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CELLULAR AND PERSONALIZED THERAPIES IN MULTIPLE MYELOMA WITH SPECIAL EMPHASIS ON RETARGETED NATURAL KILLER CELLS

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Cellular and personalized therapies in multiple myeloma with special emphasis on retargeted natural killer cells

Thesis for Doctoral Degree (Ph.D.)

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To the patients battling multiple myeloma

Popular science summary of the thesis

Multiple myeloma (MM) is a type of blood cancer affecting about 2 in 100.000 people worldwide. It forms in plasma cells, the white blood cells that help our body fight infections by producing antibodies. Malignant plasma cells, like normal plasma cells, are found in the bone marrow. There, they proliferate at a fast pace and release large amounts of abnormal antibodies causing painful and life-threatening symptoms, such as bone damage, anemia, and kidney dysfunction. MM is most often diagnosed in people around the age of 65, and thus is commonly referred to as a disease of the elderly. MM also has a higher incidence rate in Black over Caucasian populations, and men over women.

During the last decades, new treatments have significantly improved the survival of the patients, from a median of 3-4 to 8-9 years. The new treatments are based on the use of the immune system to battle cancer, hence are known as *immunotherapies*. Cell therapy is a type of immunotherapy. In this, immune cells with natural ability to recognize and eliminate cancer cells are taken from a small amount of patient's or donor's blood, processed in the laboratory and given to the patient as treatment. The 'processing' can be split into two main parts. First is the culture of the immune cells in order to increase their absolute number. Second is the modification of the cells with the aim to increase their anti-cancer properties. An example of this modification is the introduction of an artificial receptor, e.g. chimeric antigen receptor (CAR) or chimeric switch receptor (CSR), that targets specifically a protein found in excess on the malignant cells. Apart from their protein recognition part, CARs and CSRs have intricately designed domains, that transmit activating signals to the inside of the immune cells upon their interaction with the targeted protein. In turn, this triggers the exertion of cytotoxicity and mediates the killing of the cancer cell.

In this thesis, and specifically in **papers I and II**, we investigated cell therapy approaches for MM based on Natural Killer (NK) cells. NK cells are part of the innate immune system, which is the body's first defense against pathogens. They comprise 5-15% of the white blood cells in the blood circulation. Regarding cancer, NK cells hold a central place in the protection of our body. They own a unique set of mechanisms that carefully considers the health status of a cell and emits spontaneous cytotoxic activity if found abnormal. NK cell-based therapy has shown great potential in preclinical investigations and clinical trials; however, it is still not approved for broad clinical use.

In **paper I,** we investigated the feasibility of NK cell-based therapy targeting CD38, a promising immunotherapeutic target for MM, using a CAR. CD38 is also found on NK cells, making the development of this cell therapy troublesome due to potential fratricide phenomena. We found that by applying a specific cell culture protocol, based on the addition of interleukin-2 (IL-2), CD38 on the NK cells is downregulated. This decrease, in combination with a specially designed CAR, is sufficient to overcome the challenges in the development of the cell product and shows potent CD38-directed anti-myeloma activity in our study.

In **paper II**, we developed a CSR targeting a different protein, PD-L1, which is commonly upregulated in both hematological and solid tumors. For the design of the CSR, we used the receptor that naturally interacts with PD-L1, called PD1. Physiologically, the interaction

between PD1 and PD-L1 leads to the inactivation of the immune cell and the subsequent 'escape' of the cancer cell. In this study, we used PD1 as the protein recognition domain of the CSR and aimd to propagate activating signals into the NK cell, instead of inhibitory signals as it would normally occur. To achieve that, we combined the PD-L1 recognition domain with domains inspired by the naturally activating signals an NK cell receives. We generated seven CSRs and compared them in terms of their effect in increasing the targeting and the cytotoxic activity of NK cells towards MM cells. Our findings showed two CSRs to have the greatest potential.

Despite the advances of immunotherapy, chemotherapy still plays a big role in the treatment of MM. In **paper III**, we investigated the potential of Venetoclax in treating a specific group of MM patients that carry the genetic mutation t(11;14). These cancer cells have excess amounts of a protein, called BCL2, that protects them from cell death. Venetoclax specifically inhibits BCL2 making them vulnerable to treatment. We found that a daily low dose of Venetoclax is adequately safe for these patients, many of whom respond to the treatment.

Overall, this doctoral thesis discusses novel and promising immunotherapeutic strategies for the treatment of MM and aims to contribute to the search for new treatment options.

Popular science summary of the thesis (in Greek)

Εκλαϊκευμένη περίληψη διδακτορικής διατριβής

Το πολλαπλό μυέλωμα (ΠΜ) είναι ένας τύπος καρκίνου του αίματος που επηρεάζει περίπου 2 στους 100.000 ανθρώπους παγκοσμίως. Είναι καρκίνος των πλασματοκυττάρων, δηλαδή των λευκών αιμοσφαιρίων που παράγουν αντισώματα για να βοηθήσουν το σώμα μας να καταπολεμήσει λοιμώξεις. Τα κακοήθη πλασματοκύτταρα, όπως και τα φυσιολογικά πλασματοκύτταρα, βρίσκονται στον μυελό των οστών. Εκεί, πολλαπλασιάζονται με γρήγορο ρυθμό και απελευθερώνουν μεγάλες ποσότητες μη φυσιολογικών αντισωμάτων προκαλώντας επώδυνα και απειλητικά για τη ζωή συμπτώματα, όπως αναιμία, οστικές αλλοιώσεις και νεφρική δυσλειτουργία. Το ΠΜ θεωρείται μία κατ' εξοχήν πάθηση της μέσης και μεγάλης ηλικίας. Περισσότερο αυξημένα ποσοστά εμφανίζονται επίσης στους Νέγρους απ' ότι στους Καυκάσιους, και στους άνδρες παρά στις γυναίκες.

Τις τελευταίες δεκαετίες, η εμφάνιση νέων θεραπειών έχει βελτιώσει σημαντικά την επιβίωση των ασθενών, από μέσο όρο 3-4 έτη σε 8-9. Οι νέες θεραπείες βασίζονται στη χρήση του ανοσοποιητικού συστήματος για την καταπολέμηση του καρκίνου. Για τον λόγο αυτό είναι γνωστές ως ανοσοθεραπείες. Ένα είδος ανοσοθεραπείας είναι και η κυτταρική θεραπεία. Η θεραπεία αυτή εκμεταλλεύεται τα κύτταρα του ανοσοποιητικού συστήματος που εχουν φυσική ικανότητα να αναγνωρίζουν και να εξαλείφουν καρκινικά κύτταρα, όπως τα κύτταρα Τ ή τα κύτταρα Φυσικοί Φονείς (Natural Killer cells, NK). Συγκεκριμένα, κατά την κυτταρική θεραπεία, τα ανοσοκύτταρα αυτά λαμβάνονται από μία μικρή ποσότητα αίματος, υποβάλλονται σε επεξεργασία στο εργαστήριο και ακολούθως εγχέονται ξανά στον ασθενή. Η «επεξεργασία» μπορεί να χωριστεί σε δύο κύρια μέρη: α) την καλλιέργεια των κυττάρων με σκοπό την αυξηση του αριθμού τους, και β) την τροποποίηση των κυττάρων με στόχο την αύξηση των αντικαρκινικών τους ιδιοτήτων. Ένα παράδειγμα τροποποίησης αποτελεί η εισαγωγή ενός τεχνητού υποδοχέα στα κύτταρα, όπως π.χ. ενός χιμαιρικού υποδοχέα αντιγόνου (chimeric antigen receptor, CAR) ή ενός χιμαιρικού υποδοχέα μεταγωγής (chimeric switch receptor, CSR). Οι υποδοχείς αυτοί στοχεύουν πρωτεΐνες που βρίσκονται μόνο στα καρκινικά κύτταρα, ή τα καρκινικά κυτταρα τις έχουν σε περίσσεια σε σύγκριση με τα φυσιολογικά. Η αναγνώριση των πρωτεϊνών γίνεται απο το εξωτερικό τμήμα των υποδοχέων. Πέρα από αυτό, οι υποδοχείς CAR και CSR έχουν περίπλοκα σχεδιασμένα εσωτερικά τμήματα που μεταδίδουν σήματα ενεργοποίησης στο εσωτερικό των ανοσοκυττάρων κατά την αλληλεπίδρασή τους με την πρωτεΐνη-στόχο. Η ενεργοποίηση του ανοσοκυττάρου επιφέρει τη θανάτωση του καρκινικού κυττάρου.

Σε αυτήν τη διατριβή, και συγκεκριμένα στις μελέτες I και II, διερευνούμε κυτταρικές θεραπείες για το ΠΜ που βασίζονται σε κύτταρα ΝΚ. Τα κύτταρα ΝΚ αποτελούν το 5-15% των λευκών αιμοσφαιρίων στην κυκλοφορία του αίματος και συμπεριλαμβάνονται στην πρώτη γραμμή άμυνας του ανοσοποιητικού μας συστήματος ενάντια στα παθογόνα. Όσον αφορά τον καρκίνο, τα κύτταρα ΝΚ κατέχουν κεντρική θέση στην προστασία του σώματός μας. Έχουν ένα μοναδικό οπλοστάσιο μηχανισμών που εξετάζει την κατάσταση 'φυσιολογικότητας' ενός κυττάρου και ασκούν κυτταροτοξικότητα εφόσον το κρίνουν απαραίτητο. Οι θεραπείες με βάση τα κύτταρα ΝΚ έχουν δείξει μεγάλες δυνατότητες σε προκλινικές έρευνες και κλινικές δοκιμές, ωστόσο, ακόμα δεν είναι εγκεκριμένες για ευρεία κλινική χρήση.

Στη μελέτη Ι, διερευνούμε μια ΝΚ κυτταρική θεραπεία που στοχεύει την πρωτεΐνη CD38 μέσω ενός υποδοχέα CAR. Η CD38 είναι ένας πολλά υποσχόμενος στόχος ανοσοθεραπείας για το ΠΜ, καθώς βρίσκεται σε μεγαλύτερο βαθμό στα καρκινικά κύτταρα απ' ότι στα φυσιολογικά. Η πρωτεΐνη αυτή όμως βρίσκεται και στα ίδια τα κύτταρα ΝΚ. Το γεγονός αυτό δυσκολεύει την ανάπτυξη της κυτταρικής θεραπείας, λόγω της πιθανότητας τα CAR-ΝΚ κύτταρα να επιτεθούν σε αδελφικά ΝΚ κύτταρα. Η έρευνα μας επικεντρώθηκε στην εύρεση εναλλακτικής λύσης. Διαπιστώσαμε ότι με την εφαρμογή ενός ειδικού πρωτοκόλλου κυτταροκαλλιέργειας το CD38 στα ΝΚ κύτταρα μειώνεται. Αυτή η μείωση, σε συνδυασμό με ένα ειδικά σχεδιασμένο CAR, είναι αρκετά για να ξεπεραστούν οι δυσκολίες της ανάπτυξης του κυτταρικού προϊόντος. Επιπρόσθετα, αποδεικνύουμε ότι η θεραπεία έχει ισχυρή δράση κατά του μυελώματος σε πειράματα που έγιναν στο εργαστήριο.

Στη μελέτη ΙΙ επιχειρήσαμε τη δημιουργία ενός υποδοχέα CSR που στοχεύει μια διαφορετική πρωτεΐνη, την PD-L1. Η πρωτεΐνη αυτή βρίσκεται συχνά και σε μεγάλο βαθμό στα καρκινικά κύτταρα τόσο αιματολογικών όσο και συμπαγών καρκίνων. Έχει βρεθεί ότι η αλληλεπίδραση μεταξύ της PD-L1 και του υποδοχέα της στα ανοσοκύτταρα καταλήγει στην αδρανοποίηση των ανοσοκυττάρων και βοηθάει τα καρκινικά κύτταρα να αποφύγουν την εξόντωσή τους. Για τον σχεδιασμό του CSR χρησιμοποιήσαμε τον υποδοχέα που αλληλεπιδρά φυσικά με την PD-L1 και ονομάζεται PD1. Σχεδιάσαμε λοιπόν έναν τεχνητό CSR υποδοχέα με το PD1 ως εξωτερικό τομέα αναγνώρισης και αλλάξαμε τον φυσικό ανασταλτικό τομέα με ενεργοποιητικό. Κατ' αυτόν τον τρόπο, η αλληλεπίδραση της PD-L1 πρωτεΐνης με τον PD1-CSR υποδοχέα σηματοδοτεί την ενεργοποίηση του ανοσοκυττάρου ως προς την εξολόθρευση του καρκινινού. Δημιουργήσαμε συνολικά επτά CSR υποδοχείς με διαφορετικούς ενεργοποιητικούς τομείς και τους συγκρίναμε μεταξύ τους. Από αυτούς ξεχωρίσαμε δύο υποδοχείς που επιφέρουν μεγαλύτερη αύξηση της στοχευμένης κυτταροτοξικότητας ΝΚ κυττάρων κατά του ΠΜ.

Παρά την πρόοδο της ανοσοθεραπείας, η χημειοθεραπεία εξακολουθεί να παίζει μεγάλο ρόλο στη θεραπεία του ΠΜ. Στη μελέτη ΙΙΙ, διερευνούμε τη δυνατότητα του φαραμάκου Venetoclax να επιφέρει θεραπευτικό αποτέλεσμα σε άτομα με ΠΜ που φέρουν τη γενετική μετάλλαξη t(11;14). Τα καρκινικά κύτταρα των ασθενών αυτών έχουν υπερβολικές ποσότητες μιας προστατευτκής πρωτεΐνης που ονομάζεται BCL2. Το Venetoclax αναστέλλει ειδικά την BCL2, καθιστώντας τα καρκινικά κύτταρα ευάλωτα στη θεραπεία. Σε κλινική δοκιμή διαπιστώσαμε ότι ημερήσια χαμηλή δόση Venetoclax είναι επαρκώς ασφαλής για τους ασθενείς, πολλοί από τους οποίους ανταποκρίθηκαν στη θεραπεία.

Συνοψίζοντας, η παρούσα διατριβή συνδράμει στην επέκταση των θεραπευτικών επιλογών για το ΠΜ.

