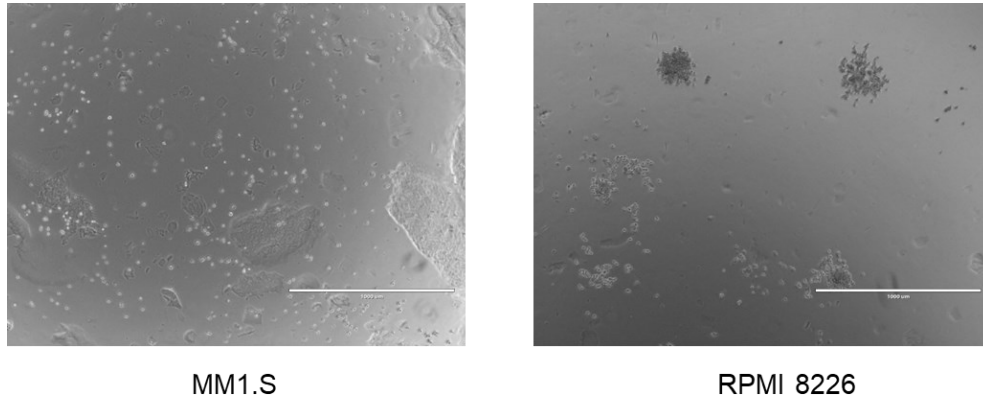
demonstrated a strong effect *in vivo*, leading to a reduction of MM1.S cells in the BM and decreased serum Ig λ levels. We interpret this outcome as a consequence of a shifted balance between pro- and anti-phagocytic signals, namely CALR and CD47, respectively. However, one has to rule out that this effect may partially result from the non-syngeneic interaction between murine macrophages and human malignant cells. The combination treatment with dexamethasone did not show additional effects on the bulk malignant cells, but a significant reduction was observed within the stem cell-like compartment. These findings suggest an additive effect of dexamethasone when combined with anti-CD47 therapy, indicating a potential strategy to overcome disease resistance by targeting the stem cell-like compartment. We also confirmed that this combinatorial approach enhances macrophage-mediated phagocytosis *in vitro* and observed a trend toward a reduction in ALDH^{pos} stem cell populations in patient-derived samples. Overall, the results support the superiority of the combination treatment over the individual monotherapies in targeting MM cell survival and resistance. However, further investigations are required to fully validate these findings and elucidate their clinical relevance.

The effect of dexamethasone treatment on macrophage polarization toward the M2 phenotype was confirmed in both *in vitro* and *in vivo* studies, consistent with the established understanding that GCs reduce inflammation and promote an anti-inflammatory macrophage phenotype. This trend was also observed in *in vivo* combination studies when dexamethasone was added to anti-CD47 treatment. Nevertheless, the activation of macrophage phagocytic function, demonstrated by phagocytosis assays and expression analyses upon anti-CD47 therapy in combination with dexamethasone formulations, supports the potential therapeutic benefit. Given that CD47–SIRP α interactions prevent macrophage-mediated phagocytosis of MM cells, interference with this axis represents a promising therapeutic strategy. Although various antibody formulations are currently being investigated in preclinical studies and show encouraging therapeutic effects, predictive biomarkers for patient selection and response prediction remain underdeveloped. To improve anti-myeloma therapies, a deeper investigation into combinatorial treatment approaches is warranted—specifically, the use of immune checkpoint inhibition in combination with nanotechnology-based delivery of standard-of-care agents. We propose that combining anti-CD47 therapy with liposomal dexamethasone represents a promising strategy to enhance anti-tumor immunity and potentially overcome dexamethasone