Abstract

Multiple myeloma (MM) is a clonal plasma cell malignancy accounting for approximately 10% of all hematological cancer cases. Despite considerable advances in MM management, which led to exceptional response and survival rates, patients still experience relapse and cure remains elusive. Personalized, antibody-based and cell-based immunotherapies have given new hope to patients with relapsed or refractory disease. The aim of this thesis was to investigate the potential of novel targeted treatments for MM, specifically those based on Natural Killer (NK) cells and patient stratification.

Studies I and II focus on retargeting applications of Natural Killer (NK) cells. NK cells have emerged as a promising alternative to current T cell-based therapies, due to their potent effector functions, safer profile and possibility for use as off-the-shelf treatments. However, the immunosuppressive microenvironment of MM drives NK cell dysfunctionality which impacts the efficacy of adoptive NK cell therapy. To address this issue we have relied on chimeric receptors; a strategy that is proven to enhance the targeting potential of NK cells while improving the exertion of cytotoxicity.

The first study centers around CD38; a protein that is highly expressed on the surface of myeloma cells. CD38-targeting with monoclonal antibodies, such as Daratumumab and Isatuximab, has revolutionized MM treatment, inducing durable responses in a fraction of patients. Targeting CD38 using Chimeric Antigen Receptor (CAR)-expressing NK has also been attempted. It is, however, met with feasibility challenges, such as the intrinsic CD38 expression on NK cells which may lead CAR-NK cells to perform fratricide. Here, we demonstrate an alternative approach by harnessing the $CD38^{dim}$ phenotype occurring during long-term cytokine stimulation of primary NK cells. Our findings show that the combination of a functional, affinity-optimized αCD38-CAR construct with a suitable NK cell expansion and activation protocol results in a promising immunotherapeutic strategy for MM.

The second study aims to improve outcomes of adoptive NK cell therapy by converting the inhibitory signals, that NK cells receive from the PD1/ PD-L1 axis, to stimulating signals. For this purpose, we designed novel PD1-based chimeric switch receptors (PD1-CSRs) by fusing the PD1 ectodomain to the activating signaling domains of NKp46, DAP10, DAP12, and CD3ζ. The results show that PD1-CSR+ NK cells exert potent anti-tumor activity against PD-L1+ cancer cell lines and primary MM cells *in vitro*, laying the foundation for improved treatment of PD-L1+ tumors.

The third study investigates the use of the BCL2 inhibitor Venetoclax in MM and AL-amyloidosis patients harboring the t(11;14) genetic mutation. This clinical study concludes that treatment with a daily low-dose of Venetoclax is adequately safe and has promising efficacy. The study also identifies resistance mechanisms associated with t(11;14), such as the downregulation of IRF5 targeted genes, which can be further exploited by therapeutic interventions. Overall, the present doctoral thesis investigates novel approaches of NK cell-based immunotherapy and stratified chemotherapy for MM. The findings of these studies provide foundation for future research in the field and contribute to the expansion of current therapeutic options.

List of scientific papers

I. **Karvouni M**, Vidal-Manrique M, Susek KH, Hussain A, Gilljam M, Zhang Y, Gray JD, Lund J, Kaufmann G, Ljunggren HG, Ji H, Lundqvist A, Wagner AK, Guo W, Alici E. Challenges in αCD38-chimeric antigen receptor (CAR)-expressing natural killer (NK) cell-based immunotherapy in multiple myeloma: Harnessing the CD38dim phenotype of cytokine-stimulated NK cells as a strategy to prevent fratricide. Cytotherapy. 2023 Apr 11:S1465-3249(23)00068-3. doi: 10.1016/j.jcyt.2023.03.006. Epub ahead of print. PMID: 37055320.

II. Susek KH, Schwietzer YA, **Karvouni M**, Gilljam M, Keszei M, Hussain A, Lund J, Kashif M, Lundqvist A, Ljunggren HG, Nahi H, Wagner AK, Alici E. Generation of NK cells with chimeric-switch receptors to overcome PD1-mediated inhibition in cancer immunotherapy. Cancer Immunol Immunother. 2022 Nov 10. doi: 10.1007/s00262-022-03317-y. Epub ahead of print. PMID: 36355079.

III. Nahi H, Kashif M, Klimkowska M, **Karvouni M**, Wallblom A, Gran C, Hauenstein J, Frengen N, Gustafsson C, Afram G, Uttervall K, Lund J, Månsson R, Wagner AK, Alici E. Low dose venetoclax as a single agent treatment of plasma cell malignancies harboring t(11;14). Am J Hematol. 2021 Aug 1;96(8):925-933. doi: 10.1002/ajh.26207. Epub 2021 May 18. PMID: 33901326.

Scientific papers not included in this thesis

I. Susek KH, **Karvouni M**, Alici E, Lundqvist A. The Role of CXC Chemokine Receptors 1-4 on Immune Cells in the Tumor Microenvironment. Front Immunol. 2018 Sep 25;9:2159. doi: 10.3389/fimmu.2018.02159. PMID: 30319622; PMCID: PMC6167945.

II. **Karvouni M**, Vidal-Manrique M, Lundqvist A, Alici E. Engineered NK Cells Against Cancer and Their Potential Applications Beyond. Front Immunol. 2022 Feb 15;13:825979. doi: 10.3389/fimmu.2022.825979. PMID: 35242135; PMCID: PMC8887605.

III. Rasul KH, Hussain A, Reilly H, **Karvouni M**, Dahlberg CIM, Al-Attar MS, Wagner AK, Alici E, Mohammad DK. Assessment of T Cell Receptor Complex Expression Kinetics in Natural Killer Cells. Curr Issues Mol Biol. 2022 Aug 25;44(9):3859-3871. doi: 10.3390/cimb44090265. PMID: 36135177; PMCID: PMC9497757.

Contents

List of abbreviations

aa	Amino acid
ACT	Adoptive cell therapy
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ADO	Adenosine
ADOR	Adenosine receptor
AE	Adverse events
AL	Amyloid light-chain
AML	Acute myeloid leukemia
APC	Antigen presenting cell
Auto-SCT	Autologous stem cell transplantation
BCMA	B-cell maturation antigen
BM	Bone marrow
BCR	B cell receptor
cADPR	Cyclic adenosine diphosphate-ribose
CAR	Chimeric antigen receptor
CB	Cord blood
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CLL	Chronic lymphocytic leukemia
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRS	Cytokine release syndrome
CSR	Chimeric switch receptor
Dara	Daratumumab
DC	Dendritic cell
EC	Extracellular
EMA	European Medicines Agency
ER	Endoplasmic reticulum
expNK	Expanded NK cells

FACS	Fluorescence-activated cell sorting
FcRH5	Fc receptor-like 5
FDA	U.S. Food and Drug Administration
GPRC5D	G protein–coupled receptor C5D
GvHD	Graft-versus-host disease
hESCs	Human embryonic stem cells
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMiDs	Immunomodulatory drugs
IN	Intracellular
iPSCs	Induced pluripotent stem cells
IRF	Interferon regulatory factor
Isa	Isatuximab
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer Immunoglobulin-like Receptor
KO	Knock out
LAG-3	Lymphocyte activation gene-3
LAMP-1	Lysosomal-associated membrane protein-1
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MGUS	Monoclonal gammopathy of uncertain significance
MHC	Major histocompatability complex
MIC	MHC-class I polypeptide-related sequence
ML	Memory-like
MNCs	Mononuclear cells
MM	Multiple myeloma
MTOC	Microtubule-organizing center
NA	Nicotinic acid
NAADP	Nicotinic acid adenine dinucleotide phosphate

NAD	Nicotinamide dinucleotide
NCR	Natural cytotoxicity receptor
ND	Newly-diagnosed
NK	Natural killer
OS	Overall survival
ORR	Overall response rate
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PD1	Programmed cell death receptor-1
PFS	Progression-free survival
PIs	Proteasome inhibitors
pNK	primary NK cells
RPMI	Roswell Park Memorial Institute
RR	Relapsed/ Refractory
RyR	Ryanodine receptor
scFv	Single-chain variable fragment
SLAMF7	Signaling lymphocyte activation molecule F7
SMM	Smoldering myeloma
TAA	Tumor-associated antigen
TCR	T cell receptor
TIM-3	T cell immunoglobulin and mucin domain 3
TM	Transmembrane
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TLR	Toll-like receptor

1. INTRODUCTION

Plasma cell dyscrasias are a spectrum of progressively more severe disorders that originate from the abnormal proliferation of plasma cells in the bone marrow (BM)[1]. This may derive from a single plasma cell clone (monoclonal) or from multiple (polyclonal), with each of those clones producing immunoglobulins (Igs) or fragments of them. Depending on the severity, Ig type and malignant state, cell dyscrasias are classified into different conditions. Examples of those are multiple myeloma, plasma cell leukemia, monoclonal gammopathy of uncertain significance (MGUS), Waldenström macroglobulinemia and primary amyloidosis. The specific diagnosis largely indicates the prognosis and the necessity of treatment. The present thesis is focusing on Multiple Myeloma (MM) and the potential of novel personalized therapeutic approaches as treatment.

Historically, the first case of MM -to be documented- was that of a 39-year-old woman that exhibited severe back pain[2]. The report was made by Samuel Solly, in 1844. The name 'multiple myeloma', however, was given by von Rustizky, in 1873, after identifying eight separate tumors in the BM of a deceased patient during autopsy[3]. Following that, pioneering work on the early description of the malignancy and its related symptoms was done by physicians Kahler, Geschickter and Copeland.

1.1 THE MULTIPLE MYELOMA MALIGNANCY

MM is a highly heterogenous plasma cell malignancy that arises at multiple sites in the BM, commonly the spine, shoulders, hips, skull, pelvis, and rib cage[4,5]. The main characteristic of the disease is the accumulation of malignant plasma cells in the BM niche and the subsequent disruption of the local bone tissue **(Figure 1)**. Physiologically, plasma cells are terminally differentiated B cells that reside in the medullary cavity and secrete antibodies upon antigen presentation. Neoplastic plasma cells display features of normal plasmablasts/ plasma cells, as they also produce monoclonal Igs (also known as M-proteins), though constitutively and in large amounts. The accumulation of M-protein in the blood or urine is a usual phenomenon among patients. It contributes to the plethora of the disease's symptoms by causing renal dysfunction or even renal failure. M-protein is often used as a diagnostic tool, although it is also detected in the precursor conditions of monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma (SMM)[6]. Patients with SMM or MGUS are asymptomatic; however, close monitoring is necessary as they are at high risk of developing active MM. MM is diagnosed when increased tumor burden, symptom manifestation, and organ damage are observed. The common symptomatology of the disease is summarized by the mnemonic acronym 'CRAB', which stands for elevated hypercalcemia, renal failure, anemia, and bone lesions.

Epidemiologically, MM is the second most common hematological malignancy, representing about 10% of all hematological cancers and 1% of all malignant diseases[7,8]. MM is often referred to as a disease of the elderly since the median age of diagnosis is about 65 years[9,10]. Studies in the US population showed a higher disease prevalence in the African American population than the Caucasian or Asian American population. These studies also revealed a 1.5-fold higher incidence in men than in women. A similar trend is

observed in Sweden, which corresponds to 7.9 per 100,000 person-years for males and 5.5 per 100,000 person-years for females, according to data from the year 2015[7].

The etiology of MM is not fully understood to this date. Lifestyle and environmental exposures, such as agriculture chemicals, radiation, and viruses (e.g. HIV and hepatitis C), have been associated with an increased risk of developing MM. Inherited genetic predisposition, however, has also been suggested[11].

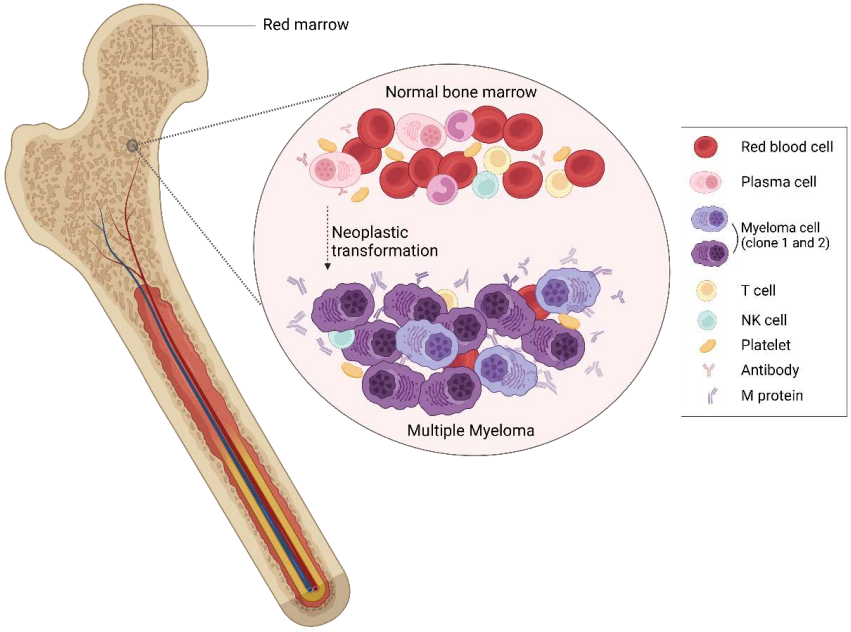

Figure 1: Schematic representation of healthy versus malignant BM in MM, inspired by an illustration of Terese Winslow[12]. Image created with BioRender.

1.2 CURRENT TREATMENT OPTIONS FOR MM

At the beginning of the century, the median overall survival (OS) for patients with MM was about 3 years[13]. It has since steadily improved due to the continuous advancements in the diagnosis, monitoring and treatment of MM[14]. Today, longer remission periods and OS of more than 5 years are routinely observed. Survival of more than 10 years in younger patients (aged <50 years) is also increasingly reported. The current treatment options for MM fall into six different categories, as depicted in **Figure 2**.

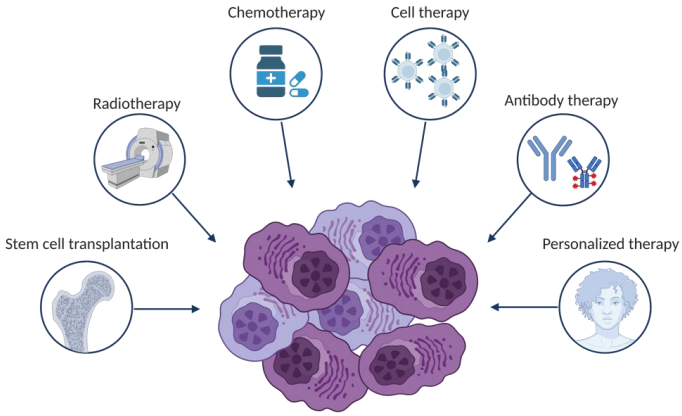

Figure 2: Types of therapeutic interventions utilized for the treatment of MM. Image created with BioRender.

1.2.1 STEM CELL TRANSPLANTATION

Autologous stem cell transplantation (auto-SCT) has marked a breakthrough in MM treatment[15]. In fact, today, more than 30 years after its incorporation into MM treatment regimens, auto-SCT is still considered standard of care[16]. It is based on the replacement of the cancer-rich hematopoietic system with a newly reconstituted one, deriving from a patient's own stem cell graft. The process has five distinct steps:

i) Induction therapy, i.e., high-dose radiation and/ or chemotherapy, for reduction of tumor burden and improvement of graft quality
ii) Stem-cell mobilization from BM and harvest from peripheral blood (PB)
iii) Preparative conditioning therapy
iv) Intravenous administration of transplant
v) Consolidation therapy.

In clinical trials, the combination of auto-SCT plus chemotherapy has shown to increase progression-free survival (PFS) and OS rates. Not all patients are eligible for this regimen, however, as it is associated with severe chemotherapy-related side effects, infection outbreaks and anemia[17]. The identification of patients that could benefit from SCT is based on factors such as age, comorbidities, performance status and disease stage. Still, even after the initial reduction of the tumor burden, all MM patients eventually relapse as cancer stem cells can be present within the graft[16,18]. In some cases, an allogeneic transplant is considered instead[19]. Allo-SCT bears the advantages of cancer-free graft and graft-versus-myeloma activity, but it is usually of higher risk, due to the transplant-related complications and hazard of graft-versus-host disease (GvHD). Moreover, the availability of suitable donors for allo-SCT is limited. In the last years, with the advent of novel therapeutic agents for MM, similar clinical outcomes are achieved with a higher benefit/risk ratio. This has brought the role of SCT into question[20]. Future directions of SCT are focusing on updating the induction and/or consolidation therapy to incorporate newly approved agents, in order to increase the safety and relevance of the procedure.

1.2.2 CHEMOTHERAPY

For many years, alkylating agents (e.g. melphalan) and glucocorticosteroids (e.g. dexamethasone) were the main treatment options for MM[21]. Later, drug repurposing led to the approval of the immunomodulatory drugs (IMiDs) thalidomide, lenalidomide, and pomalidomide for newly-diagnosed MM (NDMM) and relapsed/ refractory MM (RRMM)[22]. The mechanism of action of IMiDs is diverse. In brief, they have shown anti-angiogenic properties, direct anti-myeloma cytotoxic effect and ability to disrupt the interaction between myeloma cells and BM stromal cells by decreasing the expression of cell surface molecules. On top of that, their immunomodulatory effects include enhancement of T, NK, NK T and dendritic cell (DC) function, inhibition of regulatory T cell proliferation, decrease in TNF, IL-1β, IL-6, and IL-12 production and increased IL-2 and IFNγ synthesis[23]. Although thalidomide, lenalidomide and pomalidomide are small molecules with similar chemical structures, they differ in key pharmacological properties, such as half-life, metabolism, clearance, and side-effect profile.

Another class of drugs commonly used in MM treatment is proteasome inhibitors (PIs)[24]. PIs inhibit the degradation of excess, misfolded or unfolded proteins in proteasomes, which leads to protein build-up in the cell. This is particularly applicable in MM, as myeloma cells continuously produce and secrete large amounts of monoclonal proteins. As a result of the inhibition, proteins accumulate in the endoplasmic reticulum (ER), causing ER stress and ultimately activating cell death pathways. The first-in-class PI, bortezomib, was approved by the FDA in 2003 and it is since indicated both as a single agent and in combination with dexamethasone and IMiDs for RRMM and NDMM[25,26]. In the following years, second-generation PIs, carfilzomib and ixazomib, were introduced to the clinics for the treatment of RRMM[27,28]. Treatment regimens and indications are constantly evolving depending on the results from respective clinical trials.

Panobinostat, a non-selective histone deacetylase inhibitor, is also approved for MM[29]. Since histone deacetylases are responsible for key cell functions, such as regulation of gene transcription, cell differentiation and cell cycle progression, their inhibition induces apoptosis. Panobinostat does not have considerable effect as a monotherapy for MM. It is, however, a useful antimyeloma agent in combination with bortezomib and dexamethasone, working synergistically to promote anti-myeloma action[30].

Multiple trials are currently investigating combinational treatments. Factors dictating the final treatment regimen that each patient will follow are age, comorbidities, and general health[31]. For instance, more frail patients are typically not advised to undergo SCT, and instead, they follow a bortezomib-lenalidomide-dexamethasone strategy. Collectively, these new treatment options have resulted in prolonged remission periods, improved quality of life, and increased the OS from three to more than ten years[32]. However, unfortunately, relapse is occurring in an overwhelming majority of the patients, due to the emergence of resistant clones[33].

1.2.3 PATIENT STRATIFICATION

MM is characterized by a high occurrence of chromosomal aberrations, such as Ig heavy chain gene translocations and trisomies[34]. The majority of them arise at the onset of the disease during the early premalignant stage[35]. Primary chromosomal aberrations associated with poor prognosis are the translocations t(4;14), t(14;16) and the deletions del(17p) and del(1p32)[36,37]. t(11;14) is considered a genetic marker of intermediate risk, while patients with t(6;14), and/or chromosome trisomies are generally considered standard-risk. Genetic mutations are common in MM, further contributing to the heterogeneity of the disease[38]. Sequencing studies revealed that the mutations with the highest occurrence are observed in the RAS/MAPK pathway (activating) and NF-κB (mostly inactivating)[39]. Overall, the most common mutations are in *KRAS* (36% of cases), *NRAS* (20%), *TP53* (16%), *DIS3* (16%), *FAM46C* (12%) and *BRAF* (7%)[40]. Besides *TP53*, mutations in the DNA repair pathway genes *ATM, ATR,* and *BRCA2* have been observed[39,41]. Moreover, *MYC* dysregulation is observed in nearly one-third of patients[42] and overexpression of the Interferon Regulatory Factor-4 gene (*IRF4*), which attributes increased survival to MM cells[43]. Notably, multiple cytogenetic aberrations can be observed in one patient.

In recent years, efforts are made towards personalizing treatments according to the molecular features of the patient[44]. The need arises from the fact that clinical outcomes are highly heterogeneous and often unpredictable, with some patients experiencing long remission periods and others early relapse or even a refractory disease[45]. The idea of patient stratification can be summarized in the application of molecular signature knowledge towards matching a patient with a more effective and less toxic treatment. The translocation t(11;14) is particularly studied in this context[37]. It occurs in approximately 15-20% of patients and defines a group of patients with unique traits, e.g., lymphoplasmacytic morphology, elevated numbers of circulating plasma cells, IgG lambda and Bence-Jones isotypes, and oligo-secretory or non-secretory disease. The use of Venetoclax, as monotherapy[46] or in combination with other agents[47,48], has been suggested for these patients[49]. Although clinical evidence showed adequate safety and measurable anti-myeloma effect, Venetoclax is to date not approved for use in MM.

1.2.4 IMMUNOTHERAPY

Immune cells play a key role in cancer surveillance[50]. Using complex mechanisms, they can identify transformed cells and eliminate them, protecting our body from cancer cells that continuously arise. One of the hallmarks of cancer is the avoidance of immune destruction[51]. This is where *immunotherapy* is based, i.e., in the stimulation of the immune system towards overcoming the immunosuppressive tumor microenvironment (TME) and the defective cancer cell recognition. Immunotherapy takes advantage of the unique molecular characteristics on malignant cells and redirects effector immune cells against them. For this to be possible, the better understanding of the disease pathology and the advancement of molecular biology techniques contributed immensely. Scientists were thus able to both identify tumor-associated antigens (TAAs) and develop targeted immunotherapeutics against them. Examples of immunotherapies, particularly related to MM, include monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs) and cellular therapies[52].

Immunotherapy has been particularly successful in treating hematological malignancies. A prime example of immunotherapy in MM is Daratumumab (Dara). Dara is a fully human IgG1κ mAb that was approved by the FDA in 2015[53,54]. The target of Dara is a cell-surface protein called CD38. CD38 is overexpressed on the surface of MM cells, in contrast to its expression in normal plasma cells, other immune cell types and tissues. Dara induces the killing of myeloma cells by multiple mechanisms. These include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC) and direct cytotoxicity[55]. Treatment with Dara has shown great potential both as a monotherapy[54,56,57] and in combination with IMiDs, PIs, and corticosteroids[26,58,59.] . Specifically, in RRMM, Dara achieved a response of about 50% as a single agent during CENTAURUS clinical trial[60]. The overall response rate increases further when Dara is used in combination with chemotherapeutic agents. Indicatively, a meta-analysis of six independent clinical trials assessing Dara-including treatments showed that Dara's addition produced significant progression-free survival and clinical benefits with a complete response rate of 24%, a very good partial response of 67%, and an overall response rate of 92%[61]. More recently, Dara received approval for the treatment of newly diagnosed patients (NDMM). Another approved anti-CD38 mAb is the chimeric (human/mouse) IgG1κ antibody Isatuximab (Isa)[62]. Isa got approval in 2020 for patients with relapsed/ refractory MM, in combination with pomalidomide and dexamethasone. It targets a different epitope on the CD38 molecule and has a distinct mechanism of action compared to Dara. Specifically, Isa is dependent more on ADCC and on the inhibition of CD38's ecto-enzymatic activity rather than CDC[63].

Dara and Isa are not the only regulatory-approved mAbs for MM treatment. In 2014, FDA granted a 'breakthrough therapy' designation to the humanized IgG1κ mAb elotuzumab[64]. It targets a cell-surface glycoprotein called Signaling Lymphocyte Activation molecule F7, or SLAMF7 (also known as CS1, CD319, and CRACC) that is highly expressed on myeloma cells. SLAMF7 is also present in immune cell subsets, mainly NK cells, but also $CD8^+$ T cells, pro-B cells, monocytes, macrophages and DCs[65–69]. The success of elotuzumab lies in the fact that SLAMF7 expression is independent of treatment history, chromosomal abnormalities, and molecular subtypes. This makes it eligible for patients that are refractory to multiple lines of therapy. Elotuzumab has limited effect as a single agent, but it is promising in combination with IMiDs and corticosteroids in MM patients who had previously received one to three treatments[70].

The B-cell maturation antigen (BCMA) is another successfully targeted molecule in MM[71]. It has the unique advantage of being almost exclusively expressed in plasma cells, which decreases the on-target/off-tumor effects[72]. BCMA has been extensively investigated as a target of different immunotherapeutic approaches, such as mAbs, ADCs and cellular therapies. Several of them have received regulatory approval. The first was belantamab mafodotin, an off-the-shelf ADC comprising an anti-BCMA humanized mAb conjugated to the cytotoxic payload monomethyl auristatin F[73]. It received FDA approval in 2020, but was discontinued in 2022 based on the latest phase III clinical trial. BCMA targeting is particularly effective in the context of chimeric antigen receptor (CAR)-T therapy, where patient's T cells are expanded and edited *ex vivo* before infused back into the patient. As of today, two CAR-T products have received approval, namely idecabtagene vicleucel[74] and ciltacabtagene autoleucel[75]. Lastly, the bispecific BCMA-directed CD3 T-cell engager

teclistamab-cqyv was granted accelerated FDA approval in 2022 for the treatment of RRMM patients that received at least four prior lines of treatment[76].

1.3 MOLECULAR TARGETS FOR MM

The identification of novel molecular targets for MM continues beyond the development of immunotherapeutics against CD38, BCMA and SLAMF7. Other targets currently under investigation include CD138[77], CD47, Fc receptor like 5 (FcRL5), G protein–coupled receptor C5D (GPRC5D), as well as the programmed cell death protein-1 (PD1) receptor and its ligands (PD-L1 and PD-L2). Although the field is blooming with exciting studies of all stages of preclinical and clinical assessment for these markers, this thesis will only focus on CD38 and the PD1 axis.

1.3.1 CD38

As previously mentioned, CD38-targeting with Dara initiated a new era in MM immunotherapy. CD38 is a 45-kDa type II transmembrane glycoprotein, with both receptor and enzyme properties[78]. It was first identified in the late 1970s by E.L. Reinherz and S.F Schlossman as a T cell marker and was given the name T10 because of its reactivity with the OKT10 antibody[78–80]. Since then, the knowledge around the protein has increased immensely. The *CD38* gene is found on chromosome 4p15; it spans more than 80kb and is comprised of 8 exons[81,82]. Structural protein studies revealed that the CD38 glycoprotein has a large 257aa extracellular domain, a single transmembrane 23aa domain, and a short 20aa N-terminal cytoplasmic tail[83]. The expression of the gene is under a multilayered transcriptional regulation[84]. The first layer of control is found in the promoter region, where the presence of a CpG island indicates methylation control. Secondly, upstream of the CpG island, there are binding sites for transcription factors, like the T cell transcription factor-1 and the nuclear factor for interleukin-6. Lastly, the first intron of the *CD38* gene contains response elements to retinoic acid and peroxisome proliferator-activated receptor γ. More transcriptional factors and regulatory pathways are suggested, such as NF-κB, LXR, JAK-STAT3, and JAK-STAT1, which altogether underline CD38's key role in regulating inflammatory responses[85,86].

Under physiological conditions, CD38 is ubiquitously expressed on the surface of cells of the myeloid and lymphoid lineage, including subtypes of T cells, NK cells, monocytes, granulocytes, and DCs[84,87,88]. CD38 plays an important role in the function of these cells, as showcased first in the CD38 knockout (KO) experiments done by Cockayne DA et al., in 1998[89]. In these studies, complete KO of CD38 in mice resulted in defective humoral immune responses, insufficient T cell priming, and defects that, CD38 is also found in lower levels in non-hematopoietic tissues and organs, such as in prostate (luminal cells)[90], lung (smooth muscle cells)[91], brain (Purkinje cells)[92], kidney (renal tubules)[93] and eye (retinal ganglia cells[94] and cornea[95]).