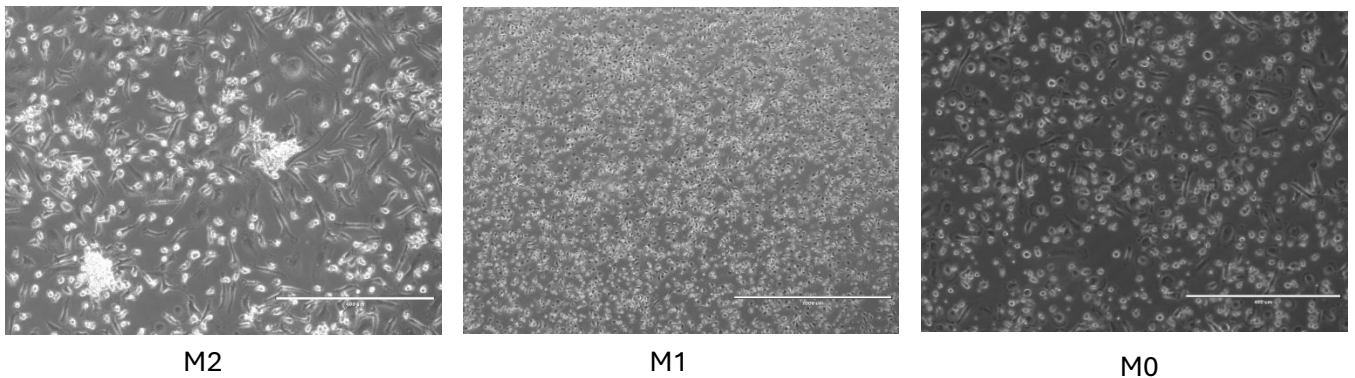
drug resistance. This may be achieved by fine-tuning the balance between promoting pro-phagocytic signals, blocking anti-phagocytic signals, and sensitizing the stem cell-like populations that contribute to disease relapse.

5 APPENDIX

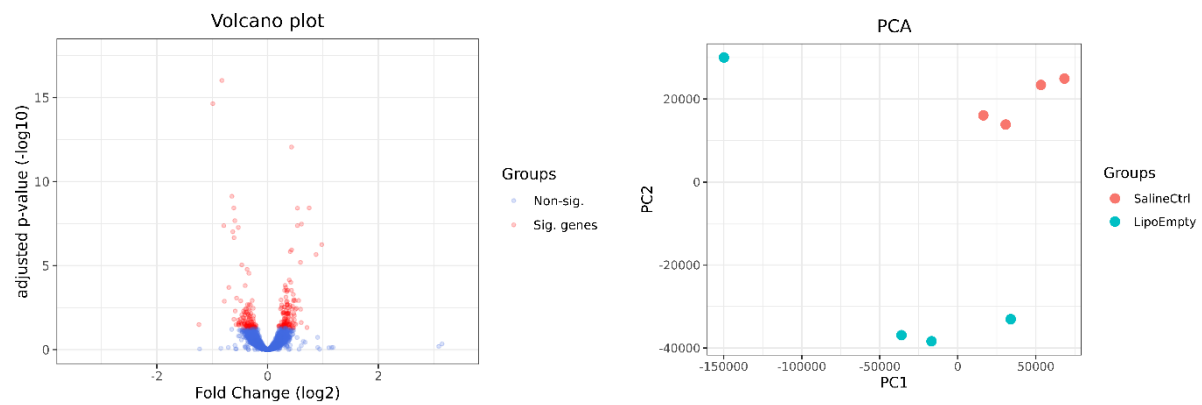
5.1 SUPPLEMENTARY FIGURES



Supplementary figure 1. Evaluation of colony forming capacity in cell lines. MM1.S and RPMI 8226 cell lines were cultivated in methylcellulose medium for 14 days to assess their colony forming potential. While MM1.S cells were not able to form colonies (left) RPMI 8226 showed CFU capacity (right)



Supplementary figure 2. Morphological differences between *in vitro* differentiated M0 and polarized M1 and M2 macrophages. M2 macrophages are characterized to exhibit protrusions and have spindle like formation, while M1 macrophages exhibit more fried egg-like morphology. M0, non-polarized macrophages have round shape, more similar to M1 macrophages although they were subjected to different cytokines during their differentiation.



Supplementary figure 3. Differences between untreated and empty liposome control. A proportion of significant differences in expression profile between the animals treated with saline vehicle and empty liposomes were observed (left). Principal component analysis didn't show major differences between two groups (PC1), but minor differences were detected in PC2 divergence. These differences are suspected to originate from the existence of one outlier sample within the empty liposome group (right).