Functional CD38 is found both as a soluble and as a cell surface-bound molecule[96]. In addition, it is present intracellularly either attached to the membranes of organelles or in its soluble form[97]. The orientation of the CD38 molecule can also vary. In 90% of the cases, the enzymatic domain is found facing extracellularly (type II orientation). However, the

molecule can also be found reversed, facing the cytoplasm (type III orientation). From the multiplicity in topology and orientation of the molecule, it is evident that CD38 is a multifaceted glycoprotein, with unique characteristics and diverse functions. The main CD38 roles are depicted in **Figure 3**.

When in type II orientation, CD38 functions as a nicotinamide adenine dinucleotide (NAD+) glycohydrolase[98]. The main substrates are NAD+ and NAD phosphate (NADP). The interaction between the enzymatic domain and its substrates is an intriguing 'topological paradox', as the first is facing towards the outside when the latter are most commonly found intracellularly[99]. This paradox was recently elucidated with findings demonstrating that apart from NAD+, CD38 also degrades circulating NAD precursors, such as nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR), before these are internalized by the cell and enter NAD biosynthetic pathways[100,101]. The enzymatic reaction is pH-dependent, and it results in the production of the second messengers adenosine diphosphate ribose (ADPR), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP)[98,102]. The metabolites function as cell signaling molecules by mobilizing calcium from intracellular stores or by activating calcium-permeable plasma membrane channels[103,104].

A secondary function attributed to CD38 is that of adhesion molecule and receptor, although its short cytoplasmic domain does not act in favor of the latter. In fact, in order to function as a receptor, CD38 needs to laterally associate with receptor complexes, such as the T cell receptor (TCR)/CD3 complex, B cell receptor (BCR) and CD16. Experiments showing its accumulation at the contact zone of T cells during the synapse formation provided additional evidence of its role as an accessory component of the immunological synapse[105]. Investigations on CD38 as ligand commenced when T cells were observed to adhere to endothelial cells via CD38 engagement[106]. Successive experiments showed that CD31 (also known as PECAM-1) was its non-substrate ligand[106]. CD31 is a member of the Ig superfamily, important for leukocyte adhesion and migration through the endothelium. The CD38/CD31 axis has been studied extensively in several cell types, and it is now known to deliver growth signals to various lymphocytic populations that drive activation and proliferation[107,108]. The axis is found to play an important role in cancer as well. Studies in chronic lymphocytic leukemia (CLL) showed that the interplay between CD38 and CD31 activates genetic signatures, that apart from inducing proliferation, survival, and differentiation of immunoblasts *in vitro,* they have an effect on the migration and homing of the malignant cells to favoring environments[109–111]. Additionally, studies in acute myelogenous leukemia (AML) patient samples highlighted the distinct characteristics that different CD31/ CD38 ratios attribute to the disease[112]. By using interaction-blocking antibodies, researchers found that an excess of CD31 leads to greater transendothelial migration, whereas higher CD38 allows cells to be better retained in the BM microenvironment through hyaluronate adhesion.

An alternative way in which CD38 affects the TME of hematological and solid tumors is via its contribution to the accumulation of immunosuppressive metabolites[113]. In fact, this notion has recently emerged as a novel immune checkpoint. Along with the enzymes CD39 and CD73, CD38 is involved in the production of adenosine (ADO) due to the ADPR that feeds into the ADO production pathway. ADO is a main immunosuppressive molecule known to impair T cell function and metabolic fitness via the recently identified

A2AR/PKA/mTORC1 pathway and negatively affects the immune control of the tumor growth[114]. CD38-mediated immunosuppression via the ADO pathway has also been suggested as a mechanism of acquired resistance to PD1/PD-L1 blockade, due to an observed CD38 upregulation in tumors treated with the respective monoclonal antibodies[115]. Co-inhibition of CD38 and PD-L1 improved the anti-tumor immune response in the same study.

Besides its role in inflammation and cancer, CD38 is involved in several other pathophysiological conditions, such as aging[99,116] and obesity[117]. One of the main reasons for that is its role in NAD+ catabolism. Several reports have shown that during aging, there is a noticeable NAD+ decline, which results in pseudohypoxia, mitochondrial dysfunction, and metabolic abnormalities. Although this could also be a result of a decrease in NAD-synthesizing enzymes, Camacho-Pereira et al. attributed the phenomenon to increased SIRT3-dependent catabolism driven by CD38[100]. Interestingly, investigations using a CD38 KO mouse model showed that CD38 ablation increased the NAD+ levels and improved the response to NAD-replacement therapy. The same research group has also investigated the role of CD38 in obesity using their established animal models. More specifically, in CD38 KO mice, the higher NAD+ levels were found to act prophylactically against obesity and metabolic syndrome[117,118], while the treatment of obese mice with CD38 inhibitors led to a significant improvement in glucose and lipid homeostasis[118]. The implication of CD38 in chronic inflammatory diseases, like rheumatoid arthritis[119,120] and asthma[121], has also been investigated, and it provides further evidence to the fact that aberrant expression of CD38 can tip immune responses towards pathology.

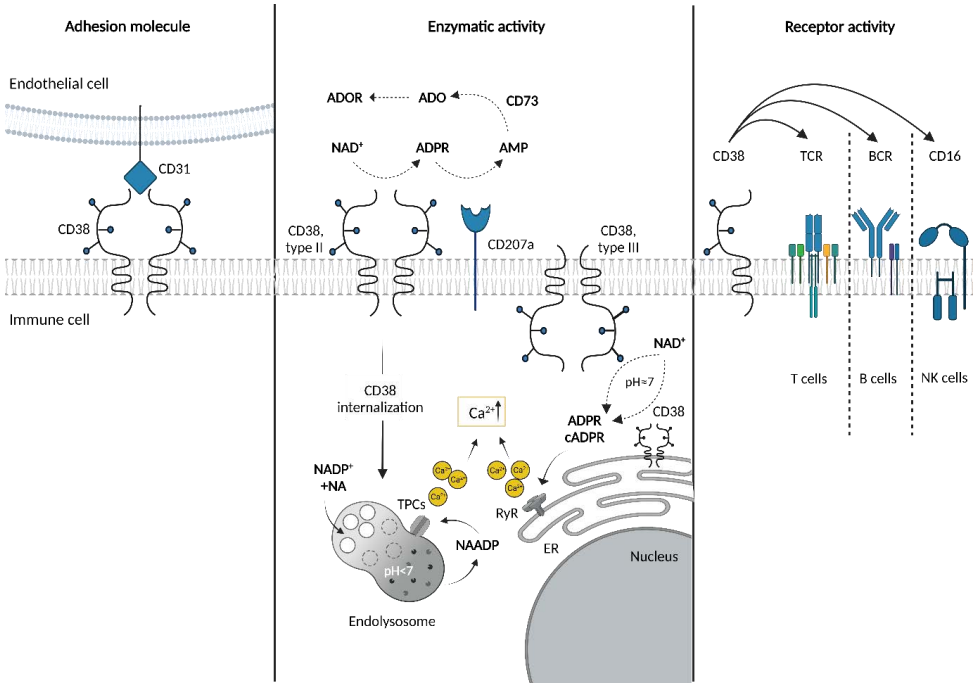

Figure 3: Schematic representation of the CD38 protein functions. From left to right is depicted the role of CD38 as i) Adhesion molecule, mediating cell-to-cell interaction, ii) Enzyme, mediating intracellular calcium regulation and adenosine production, and iii) Receptor, interacting laterally with big receptor complexes, such as TCR, BCR and CD16. Image created with BioRender.

1.3.2 THE PD1/ PD-Ls AXIS

PD1 (CD279) is a type I transmembrane protein of 288aa encoded by the *PDCD1* gene on the 2q37.3 chromosome[122]. It is an extended member of the CD28/ CTLA-4 family and the Ig superfamily. Structurally, the protein consists of an extracellular N-terminal domain (IgV-like), a membrane-permeating domain and an intracellular tail where two phosphorylation sites are located in an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). PD1 is predominately expressed on activated T cells, but it can also be found on other immune cells, such as B cells, macrophages, DCs and NK cells[123]. It was first described in the early 1990s by Tasuku Honjo's lab as a protein involved in the programmed cell death of immature T cells[124]. This notion was abandoned a few years later, when the immunomodulatory function of PD1 was discovered by the same research group[125,126].

Ligands of PD1 are the transmembrane proteins PD-L1 (B7-H1, CD274) and the PD-L2 (B7-DC, CD273) (also referred to as PD-Ls)[126,127]. PD-L1 is expressed in a variety of normal and immune cell types, including T cells, B cells, DCs, macrophages, mesenchymal stem cells and endothelial cells, and it is upregulated upon immune cell activation or malignant transformation. PD-L2, on the other hand, is almost exclusively expressed on antigen presenting cells. Upon interaction with its ligands, PD1 transmits inhibitory signals to the immune cells, decreasing their effector functions and proliferative capacity[127]. This mechanism is best described in T cells. Specifically, ligation of the PD1 leads to the recruitment of the SHP-1 and SHP-2 phosphatases to the ITIM and ITSM. In turn, these terminate ZAP70 and PI3K phosphorylation on the TCR and the T cell co-stimulatory molecule CD28 respectively, diminishing their downstream activation signals. In addition, PD1 ligation induces the expression of the transcription factor BATF, known to impair T cell proliferation and cytokine production, and decreases the expression of the pro-survival gene BCLXL[128]. The PD1 pathway further suppresses effector T cells by promoting the differentiation of regulatory T cells from naïve and helper T cells.

Due to the impact that the PD1/ PD-Ls signaling axis has in immune cell regulation it is considered an 'immune checkpoint'[129]. Physiologically, the axis plays a central role in regulating peripheral T cell tolerance, serving as a negative feedback mechanism to prevent excessive immune response and inflammatory damage[123]. In cancer, however, the upregulation of PD-Ls contributes to the immune escape of many solid and liquid tumors. Inhibition of the axis using mAbs has demonstrated unprecedented clinical efficacy in treating metastatic melanoma, non-small cell lung cancer, Hodgkin's lymphoma and renal cell carcinoma, amongst others. Several mAbs are approved by FDA, e.g. the anti-PD1 mAbs nivolumab, pembrolizumab, cemiplimab, and the anti-PD-L1 mAbs atezolizumab, avelumab, and durvalumab[130]. As of 2020, these mAbs have resulted in 67 FDA approvals across 17 different cancer types and two tissue-agnostic conditions.

Although PD1 and PDL-1 inhibitors show outstanding, long-lasting responses in patients that do respond, the majority of the patients (40-80%) are non-responders[131,132]. The underlying mechanism of this remains unclear. To stratify the patients, predictive biomarkers, such as PD-L1 expression levels on cancer cells, are suggested as companion diagnostics[133,134]. Still, clinical trials selecting patients based on the PD-L1 status were altogether inconclusive, even after the standardization of the technical steps. Consequently, to further increase the fraction of responders, the focus of the field is shifting towards developing combination therapies using PD1/ PD-L1 blockade as the backbone[135]. Possible routes include combinations with other immunotherapeutic agents, cell therapy, chemotherapy and radiation therapy, each of which has a rationale for achieving synergy.

Targeting the PD1/PD-L1 axis is relevant in MM, as both molecules show upregulation, compared to the physiological levels. Specifically, PD1 is overexpressed in CD4+ T cells, CD8+ T cells and NK cells[136], while PD-L1 is upregulated in myeloma cells and DCs[137]. PD-L1 expression also showed a further increase with disease progression, compared to the expression on plasma cells of MGUS and healthy individuals[138]. PD-L1 expression is induced by different cells and soluble factors present in the myeloma BM microenvironment. For instance, it is induced by IFN-γ or toll-like receptor (TLR)-stimulated STAT1 activation through the MyD88/TRAF6 or MEK/ERK pathway[139]. Induction of PD-L1 is also mediated by soluble IL-6 produced by BM stroma cells and the proliferation-inducing ligand (APRIL) secreted by eosinophils, osteoclasts and myeloid cells[140]. IL-6 is known to induce PD-L1 by activating the JAK2, STAT3 and MEK1/2 pathways, whereas APRIL activates MEK1/2 by binding to BCMA. PD-L1 expression impacts the pathophysiology of MM greatly. In *in vitro* studies, PD-L1$^+$ plasma cells showed a proliferative advantage and exhibited resistance to killing by myeloma-specific T cells and traditional myeloma drugs, like melphalan. In a clinical setting, expression of PD-L1 was found to correlate with an increased risk of progression from SMM to symptomatic myeloma[141]. In addition, statistically higher PD-L1 was reported on plasma cells from MM patients with minimal residual disease (MRD), compared to healthy donors.

PD1/ PD-L1 inhibitors have been extensively assessed in MM but met with limited success so far[138]. In clinical trials, both anti-PD1 and anti-PD-L1 mAbs were ineffective as single agents, which was partly explained by the senescent rather than exhausted phenotype of T cells in the MM microenvironment[142]. Combination treatments appear more promising. Indicatively, pembrolizumab plus IMiDs (lenalidomide or pomalidomide) and low-dose dexamethasone showed ORR of 50-60% in heavily pretreated RRMM patients[143]. Due to the severe toxicities that were reported, however, two phase III clinical trials investigating this combination were suspended (namely Keynote-183 and Keynote-185), and several others were put on hold. Today, investigations on the combination of nivolumab and atezolizumab with IMiDs have resumed. Under evaluation are also combinations of PD1/ PD-L1 mAbs with: i) auto-SCT[144], as part of the consolidation therapy, ii) anti-CD38 and anti-SLAMF7 mAbs[145,146], and iii) radiation therapy[147]. Besides finding the appropriate combinatorial approach, investigations on the timing of the administration of PD1/ PD-L1 inhibitors appear to also be important in reducing side effects.

1.4 NK CELL-BASED THERAPY

In the past decade, cell therapy has emerged as a powerful treatment option for hematological cancers. However, the severe side effects that T cell-based therapy is associated with, such as cytokine release syndrome (CRS) and neurotoxicity, have led scientists to seek alternatives. NK cells were quickly seen as a candidate for cell therapy, due to their intrinsic ability to exert cytotoxicity against transformed cells[148].

1.4.1 NK CELL BIOLOGY

NK cells are effector cells of the innate immune system. They are phenotypically defined as CD3-CD56+NKp46+ and constitute 5-15% of the circulating mononuclear lymphocytes[149,150149,150]. In addition to PB, NK cells are most commonly found in the BM and lymphoid tissues, although they can also be tissue-resident[151]. They were first discovered by Kiessling et al.[152,153] and Herberman et al.[154], circa 1975, during experiments on reactive lymphocytes against leukemia cells. The name 'natural killers' was given due to the notion that they do not require a sensitization step before eliciting cytotoxic actions. Indeed, NK cells are known to eliminate virus-infected and tumor cells without prior sensitization, unlike T cells, which require priming by antigen-presenting cells. NK cells develop from CD34+ hematopoietic progenitor cells (HPCs), and they are found in five maturation stages, making them a rather diverse cell population[155]. Different phenotypic markers are used to distinguish between the NK cell subtypes, such as CD34, CD94, CD117, CD56, and CD16A. The maturation state, combined with the environmental stimulus, is what will ultimately drive the activation or inhibition of the cell and define the type/magnitude of the effector function. For instance, $CD56^{bright}$ NK cells are known to be potent producers of pro-inflammatory cytokines, whereas $CD56^{dim}$ NK cells show significantly higher cytotoxic activity[156].

NK cells regulate their activation state by balancing activating and inhibitory signals thanks to their large repertoire of germline-encoded receptors[157] **(Figure 4)**. Activating NK cell receptors are include the Natural Cytotoxicity Receptors (NCRs) NKp44 (CD336), NKp46 (CD335), and NKp30 (CD337), as well as the Killer Immunoglobulin-like Receptors (KIRs) KIR2DS1, KIR2DS2, KIR2DS4, KIR2DS5, and KIR3DS1. Other important activating receptors are NKG2D, CD16A (FcγRIIIA), CD224 (2B4) and CD226 (DNAM-1). There is a plethora of activating receptor ligands, which are commonly expressed upon cellular stress, infection, or neoplastic transformation. In cancer, the most commonly upregulated ligands are MHC-class I polypeptide-related sequence A and B (MICA/B), the UL16-binding proteins (ULBPs), and the adhesion molecules CD112 and CD155 (also known as nectin-2 and poliovirus receptor, PVR, respectively). CD16A is one of the most potent activating receptors in the NK cell repertoire. It binds to the Fc part of antibodies coating target cells and triggers ADCC[158]. This function is particularly important for the mechanism of action of therapeutic antibodies, as mentioned above.

Many of the KIR group receptors propagate inhibitory signals, such as the KIR2DL-1, -2, and -3 and the KIR3DL1. Structurally, inhibitory KIRs have long cytoplasmic tails that contain ITIMs, in comparison to the activating KIRs. Ligands of the inhibitory KIRs are self-MHC (major histocompatibility complex)-class I molecules, which are present in all

nucleated cell types in the human body and play a vital role in the prevention of autoimmunity. Downregulation of the MHC class I molecules occurs under similar cellular stress conditions, as previously mentioned, and results in the recognition of the cell as a target. Recognition of a target via this pathway is known as 'missing-self recognition' and is a unique feature of NK cells. Other inhibitory receptors are the complex NKG2A/CD94 and the receptors CD161 and KLRG1, which bind HLA-E, lectin-like transcript 1, and cadherins, respectively.

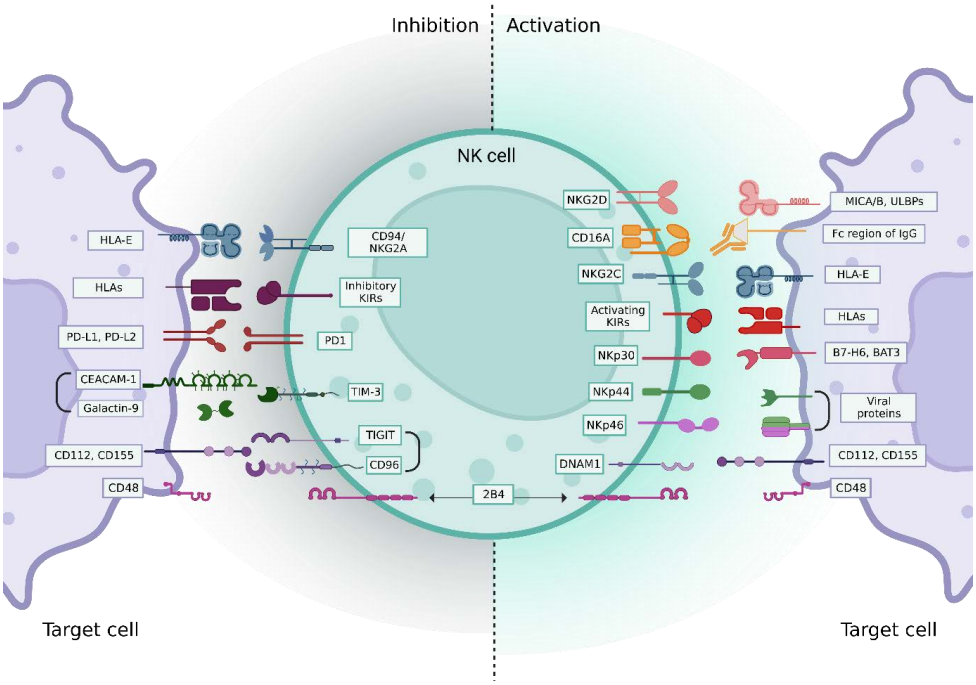

Figure 4: Main activating (right) and inhibitory (left) NK cell receptors, as well as their respective ligands on target cells. Image created with BioRender.

The effector functions that follow NK cell activation may vary from cytokine release to direct cytotoxicity[159]. NK cells have two distinct ways of mediating their cytotoxicity, namely degranulation and death receptor-mediated apoptosis. Degranulation is the process by which an activated NK cell releases cytotoxic granules containing perforin, granzymes (e.g., Granzyme B) and granulysin against the target cell[160]. The pre-loaded granules are found in the cytoplasm and are transferred to the cell surface aided by the polarization of the microtubule-organizing center (MTOC) and the reorganization of the actin cytoskeleton, triggered by the activation[161]. Perforin and granzyme act in concert to lyse the target cell, with the first forming pores on the cell membrane and the second by using these pores to enter the target cell and activate caspase-mediated cellular

apoptosis. The surrogate marker for degranulation in flow cytometric analyses is the lysosomal-associated membrane protein 1 (LAMP-1, or CD107a), which gets exposed upon the fusion of the lytic granules to the NK cell membrane[162]. Degranulation is a fast-occurring process, happening in minutes, in comparison to death receptor-mediated apoptosis. The second pathway involves the interaction of the tumor necrosis factor (TNF) ligand superfamily 6 (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), with their respective receptors, CD95 and TRAIL-receptor 1 and 2, found on targeted cells[159] .

1.4.2 ADOPTIVE NK CELL THERAPY

Similar to T cell-based therapy, adoptive NK cell therapy requires the *ex vivo* expansion of the cells to reach clinically relevant doses. In the NK cell setting, two main expansion systems are used, i.e. the cytokine-based[163,164] and the feeder cell-based system[165–168]. The first involves long-term stimulation with one or more cytokines that are known to provide activating and proliferating stimuli to NK cells. Such cytokines are IL-2, IL-12, IL-18, IL-15 and IL-21[169–172]. Alternatively, the stimulus can originate from irradiated feeder cells. Examples of feeder cell systems include Epstein-Barr virus-transformed lymphoblastoid cell lines[173,174], recombinant human fibronectin fragment-stimulated T cells[175], as well as the cell line K562 genetically modified to express membrane-bound IL-15 (mbIL-15), mbIL-21, MICA and/or 4-1BB ligands[176,177]. Although both strategies result in high amounts of activated NK cells, notable differences in their phenotype, cytotoxic potency and proliferative capacity are observed depending on the method[178].

Initial studies of adoptive NK cell therapy used autologous peripheral blood (PB) NK cells that were pre-activated with interleukin-2 (IL-2)[179]. Unfortunately, these treatments did not have the anticipated clinical outcomes, partly due to immunosuppressive TME that was already upregulating NK cell inhibitory ligands[180]. Nevertheless, the adoptive transfer of NK cells was deemed safe to administrate and, therefore, new ways of improving the cells' performance were sought. Indeed, by using allogeneic or haploidentical NK cells, the inhibition was overcome due to the KIR ligand-mismatch that increased the recognition of tumor cells and skewed the NK cell balance towards activation[181–183]. The off-the-shelf potential of NK cells has inspired the exploration of alternative NK sources. Cord blood-derived NK cells (CB NK cells) are a viable option due to the advantage of being readily available in cell banks and having a higher proliferative capacity compared to PB NK cells[182,184]. Other investigations are focusing on stem cell-derived NK cells. They are commonly generated by the genetic reprogramming of human embryonic stem cells (hESCs), CD34+ HSCs or induced pluripotent stem cells (iPSCs)[185]. These cells share the proliferative advantage of CB NK cells, but are additionally known to be easier to genetically engineer. A newer addition to the NK cell sources is the memory-like (ML) NK cells, which exhibit characteristics of adaptive immunity. ML-NK cells are generated following viral infection, exposure to haptens or cytokines, such as IL-12, IL-15 and IL-18[186–189]. Besides primary NK cells, NK cell lines are also being used in the context of NK cell-based therapy. NK cell lines were initially investigated due to challenges in genetic modification and *ex vivo* expansion of allogeneic NK cells. The most common one is the NK92 cell line, an IL-2-dependent non-Hodgkin lymphoma NK cell line[190]. NK92 has been FDA-approved for use in clinical trials following an irradiation step. Nevertheless, the use of NK92 is hampered due to the risk of tumorigenicity by undesired clonal expansion and compromised function following irradiation[191]. Other NK cell lines, that however have not

been investigated in a clinical setting, are the YT, YT-S, KHYG-1, NK3.3, NK-YS, NKL, NKG, SNK-6 and IMC-1[192].

Regardless of the NK cell source, the targeting as well as the cytotoxic potential of NK cells can be further augmented by the expression of chimeric receptors, such as CARs or chimeric switch receptors (CSRs).

1.4.2.1 CHIMERIC ANTIGEN RECEPTORS

CARs are artificial receptors, comprising of an antigen-binding single-chain variable fragment (scFv), a short transmembrane (TM) region and one or more signaling domains[193]. CAR deign has progressed over the years, from having one signal transduction domain (1st generation CARs)[194], to having one or two additional co-stimulatory domains (2nd and 3rd generation)[195]. The most commonly used co-stimulatory domains are CD28 and 4-1BB (CD137), whereas the most widely used signal transduction module is the CD3ζ. Although both CD28 and 4-1BB recapitulate natural co-stimulation and attribute increased potency to the transduced cells, comparative studies revealed a different set of advantages between the two[196]. Specifically, CD28 is associated with a stronger cytotoxic potential, while 4-1BB with enhanced survival and proliferation[197].

The initial CAR design was inspired by the signaling moieties of T cells. Although the same CARs proved functional in the NK cell setting, further advances followed[198,199]. Novel constructs employed domains that NK cells typically use for downstream signaling, such as CD3ζ, DAP10, DAP12 and FcRγ chains. Of note, unlike CD3ζ which has three immunoreceptor tyrosine-based activation motifs (ITAMs), the rest have one. DAP10, the adapter protein of the NK cell activating receptor NKG2D, proved functional only when in combination with the NKG2D's ectodomain, while the use of the TM domain alone had a negative impact on the exerted cytotoxicity[200]. On the other hand, DAP12, the adapter protein of activating KIRs, NKG2C and NKp44 receptors, outperformed CD3ζ-based CARs *in vitro* and *in vivo*[201]. NK-like CARs comprising of the activating receptor 2B4 have also been successfully investigated, attributing superior anti-tumor responses, compared to 4-1BB and CD28 CAR constructs[202]. The design of 3rd and 4rth generation CARs with NK-like signaling domains is currently under investigation, as different combinations are being tested.

In the last years, CAR-NK cells have marked considerable successes in clinical trials. For instance, in a phase I/II trial, CD19-CAR CB NK expressing the IL-15 transgene and the inducible caspase-9-based suicide gene (iC9), were infused in patients with relapsed or refractory B-cell malignancies[165]. The CAR-NK product induced response in 8 out of 11 patients, with 7 of them experiencing a complete response. Of note, CAR-NKs were detected in the periphery blood at least one year post-treatment. CAR-NK92 cells have also been assessed clinically in the context of acute myeloid leukemia (αCD33-CAR)[203] and pancreatic ductal adenocarcinoma (anti-Robo1-CAR)[204]. Both clinical trials concluded that the NK cell products were well-tolerated, though did not manage to sustain durable responses.

1.4.2.2 CHIMERIC SWITCH RECEPTORS

The immunosuppressive TME is a significant hurdle in ACT, as it could compromise the effectiveness of the therapy even after the successful trafficking of the infused cells to the cancer niche[205,206]. Our growing understanding of the immune checkpoints and inhibitory molecules has led to the development of a new class of chimeric receptors that could overcome this issue; the chimeric switch receptors (CSRs). CSRs are specifically designed to convert suppressive signals into stimulating ones, hence restoring the anti-tumor effector functions of immune cells[207]. This is achieved by exchanging the cytoplasmic tail of the inhibitory receptors with activating signaling components. Specifically, CSRs consist of:

i) the ectodomain of PD1, CTLA-4[208], or other inhibitory receptors, like TIGIT,[209]
ii) a transmembrane domain, usually from the receptor inhibitor receptor or CD28[207],
iii) one or more intracellular activating domains, such as CD28, 4-1BB, DAP10, NKG2D and CD3ζ[210–212].

In this way, the natural inhibitory molecules are competing with the artificial CSRs, which balance the signals and prevent immune cell exhaustion in the TME.

CSRs are more extensively tested in T cells. They have been assessed alone[213] or co-expressed with CARs[214] in a variety of *in vitro* and *in vivo* tumor settings. The majority of these studies concern PD1. PD1/CD28 constructs have been generated by different groups. Collectively, expression of PD1/CD28 CSRs was associated with improved tumor infiltration, targeted cytotoxicity and cytokine release[207,215]. Importantly, CSRs attributed higher proliferative capacity to T cells within the TME, which rationalizes their application in solid tumors. The specific design of the CSR, i.e., the size of the extracellular domain and the choice of transmembrane domains (PD1 versus CD28), had a low impact on the observed effect[216]. PD1/CD28/4-1BB constructs have also been explored in murine models of glioblastoma with similar encouraging results regarding tumor growth control[217]. Other groups have fused PD1-ectodomain with NK2GD or DAP10 signaling domains[210]. These constructs were efficiently expressed in T cells and succeeded in transducing activating signals upon interaction with PD-L1. Of note, a study comparing a PD1-directed CSR with CD28 transmembrane domain, CD28 or DAP10 intracellular domain and CD3ζ, found that the presence of CD28 or DAP10 changed T cell behavior[218]. Specifically, CD28 induced an effector memory phenotype and production of anti-inflammatory cytokines like IL-10. On the other hand, DAP10 was associated with higher anti-tumor activity. To date, only one clinical trial including PD1-CSR has been completed[219]. In a study of RR diffuse large cell B-cell lymphoma, infusion of CD19-CAR-T cells that co-express a PD1/CD28 CSR, resulted in complete remission in 3 out of 6 patients and lack of severe neurologic toxicity or cytokine release syndrome. Various CSRs with ectodomains from CTLA-4[220–222], PD-L1, TIGIT[223] and CD200R[224] have also been investigated. Furthermore, the CSR approach has been applied for the prevention of the effects of TME-related immunosuppressive cytokines, such as IL-7 and TGF-β[225]. Although these constructs appear promising in a laboratory setting, clinical studies are yet to be conducted.