5.2 LIST OF ABBREVIATIONS

7-AAD 7-amino-actinomycin D

β 2M β 2-microglobulin

A

ADCP Antibody depended cellular phagocytosis

ADCP Antibody-dependent cellular phagocytosis

ADCs Antibody–drug conjugates

ALDH Aldehyde dehydrogenase

Ann V Annexin V

APRIL A proliferation induced ligand

ASCT Autologous stem cell transplantation

B

BAFF B-cell activating factor

BAFF B-cell activating factor

BCMA B-cell maturation antigen

BLT/CT Hybrid bioluminescent tomography and computed tomography

BM Bone marrow

C

BTK Bruton's tyrosine kinase

CALR Calreticulin

CAR Chimeric antigen receptor

CCL12 C-C motif chemokine ligand 12

CD Cluster of differentiation

CFU Colony forming unit

CR Complete response

CSCs	Cancer stem cells
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C motif chemokine receptor 4
Cy7	Cyanine 7
D	
DRd	Daratumumab-lenalidomide-dexamethasone
E	
ECM	Extracellular matrix
F	
FACS	Fluorescence-Activated Cell Sorting
FcRH5	Fc receptor homolog 5
FCS	Fetal calf serum
FLT/ μ CT	Hybrid micro-computed tomography and fluorescence tomography
G	
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPRC5D	G protein-coupled receptor class C group 5 member D
gRNAs	Guide RNAs
GSEA	Gene set enrichment analysis
GWAS	Genome-wide association studies
H	
Hh	Hedgehog
I	
ICD	Immunogenic cell death
IFN- γ	Interferone γ

IGF-1	Insulin-like growth factor 1
IGH	Immunoglobulin heavy chain
IL	Interleukin
IMiDs	Immunomodulatory drugs
IMWG	International myeloma working group
ISS	International staging system
K	
KEGG	Kyoto encyclopaedia of genes and genomes
KO	knock-out
KRd	Carfilzomib-based regimens
L	
LPS	Lipopolysayharide
LRP	Low-density lipoprotein receptor-related protein
M	
mAbs	monoclonal antibodies
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Monocyte-colony stimulating factor
MDSCs	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma
MMPs	Matrix metalloproteinases
MNCs	Mononuclear cells
MRD	Minimal residual disease
MYC	Myelocytomatosis oncogene
N	

NK	Natural killer
NSG	NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ, NOD-scid IL2Rgamma ^{null} , NOD-scid IL2Rg ^{null}
NSP	Non-side population
O	
OS	Overall survival
P	
P/S	Penicillin / streptomycin
PB	Peripheral blood
PCA	Principal component analysis
PD-1	Programmed cell death protein 1
PFS	Progression-free survival
PHA-L	Phytohemagglutinin-L
PI	Proteasome inhibitor
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
R	
RARα2	Retinoic acid receptor alpha 2
Rd	Lenalidomide-dexamethasone
RFP	Red fluorescent protein
RFS	Relapse-free survival
RNP	Ribonucleoprotein
ROI	Regions of interest
RVd	Lenalidomide-bortezomib-dexamethasone
S	
SA-HRP	Streptavidin-conjugated horseradish peroxidase

SEER	Surveillance, epidemiology, and end results data
sFLC	Serum free light chain
SIRP α	Signal regulatory protein α
SLAMF7	Signalling lymphocytic activation molecule F7
SMM	Smoldering myeloma
SP	Side population
T	
TAM	Tumor associated macrophages
TAM	Tumor associated macrophages
TGF- β	Transforming growth factor-beta
Th	T helper
TMB	3,3',5,5'-tetramethylbenzidine
TME	Tumor microenvironment
TNF- α	Tumor necrosis factor alpha
TP53	Tumor suppressor 53
Tregs	Regulatory T cells
V	
VCAM1	Vascular cell adhesion molecule 1
Vd	Bortezomib-dexamethasone
W	
WT	Wild type

5.3 METRIC UNITS

SI prefixes

p	pico
n	nano
μ	micro
m	milli
c	centi
k	kilo

SI units

m	metre
l	liter
g	gram
s	second
min	minute
h	hour
A	ampere
V	volt
Gy	gray
mol	mole (6.02214076 e23 particles)

5.4 DATA AVAILABILITY

RNAseq data are deposited in the Sciebo database under the following links:

<https://rwth-aachen.sciebo.de/f/6127328352>

<https://rwth-aachen.sciebo.de/f/6127325487>

<https://rwth-aachen.sciebo.de/f/6127380372>

Username and password are available on request.

6 LITERATURE

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