The published studies on CSR-expressing NK cells are only a few. It is clear, however, that based on the plethora of inhibitory signals NK cells receive in the TME such studies are relevant. A study on NK92 cells expressing a PD1/DAP10/4-1BB CSR showed improved

cytotoxic activity against the human lung cancer cell line H199 *in vitro* and *in vivo*[210]. Increased intracellular concentration of cytotoxic molecules, such as perforin and granzymes, was also reported in the same study as a result of the CSR expression. Similar encouraging results were generated in a study of a novel bispecific PD1 and NKG2D/DAP10 chimeric receptor expressed on NK92 cells, against the human gastric cancer cell line SGC-7901 [212]. A recent publication from our group (paper II) added further to this field, by developing various PD1-CSRs with the signaling domains of DAP10 and DAP12 and showing increased infiltration and cytotoxicity against PD-L1 expressing target cells [226].

1.4.2 NK CELLS IN MULTIPLE MYELOMA

Myeloma cells develop intricate mechanisms for evading immune surveillance. One of them involves the transformation of the microenvironment to an inflammatory/immunosuppressive milieu. Progression from the pre-malignant stage of MGUS to active MM largely depends on this[227,228]. In the neoplastic BM microenvironment, transformation is promoted by both cellular and non-cellular drivers[229,230]. Cellular drivers include hematopoietic cells, immune cells, mesenchymal stem cells, endothelial cells, adipocytes, osteoclasts and osteoblasts. Non-cellular drivers consist of extracellular matrix proteins, cytokines, growth factors, chemokines and extracellular vesicles. Collectively, these factors impede physiological BM function, promote malignant cell growth, support resistance to treatment and shape a hostile environment for effector immune cells.

Important NK cell receptors in myeloma cell recognition are the NCRs, NKG2D and DNAM1[231]. However, the functionality and distribution of NK cell subsets are negatively impacted during MM. In fact, discrepancies are apparent already from the MGUS stage and less mature subsets ($CD56^{bright}$ and $CD56^{dim}CD16^{-}KIR2DL1/S1^{-}$) prevail in RR and post-SCT patients[232]. Phenotypically, NK cells from patients with MM have decreased expression of the activating receptors 2B4, CD16, DNAM1 and NKG2D[233,234]. In parallel, NK cells of some patients show upregulation of PD1, TIM3 and TIGIT receptors, which suppress NK cell activity[136,234]. Importantly, these changes are more prominent in BM rather than PB NK cells, as well as in RR and post-SCT patients compared to newly diagnosed. Although increased expression of the co-stimulatory receptors ICOS and GITR have also been reported, collectively the phenotypic changes that NK cells undergo during the progression of MM suggest the emergence of immature/exhausted cells[234]. In parallel, MM cells express NK-activating ligands poorly, while upregulating inhibitory ligands. In contrast to other cancer types, where MHC class I is lost or downregulated, MM cells upregulate it impeding recognition by NK cells. Myeloma cells are also characterized by low expression, or even complete absence, of MICA/B and Fas receptor, which interact with the potent NK cell receptor NKG2D and Fas ligand, respectively.

NK cell-based immunotherapies have been established as safe off-the-shelf therapies via clinical assessments in hematological and solid tumors. Regarding MM, multiple approaches have been explored, as there is a strong rationale for their use[235,236]. At the moment there are 22 clinical studies registered on clinicaltrials.gov. These involve ACTs of *ex vivo* expanded and activated NK cells, that are administered either alone, in combination with FDA-approved agents, or in the form of genetically modified NK cells [237]. A co-administration of activating cytokines, such as IL-2 and IL-15 is usually

recommended for NK cells to reach their full anti-myeloma potential. NK cell-based approaches can be further categorized as autologous or allogeneic depending on the NK cell source. The team of Dr. Frits van Rhee at the University of Arkansas has led three clinical trials involving NK cells from heavily pre-treated MM patients. The trial reported efficient *ex vivo* expansion using a feeder cell system and provided evidence that co-administration of NK cells with bortezomib improved clinical outcomes[238]. Furthermore, FDA and EMA recently gave orphan drug status to CellProtect, an autologous NK cell product that was trialed at Karolinska University hospital in newly diagnosed MM patients who had undergone auto-SCT[239]. The product showed promising results with no severe adverse events reported. Its combination with Isa are currently underway (NCT04558931). The combination of autologous NK cells and bortezomib was also studied in two further trials with positive results[240]. The completed studies demonstrate that autologous activated NK cells have anti-myeloma activity, and infusions from patient NK cells are feasible.

2. RESEARCH AIMS

This thesis aimed to investigate the potential of novel targeted treatments for MM and contribute to the expansion of current therapeutic options. Each study was focusing on a different molecular target, namely CD38 **(paper I)**, PD-L1 **(paper II)** and BCL2 **(paper III)**, the rationale for each of which is well-established in the MM field. **Papers I and II** explore NK cell-based approaches, whereas **paper III** focuses on providing new insights into the use of personalized chemotherapy. The specific research aims are listed below:

Paper I. CD38-targeting antibody-based immunotherapies are routinely used in clinics leading to positive clinical outcomes for many patients. However, the exploitation of CD38-targeting in the context of NK cell-based therapy is met with limitations. The aim of this study was to suggest an alternative approach to αCD38-CAR-NK cell therapy and explore its feasibility in a preclinical setting.

Paper II. The PD1/ PD-L1 axis plays a critical role in regulating the effector activity of immune cells in the MM TME. This is suggested to have a negative impact on the efficacy of NK cell-based cellular therapies. The study aimed to investigate the potential of *ex vivo* expanded NK cells expressing PD1-CSRs in a preclinical setting of MM, and draw conclusions on the optimal CSR design.

Paper III. Chromosomal aberrations are common among patients with MM. Out of them, translocation t(11;14) is known to upregulate the anti-apoptotic protein BCL2 on the myeloma cells. In this clinical study we aimed to assess the safety and efficacy of monotherapy with low-dose Venetoclax, a specific BCL2 inhibitor, in patients with relapsed MM and amyloid light-chain (AL) amyloidosis harboring the t(11;14) mutation.

3. ETHICAL CONSIDERATIONS

It is essential one to reflect on the ethical aspects of their research and acquire in-depth knowledge of the regulations that need to be followed. My studies centered around the MM malignancy. In **studies I and II**, we used PBMCs from healthy individuals and patients and BM-derived mononuclear cells (MNCs) from MM patients (ethical permit No: 2019-04873). Both studies were solely *in vitro*. In **study III**, we conducted a clinical trial on patients with MM and amyloidosis, for which the ethical permits 2014-526-31/3, 2019-02638 and 2020-00175 were issued.

The handling and storage of primary specimens have been a main topic in research ethics, especially in the genomic era that we currently live in. Over the years many patients have expressed concerns regarding the misuse of their material, their potential identification through the DNA and the use of their information for commercial purposes. To avoid these concerns and protect the rights of the individuals involved, we carefully wrote and followed ethical permits. We were sensitive and thorough in respecting donor anonymization and kept the essential information of MM patients confidential, according to General Data Protection Regulation. Moreover, given the invasiveness of blood and BM collection, research samples were ethically collected only during routine or diagnostic proceedings, to prevent further pain. The use of the material was always preceded by a signed informed consent.

Besides respecting the principles of preclinical and clinical research conduct, I would like to elaborate on the translational and social aspects of the studies. My research focus is to explore the potential of novel therapeutic approaches for MM, with special emphasis on NK cell-based cell therapy and personalized treatments. Taking the example of FDA-approved CAR-T cell products, it is clear that the cost of cell-based therapies is high. For instance, each Kymriah (an approved CAR-T cell product) treatment costs 475.000 USD, whereas the total cost per patient is estimated at 1-1.5 million USD. Similarly, personalized treatments require genetic testing and specially trained personnel capable of interpreting the results. If one considers the restricted healthcare budget of each country and the limitations that health insurance companies set, ethical questions arise regarding the financing of the treatments by the poorer population groups and their overall accessibility. These questions are often downgraded in the laboratory environment, where researchers are accustomed to expensive techniques. My thoughts on this issue are that, as researchers and part of the medical community, we should strive to provide equal opportunities by improving our techniques using a cost-effective mindset. One step towards this direction would be the development of automated cell expansion systems and further optimization of large-scale processes. This would greatly cut the cost of cell therapy production, both in terms of reagents and labour. With the positive clinical trial results motivating such research, I am optimistic that the constant advances in the field would eventually reduce the production cost, making personalized treatments widely available and allowing equal access to treatment.

4. RESULTS AND DISCUSSION

4.1 STUDY I

As previously discussed, the development of CD38-targeting therapies has completely redefined the standard of care for MM patients. Dara and Isa, the two FDA-approved antiCD38 mAbs, have shown remarkable efficacy and improved the survival rates for a significant fraction of patients[241]. Yet many patients are refractory, respond partially or relapse. Many theories are investigated regarding the reason behind this effect. Most of them take Dara treatment as reference and study its mechanisms of resistance.

Dara-mediated cytotoxic activities are highly dependent on CD38 expression levels on target cells, with higher expression leading to more potent reactions[242]. Clinical response to Dara does not always correlate with surface CD38, however, as baseline expression levels between responders and non-responders often overlap[243]. It has been additionally shown that CD38 rapidly decreases on myeloma cells after the first dose; an effect that occurs irrespective of the degree of response[243,244]. Some reports have shown that CD38 has the tendency to rapidly internalize upon antibody binding. This argument is supported by the CD38 rescue when the endocytosis inhibitor Dynasore is applied[245]. Other published data demonstrate that loss of CD38 expression is mediated by monocyte and granulocyte trogocytosis[244]. Although the decrease is transient (lasting for about 3-6 months) and it does not necessarily lead to treatment resistance[244], the efficacy of the following doses and their timing can be affected.

AntiCD38-mAbs eliminate CD38+ cells. Selective expansion of resistant $CD38^{dim/-}$ myeloma clones can also occur at this stage[246]. In parallel to the MM cell eradication, normal CD38+ cells, such as NK cells, are also affected[247]. This does not only imply reduced treatment effect due to the reduced NK cell mediated-ADCC but can lead to severe infectious complications[248]. Indicatively, 9 out of 23 patients with progressive MM had either viral, bacterial or both viral and bacterial infections after Dara.

The aforementioned challenges in CD38-targeting by mAbs rationalize the exploration of alternative CD38-targeting modalities. NK cell therapy holds great potential in this setting. Specifically, the rationale is based on the following four pillars:

i) NK cells exert anti-myeloma activity *in vitro*[249,250].
ii) NK cells can exert CAR-dependent, as well as CAR-independent target recognition, making them valuable when the targeted TAA is lost.
iii) NK cell-based therapy has potential to control minimal residual disease.
iv) Adoptively transferred NK cells could control infectious complications.

In study I, we aim to explore the feasibility of αCD38-CAR-NK therapy. Besides the established rationale, the generation of αCD38-CAR-NK is problematic due to the inherent expression of CD38 on NK cells, which can lead to fratricide **(Figure 5).** So far, strategies have mainly focused on the use of genetic engineering tools to KO CD38[251], or employed $CD38^{low}$ NK cell lines [252], which showed encouraging results. Given the key role that CD38 plays in NK cells, we investigate the use of $CD38^{dim}$ NK cells, generated without genetic engineering, as the basis of our αCD38-CAR approach.

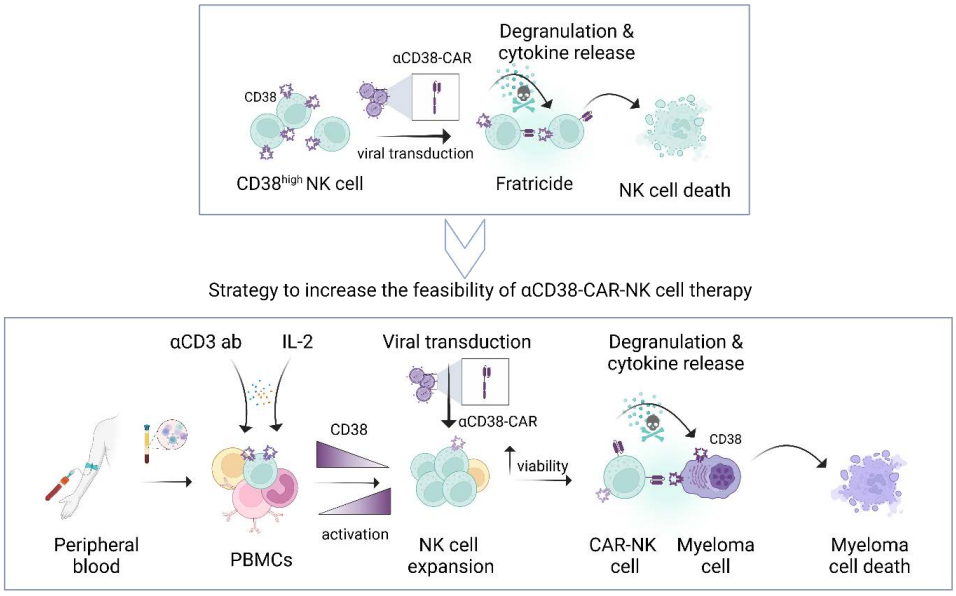

Figure 5: Graphical abstract of **Study I**. Image created with BioRender.

4.1.1 Primary expanded NK cells downregulate CD38 during long-term IL-2 stimulation.

An important aspect of adoptive NK cell therapy is the generation of adequate amounts of NK cells to cover for the therapeutic doses. In studies I and II we implemented a cytokine-based approach, that leads to high fold expansions of activated NK cells after approximately 20 days **(Paper I, Fig. 2A)**, starting from a small amount of peripheral blood mononuclear cells (PBMCs). This approach, that is based on the long-term IL-2 stimulation, has also been utilized in a clinical trial, showing adequate safety across the patients[253].

By performing routine phenotypic analyses, using flow cytometry, we observed a gradual downregulation in both the percentage of CD38+ NK cells, and in their mean fluorescence intensity (MFI) **(Paper I, Fig. 2B)**. This observation contrasts with findings from other groups, that use feeder cell-based systems, and show significant CD38 upregulation[251].

CD38 downregulation or deletion, is associated with improved metabolic function and effector activity in NK cells[251,254,255]. Although CD38 is considered a marker that upregulates during NK cell activation, and thus its downregulation could imply NK cell dysfunctionality, phenotypic analyses on critical activation and proliferation markers showed acceptable levels of expression overall. Specifically, after 20 days of expansion, NK cells exhibited an activated phenotype, as seen by the increased expression of CD25, CD69, HLA-DR, NKG2D and NKp44, as well as the continuous high expression of CRACC **(Paper I, Sup. Fig. 3C, D)**. The same phenotypic analysis revealed a decrease in the expression of the receptors CD16, DNAM-1, NKp30 and NKp46, which however did not result in impairment

of the degranulation and cytokine release capacity against the commonly used target cell line K562 **(Paper I, Fig. 2F)**.

4.1.2 CD38^{dim} NK cells can be efficiently transduced using an affinity-optimized aCD38-CAR, maintaining high viability and effector functions.

The intrinsic expression of CD38 on NK cells in combination with the exertion of ADCC, is known to induce fratricide phenomena during treatments with αCD38 mAbs. Therefore, we set out to test if the downregulation of CD38 on the expanded NK cells translated into reduced fratricide. Indeed, treatment with the αCD38 mAb Daratumumab, led to no change in the viability of expanded NK cells, unlike non-expanded NK cells **(Paper I, Sup. Fig. 3B)**.

Based on this, we rationalized the transduction of expanded NK cells (expNK) cells with a 2nd generation affinity-optimized αCD38-CAR using a retroviral vector. The expression of the CAR construct was confirmed four days post-transduction, by a two-step staining, using a Fc-tagged recombinant CD38 protein first and a secondary anti-Fc antibody second. Usually, genetic manipulation of NK cells is a challenge due to their natural anti-viral mechanisms[256]. We optimized NK cell transduction with αCD38-CAR construct by:

i) finding the optimal timepoint for retroviral vector transduction, based on the peak of NK cell expansion **(Paper I, Fig. 2A)**
ii) identifying the timepoint when CD38 expression is at its lowest and, thus, has a lower probability of fratricide events **(Paper I, Fig. 2C, D)**
iii) optimized the transduction method by employing Retronectin-coated plates **(Paper I, Fig. 2E)**.

Optimal transduction efficacy, sustained NK cell frequency and highest viability were achieved when transduction was performed on day 13 of expansion. Importantly, we observed that the cytotoxic potential of transduced NK cells was sustained and reflected that of the parental unmodified cells against the K562 cell line. Based on these findings we developed a combinational protocol, that includes the NK cell expansion, viral transduction, and assessment of cytotoxicity **(Paper I, Fig. 3A).**

We applied the combinational protocol to generate CAR-expressing and control GFP+ NK cells from PBMCs of healthy donors. To better investigate the functionality of the CAR construct, we generated CD38 KO cell lines from the CD38^{high} MM cell lines RPMI-8226 and MM.1S, using CRISPR. We performed three different assays to assess the exertion of specific cytotoxicity and induction of NK cell effector functions upon target cell recognition. In the *in vitro* activation assay, CAR-NK cells showed increased degranulation (as shown by the surrogate marker CD107a) and production of IFNγ and TNFα cytokines, against the wild-type myeloma cell lines, compared to the unmodified expanded NK cells **(Paper I, Fig. 3B-D)**. To see if the enhanced activation resulted in increased target cell lysis, we performed a chromium release assay **(Paper I, Fig. 3E, Sup. Fig. 6A)**. Indeed, across all assessed effector: target cell ratios, αCD38-CAR-NK cells outperformed the control NK cells. Lastly, we sought to evaluate the proliferative capacity of αCD38-CAR-NK cells upon interaction with CD38+ positive cells. Our results showed that αCD38-CAR-dependent increase in the proliferation of NK cells upon interaction with CD38+ target

cells **(Paper I, Sup. Fig. 5)**. Altogether, these results indicate a functional αCD38-CAR construct, which upon expression by the primary expanded NK cells, provides significant advantages against CD38+ myeloma cells lines.

3.1.3 Patient-derived aCD38-CAR-NK cells show increased reactivity against autologous myeloma cells.

Our findings on donor aCD38-CAR-NK encouraged further investigations. This time we assessed the approach in an autologous MM setting. PB NK cells from 3 patients with RRMM, previously refractory to Dara, were expanded using our optimized protocol and transduced with the therapeutic retroviral vector on day 13. BM-derived mononuclear cells (MNCs) were also sampled and isolated from the same patients. MNC samples had a frequency of 2-20% malignant cells **(Paper I, Fig. 4A)**. The expansion of patient-derived NK cells differed from that of healthy donors **(Paper I, Fig. 5A)**, in terms of reaching the expansion milestones on the assessed time points. Moreover, CD38 expression on NK cells marked only a small decline, which was uncommon based on our observations on PB NK expansions, as well as the expansions of other RRMM patients found in the literature. Possible explanations could be the heavy pretreatment of these patients prior to the peripheral blood collection or the cryopreservation step. Still, the introduction of the aCD38-CAR construct to the patient-expanded NK cells, induced higher degranulation and IFNγ release, compared to the control cells, when co-cultured with autologous MNCs. This experiment aimed to assess the feasibility of the approach *in vitro* and highlighted the optimizations that are needed before translating the findings to the clinic.

4.2 STUDY II

The PD1/ PD-Ls axis plays a critical role in the immune evasion of many cancer types. However, its role in MM is debated. Contradicting reports on MM and immune cell phenotype, in regard to PD-L1 and PD1 expression respectively, have been published throughout the years[257]. Lately, with the standardization of the flow cytometric guidelines for myeloma cell phenotyping, PD-L1 expression on MM cells has been confirmed[258]. In addition, *in vitro* and *in vivo* studies employing anti-PD1 mAbs showed increased anti-myeloma activity by T and NK cells, further strengthening the rationale for targeting the axis in MM.

As previously discussed, trials including anti-PD1/ PD-L1 mAbs were met with partial responses and unpredicted side effects. Blocking the axis with a retargeted cellular therapy approach has also been attempted, although not in MM. For instance, CAR-T cells with disrupted PD1 were investigated in ovarian cancer[259], while PD1 KO was assessed in B cell lymphomas[260]. Although the loss of PD1 relieved immunosuppression, T cells exhibited functional impairment, decreased survival and acquired exhausted phenotypes[260,261]. In light of this, balancing the immunosuppressive and activating stimuli, instead of interfering with existing PD1 pathways, is rationalized.

In this study we investigated the potential of PD1-CSRs in the context of MM. CSRs were preferred over CARs, due to the risk of on-target off-tumor effects that can occur[262]. CSRs

have proven to efficiently redirect adoptively transferred cells to the tumor site and compete with immune checkpoints[209]. Due to the safer clinical profile and anti-myeloma rationale mentioned above, we employed NK cells as the cell basis of our approach, and studied the potential of PD1-CSR-NK cells in an in vitro MM model. The rationale of the study, as well as the design of the CSRs is depicted below (**Figure 6)**.

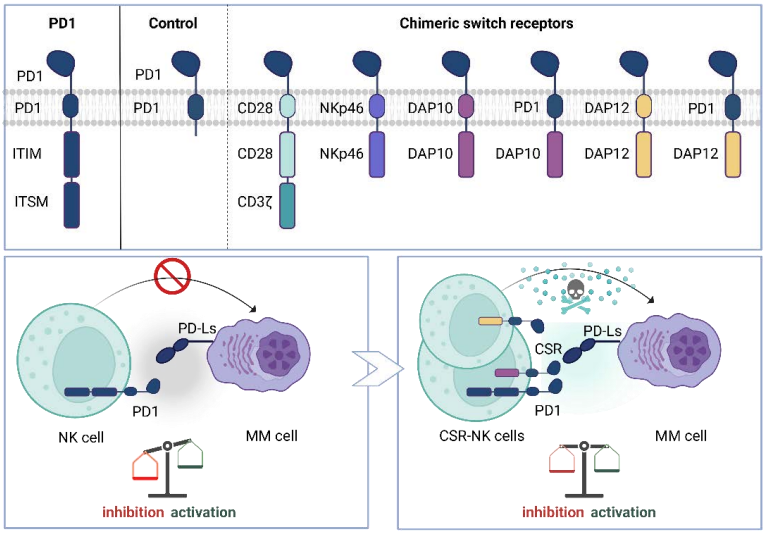

Figure 6: Graphical abstract of **study II**. Image created with BioRender.

4.2.1 Generation of PD1-CSR constructs

CSRs have been investigated more in the context of T cells, rather than NK cells, which reflects on the design of the receptors discussed in literature. Based on findings from CAR-NK studies, demonstrating the superiority of NK cell-associated activating moieties, we designed six different CSRs **(Paper II, Fig. 1A-B)**. The full length of the extracellular part of the PD1 receptor was used as the respective domain in all constructs. This was an additional step towards safety, since it reduces the chances of immunogenicity[263]. As TM and intracellular domains either the continuation of the PD1 receptor sequence or domains from the NKp46 receptor and the adaptor proteins DAP10 and DAP12 were used. In addition, a CAR-like CSR was generated, harboring the commonly used 2nd generation CAR domains CD28 and CD3ζ. In this way we generated a spectrum of CSRs able to provide a well-rounded comparison between the signal transduction domains. Of note, a CSR with truncated intracellular activating domains served as universal control.

Going more in-depth on the selection of the domains, it is worth mentioning the rationale. NKp46 is a potent, highly conserved NK cell cytotoxicity receptor, present in both resting and activated NK cells. NKp46 lacks ITAMs in its cytoplasmic tail, however, its TM region contains an Arg residue able to interact with the ITAM-bearing downstream signaling adapters FcεRI and CD3ζ [264,265]. NKp46-based CARs have been generated before [266,267]. In

our NKp46-based CSR designs we incorporate both the TM and the intracellular domains of the receptor.

DAP10 and DAP12 are membrane-bound signaling adaptor proteins that associate with many NK cell activating receptors in a noncovalent manner[268]. The two proteins have a 20% amino acid homology and remarkably similar TM regions. However, unlike DAP10, DAP12 possesses an ITAM domain. As previously mentioned, DAP10 has been assessed in the context of PD1-chimeric receptor, showing efficient triggering of NK92 cytotoxicity [212]. DAP12, on the other hand, had only been assessed in CARs for NK cells, where it was proven superior to CD3ζ, despite the latter having three ITAMs.

4.2.2 $PD1_{ECTM}DAP10_{IC}$ and $PD1_{ECTM}DAP12_{IC}$-*expressing NK cells efficiently target PD-L1+ target cell lines in 2D and 3D in vitro cytotoxicity assays*.

NK92 and primary NK cells were stably transduced with lentiviral vectors encoding for the CSRs **(Paper II, Fig. 1C)**. For all CSRs, construct expression was assessed by staining for the PD1 ectodomain using a commercially available antibody for flow cytometry. Of note, native PD1 expression of NK92 was always <2%. PD1-CSR-NK cells were evaluated for the targeting capacity and the sustaining of their anti-tumor activity within a PD-L1-rich environment. As target cells were used the WT hypertriploid renal cell carcinoma cell line 786-O (PD-L1+) and the Burkitt lymphoma cell line Raji (PD-L1-). In addition, we generated PD-L1 KO 786-O cells using the CRISPR technology and PD-L1+ Raji cells by electroporation of the PD-L1 plasmid.

CSRs were first compared in an *in vitro* NK92 activation experiment against the generated 786-O cell lines, which are known to be resistant to NK cell-mediated cytotoxicity. Out of the six CSRs, NK92 cells expressing $PD1_{ECTM}DAP12_{IC}$ instigated the highest increase in degranulation, IFNγ and TNF release against wild-type PD-L1+ 786-O cells in a 2D model **(Paper II, Fig. 2A)**. To further confirm the anti-PD-L1 effect, we employed a live cell imaging-based 3D cytotoxicity assay[269] using large spheroids from PD-L1+ and control PD-L1 KO 786-O cells. Tumor spheroids are better at resembling the TME and can additionally provide information on the infiltrating capacity of NK cells[270]. $PD1_{ECTM}DAP12_{IC}$ and $PD1_{ECTM}DAP10_{IC}$ cell lines were more effective in eradicating PD-L1+ 786-O cells, compared to the control **(Paper II, Fig. 3A-D)**, and showed better infiltration within the spheroid **(Paper II, Fig. 3E-G)**.

Following the proof-of-concept experiments using the NK92 cell line, we evaluated the function of primary NK cells (pNK) transduced with the selected CSRs. Two distinct populations emerged, namely $PD1^{bright}$ and $PD1^{dim}$, according to the level of CSR expression **(Paper II, Fig. 4A, B)**. Both $PD1_{ECTM}DAP12_{IC}$ and $PD1_{ECTM}DAP10_{IC}$ attributed higher cytotoxicity to pNK cells against PD-L1+ Raji cells **(Paper II, Fig. 4C-E)**, which was further enhanced in the presence of the antiCD20 mAb Rituximab **(Paper II, Fig. 4-H)**. This effect was more prevalent in the $PD1^{bright}$ cells. In contrast to the observations in Raji cells and previous findings from NK92 cells, only $PD1^{bright}$ PD1 $_{ECTM}DAP12_{IC}^{+}$ NK cells showed improved degranulation and cytokine release against 786-O cells **(Paper II, Fig. S8D-F).** However, this still did not translate to improved target cell lysis in 2D and 3D cytotoxicity assays.

The lack of sorting of the $PD1^{bright}$ population prior to the assays could explain these results, as the heterogenous population may prevent scientific conclusions to be drawn.

4.2.3 Patient-derived expanded NK cells expressing $PD1_{ECTM}DAP12_{IC}$ and $PD1_{ECTM}DAP10_{IC}$ *CSRs show potential against autologous myeloma cells.*

In a more direct effort to study the potential of autologous PD1-CSR-NK cells in MM, we expanded the NK cell population from PBMCs of heavily pre-treated patients, transduced them with $PD1_{ECTM}DAP12_{IC}$ and $PD1_{ECTM}DAP10_{IC}$ CSRs, and assessed their *in vitro* activation against autologous BM-derived MNCs. Although the overall malignant cell fraction was low across all patients, PD-L1 expression was detected in two of the three samples **(Paper II, Fig. 5A)**. Co-culture of CSR^{+} NK cells with the autologous MNCs, resulted in increased degranulation in one patient and increased cytokine production in all patients **(Paper II, Fig. 5C-M)**. Although these results are preliminary and the inclusion of more patient samples is needed, our study presented evidence of the potential of $PD1_{ECTM}DAP12_{IC}$ and $PD1_{ECTM}DAP10_{IC}$ CSRs in MM.

4.3 STUDY III

Beyond antibody and cellular approaches, targeted therapy can also be achieved using chemotherapy. In the last years, genetic testing has facilitated the identification of patient groups with distinct characteristics. Patient stratification has further intensified drug repurposing efforts, with the aim to offer more personalized treatments. A successful application of personalized medicine benefits not only the patients, via fewer side effects and lines of treatment, but also healthcare systems due to lower treatment costs. Driven by this, we focus on MM and Ig light chain (AL) amyloidosis patients, two plasma cell disorders that commonly harbor the t(11;14) genetic aberration, and investigate the potential of Venetoclax. On a molecular level, myeloma cells harboring the t(11;14) mutation present differential expression in members of the BCL2 regulator protein family. The BCL2 family comprises of the anti-apoptotic proteins MCL1, BCLW, BCLXL, BFL1, and BCL2, and the pro-apoptotic proteins BIM, BAD, PUMA, BID, BMF, HRK, NOXA, BAK, BAX, and BIK[271]. Specifically, MM cells with t(11;14) are associated with high BCL2 and low MCL1/BCLXL ratio, which attributes increased resistance to apoptosis. In this context, the use of Venetoclax, an oral selective BCL2 inhibitor, is rationalized.

Venetoclax is a first-in-class BH3 mimetic, approved for the treatment of adult chronic lymphocytic leukemia, small lymphocytic lymphoma and certain cases of acute myeloid leukemia. In MM, Venetoclax has shown efficacy against MM cell lines and primary MM cells in *in vitro* studieXL.. Moreover, it has been investigated both as a single agent[272] and as part of a larger regimen[273–275]. Out of the 66 patients with RRMM that received a daily dose of Venetoclax monotherapy, in dose-escalating cohorts, only 14 patients responded (ORR 21%)[272]. Notably, 12 out of these 14 patients were harboring the t(11;14) translocation. Although the majority of patients harboring the mutation did not respond to the treatment, the likelihood to achieve a response within the t(11;14) group was significantly higher. Common adverse effects included gastrointestinal toxicities, and hematologic toxicities, such as thrombocytopenia and neutropenia. In the combinational approach

assessed in the BELLINI trial, Venetoclax or placebo was administrated with bortezomib and dexamethasone[274]. PFS was substantially higher in the t(11;14) subset in the Venetoclax arm, although increased mortality was also reported. Similar, or even greater, is the potential of Venetoclax in AL amyloidosis, where abnormal plasma cells upregulate BCL2, irrespective of the t(11;14) status[276]. Indeed, in clinical trials, RR patients showed deep responses to Venetoclax[277,278].

In this clinical study we investigated further the safety and efficacy of low-dose Venetoclax monotherapy in connection to the t(11;14) biomarker, in MM and AL-amyloidosis. In parallel, we studied the resistance mechanisms of the MM patients harboring the t(11;14) mutation.

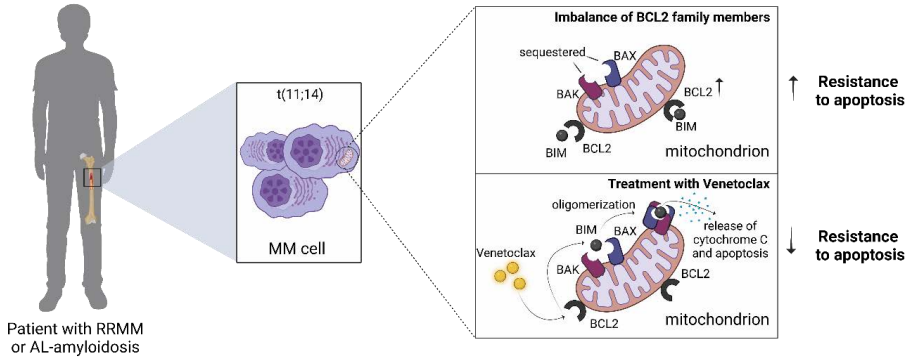

Figure 7: Graphical abstract of **study III.** Image created with BioRender.

4.3.1. Higher ratio of BCL2/ MCL1 at baseline correlates with enhanced response to the selective BCL2 inhibitor Venetoclax

In this clinical trial, 25 patients (17 with RRMM and 8 with AL-amyloidosis) participated after experiencing refractory disease **(Paper III, Table 1)**. Low-dose Venetoclax was administrated orally on a daily basis, in a dose-escalating format, reaching up to 400mg. Results showed a response rate of 33% in patients with MM, versus 71% response in patients with AL-amyloidosis **(Paper III, Fig. 1A)**. In regard to adverse events (AE), although 80% of the treated patients experienced at least one, in all cases they were manageable and no Venetoclax-related deaths were reported **(Paper III, Sup. Fig. 2)**.

The predictive value of BCL2, MCL1 and BCLXL regarding response to Venetoclax was also evaluated. Indeed, higher BCL2/MCL1 and BCL2/BCLXL ratios at diagnosis and relapse correlated with a better response **(Paper III, Fig. 1C,D)**, highlighting the importance of protein expression assessment before or during the course of the treatment.

4.3.2 The interferon regulatory factor 5 (IRF5) is implicated in resistance mechanisms of MM patients harboring the t(11;14)

To investigate the resistance mechanisms of the MM patients harboring the t(11;14) mutation, RNAseq was performed in samples from a refractory cohort of 12 patients with NDMM and RRMM. Results showed that IRF5, a transcription factor regulating interferon genes, was enriched in the downregulated genes of RRMM but not NDMM samples **(Paper III, Fig. 2)**. Given the fact that IRF5 is a direct target of the P53 tumor suppressor gene, it is plausible that IRF5 has oncogenic activities. Furthermore, the implication of IRF5 in the regulation of inflammatory response and cytokine (IL-6, IL-12, IL-33 and TNF) production implies negative modulation of the inflammatory status of the malignant cells which subsequently may lead to an additional evasion mechanism.

5. CONCLUSIONS

The studies presented in this thesis aim to investigate novel treatment options for MM. Study I explored the feasibility of αCD38-CAR-NK therapy, study II focused on the potential of PD1-CSR-NK cell therapy, while study III assessed the safety and efficacy of Venetoclax as monotherapy in patients with MM and AL-amyloidosis harboring the t(11;14) mutation. The conclusions from each individual study are summarized below:

Study I

- CD38 downregulation, occurring during NK cell expansion by long-term IL-2 stimulation, can be used as an alternative to CD38 KO strategies in CD38-targeting NK cell-based approaches.
- The combination of an affinity-optimized αCD38-CAR with *ex vivo* expanded $CD38^{dim}$ NK cells increased the cytotoxic potential of NK cells against CD38+ MM cell lines *in vitro*.
- αCD38-CAR-NK cells generated from PBMCs of patients refractory to Dara treatment showed improved degranulation and cytokine release against autologous BM-derived MNCs.

Study II

- PD1-CSRs encoding for NK cell-related intracellular activation domains of NKp46, DAP10 and DAP12 can be generated and stably expressed on NK92 cells.
- Two of the six generated CSRs, namely $PD1_{ECTM}DAP12_{IC}$ and $PD1_{ECTM}DAP10_{IC}$, showed potential in enhancing NK92 and primary NK cell anti-PD-L1 activity in *in vitro* 2D and 3D experimental models.
- In an *in vitro* autologous MM study, $PD1_{ECTM}DAP12_{IC}$ and $PD1_{ECTM}DAP10_{IC}$ -expressing NK cells had increased activation against BM-derived MNCs.

Study III

- Daily administration of low-dose Venetoclax was well-tolerated by RRMM and AL-amyloidosis patients.
- The treatment resulted in a response rate of 33% among patients with RRMM and 71% in patients with AL-amyloidosis.
- A higher ratio of BCL2/ MCL1 at baseline correlates with enhanced response to the selective BCL2 inhibitor Venetoclax, in patients with RRMM and RR AL-amyloidosis.
- IRF5 is implicated in the resistance mechanisms of MM patients harboring the t(11;14) mutation.

Overall, the results of the individual studies demonstrate that NK cells hold great promise as a therapeutic tool in the fight against multiple myeloma and emphasize the need for tailored treatment strategies that take into account individual patient characteristics.

6. POINTS OF PERSPECTIVE

MM is an incurable plasma cell malignancy from which over 160.000 people are diagnosed per year worldwide[279]. Although MM incidence has risen over the last decades, the 5-year survival has more than doubled. This is greatly thanks to the introduction of novel targeted treatments that have shown unprecedented clinical responses and significantly prolonged remission periods. However, the need for continuing these efforts remains[10]. This thesis discussed three targeted approaches, two of which rely on NK cell-based therapy and one on chemotherapy, with the hope to add to the general knowledge and contribute to the expansion of treatment options for poly-refractory patients.

NK cell therapies have a strong rationale for use in cancer treatment, as they exploit a highly cytotoxic effector lymphocyte, suitable for allogeneic approaches and able to recognize transformed cells without prior sensitization. Moreover, their functionality can be further augmented by the introduction of stably expressed chimeric receptors. Despite the theoretic appeal, adapting CAR or CSR-based approaches from T to NK cells was challenging in the early years. Today, one by one, the hurdles of NK cell therapy are overcome. Viral transduction and genetic engineering of NK cells have been optimized, adequate amounts of NK cells for therapeutic dosing are routinely reached[174,186,280] and *in vivo* persistence of cells has been improved by cytokine support or genetic manipulation[184]. In addition, accumulative evidence suggests that NK cell-based therapeutics are safe in a clinical setting and induce potent anti-tumor effects leading to remission in hematological malignancies[184].

Study I focused on investigating an alternative approach to αCD38-CAR-NK therapy, that bypasses the need for CD38 KO in pNK cells, as previous studies utilized. The discussed methodology takes advantage of the $CD38^{dim}$ phenotype that naturally and universally arises during *ex vivo* expansion. This not only speeds up the development of the cellular therapy by having a simultaneously expanded and $CD38^{dim}$ NK cell source, but also allows expNK cells to benefit from their post-expansion activated phenotype. CD38 is a multifaceted protein, that mediates transendothelial NK cell migration, intracellular calcium mobilization and signaling events. Therefore, we hypothesized that finding a balance between maintaining these functions and diminishing the CAR-mediated fratricide is optimal. Although our study did not directly compare CD38 KO, $CD38^{dim}$ and CD38+ NK cells, in terms of functionality and metabolic fitness, we proved in well-controlled *in vitro* assays that the use of $CD38^{dim}$ cells is feasible in this context. The next steps should focus on performing further validating studies, incorporating PBMCs from treatment-naïve patients as the starting material and assessing the use of lentiviruses for acquiring higher CAR transduction efficacies in patient expNK cells. In addition, shedding light on the mechanism behind CD38 downregulation in our expansion protocol is of great interest.

Study II explored the potential of PD1-CSR-NK cell therapy in an *in vitro* setting, by employing NK cell-like CSR designs. The topic of this study is controversial, as the benefit-to-risk ratio of PD-1/PD-L1 targeting is debated in MM. The study paid special attention to the safety concerns implicated in targeting the axis, by using the natural PD1 ectodomain and a single intracellular activating domain. This allows for the generation of CSRs with

reduced immunogenicity that have less chances of inducing NK cell over-activation in case of recognition of PD-Ls in normal tissue, compared to constructs with additional co-stimulatory domains. Although two of the CSRs, namely $PD1_{ECTM}DAP12_{IC}$ and $PD1_{ECTM}DAP10_{IC}$, showed clear potential against PD-L1+ tumor cell lines, further experiments are necessary to prove the same in an autologous MM setting. Specifically, sorting of the CSR^{high} NK cell population prior to the experiment and having a larger cohort of MM samples would enable better validation of the approach. Further studies should also focus on the combination of CSR+ NK cells with monoclonal antibodies, taken by our encouraging results with Rituximab. The results from the ongoing clinical trials are long awaited in order to see the clinical potential of CSRs..

Study III investigated the safety and efficacy of daily, low-dose Venetoclax in patients with RRMM and AL-amyloidosis. Based on previous findings suggesting that Venetoclax treatment was beneficial in patients harboring the t(11;14) mutation, a cohort of only t(11;14) bearing patients was selected. This allowed for a specific investigation of the potential of Venetoclax as a stratified approach. In terms of the dose, 400mg Venetoclax/daily was administrated. This is half in comparison to the dose in the BELLINI trial that reported higher mortality rate in the Venetoclax-treated arm[274]. Our trial showed that the treatment was well-tolerated and no Venetoclax-related mortalities were observed. Collectively, our results from study III show the potential of Venetoclax in the treatment of t(11;14)-positive RR patients and underscore the need for using molecular markers to predict response to treatment. However, not all patients responded, despite having t(11;14) mutation. Given the heterogeneity of the cytogenetic aberrations and the signatures that myeloma clones acquire because of them, it is possible that t(11;14) alone is not the exclusive mutation we should look into. Future directions should include further elucidation of resistance mechanisms and investigations on the optimal Venetoclax-including regimen to increase responses. In line with this, the results from the phase III trial CANOVA (NCT03539744) comparing Venetoclax-Dexamethasone with Pomalidomide-Dexamethasone, in t(11;14) patients with RRMM, are highly anticipated[281].

In conclusion, given the high relapse rate of MM, further improvement of the myeloma armamentarium with effective novel treatments is warranted. The present thesis investigates targeted immunotherapies and tailored treatments in MM with the aim to contribute to further extension of the available options.

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