Using a multi-omics approach to investigate the reversal of proteasome inhibitor drug resistance in multiple myeloma with epigenetic inhibitors

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Abstract

Background: Multiple myeloma (MM) is an incurable cancer of plasma cells. Novel therapeutics, including proteasome inhibitors (PI) and immunomodulatory imide drugs, have almost doubled median survival time of MM patients. However, most patients relapse and become resistant to drugs they previously have been treated with. Acquired anti-cancer drug resistance remains one of the biggest barriers in the treatment of myeloma.

Aims: PI resistance mechanisms in MM will be investigated with the aim of reversing the resistance phenotype, making MM cells sensitive to proteasome inhibition. Standardised robust wet-lab and computational single-cell workflows will be established to characterise drug-resistant MM cells and their surrounding microenvironment at different points in disease progression.

Results: Cured cancer mate

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List of Abbreviations

MM . . . . . Multiple Myeloma

BM . . . . . Bone marrow

MGUS . . . . Monoclonal gammopathy of unknown significance

SMM . . . . . Smoldering multiple myeloma

PI . . . . . Proteasome inhibitor

IMiDs . . . . . Immunomodulatory imide drugs

ER . . . . . Endoplasmic reticulum

UPS . . . . . Ubiquitin proteasome system

UPR . . . . . Unfolded protein response

RNA-Seq . . . Ribonucleic acid sequencing

scRNA-Seq . . Single cell RNA-Seq

dscRNA-Seq . Droplet-based scRNA-Seq

CB . . . . . Cellular barcode

UMI . . . . . Unique molecular identifier

WGS . . . . . Whole genome sequencing

1 Introduction

1.1 Overview

Multiple myeloma accounts for 1-2% of all cancers and has the second highest incidence of hematological malignancies, after non-Hodgkin's lymphoma [1].

1.2 The adaptive immune system

Humans are exposed to millions of potential pathogens every day and therefore require defences to be able to protect themselves against infection. These defences can be innate or adaptive. An example of an innate defence is the skin acting as a physical barrier between the outside world and the body. Another example of an innate defence is non-specific engulfing (phagocytosis) of foreign pathogens by macrophages (a type of white blood cell). Innate responses are relied upon as the first line of defence, however sometimes a more sophisticated, specialised response is required- called the adaptive immune response. (REF-mol biology of the cell).

Adaptive immune responses are specific to the pathogen that induced the response and are dependent on B cells and T cells, two major classes of lymphocytes (a class of white blood cell). Two classes of adaptive immune responses exist: antibody responses, co-ordinated by B cells, and cell mediated immune responses, co-ordinated by T cells. T-cell-mediated immune responses recognise foreign antigens (antibody generators; substances capable of eliciting an immune response by stimulating B or T cell activation) on the surface of cells and can either kill

the pathogen-infected cells or stimulate B cells or phagocytes to help eliminate the pathogen. In antibody responses, B cells and plasma cells secrete antibodies, also known as immunoglobulins. Immunoglobulins are large Y-shaped proteins, which recognise and bind to the specific foreign antigen on the pathogen which stimulated their production. Binding of immunoglobulins to antigens renders the virus or microbial toxin inactive as it blocks their ability to bind to host cells. Additionally, antibody binding makes it easier for phagocytic cells to ingest the pathogen.

1.2.1 Plasma cells

Plasma cell development

Stem cells are precursor cells which can give rise to at least one type of differentiated (mature) cell, with the capability of indefinite self-renewal. Hematopoietic stem cells (HSC) are stem cells that give rise to all the cells of the hematopoietic system. Two predominant cell populations are produced by HSCs: the common myeloid progenitor (CMP) and the common lymphocyte progenitor (CLP). CMP differentiation produces erythrocytes (red blood cells), mast cells, monocytes, macrophages, neutrophils, eosinophils, basophils and myeloid dendritic cells. CLP differentiation results in B cells, T cells, natural killer (NK) cells and lymphoid dendritic cells.

Most B cells die in the bone marrow soon after developing, however some will develop in the bone marrow, where initial stages of maturation occur and then migrate to secondary lymphoid organs, such as the spleen. Within secondary lymphoid organs, numerous critical decisions on B cell fate are made, involving complex transcriptional networks, cell interactions, gene rearrangements, and mutations (roth2014tracking; jourdan2011characterization). Terminally differentiated plasma cells are the final effectors of the B cell lineage, each dedicated to secreting large amounts of a single type of antibody. Plasma cells have an extensive rough endoplasmic reticulum (ER), and have numerous genes involved in immunoglobulin secretion upregulated, including XBP-1 and CHOP (shapiro2004plasma), to enable the production of the copious amounts of antibody required.

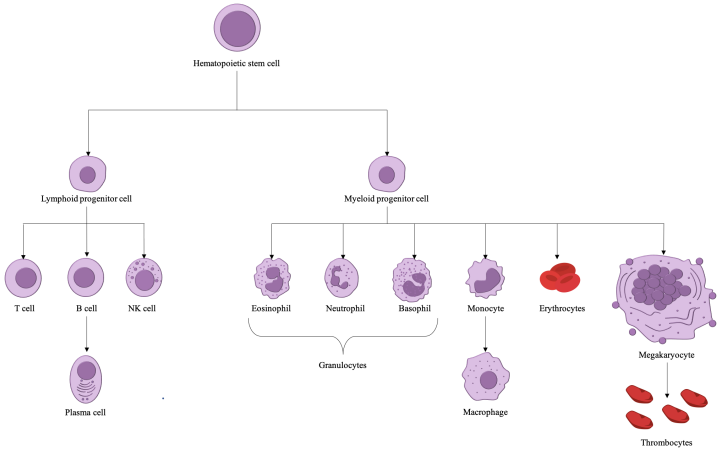

Figure 1.1: Hematopoietic stem cell (HSC) cell differentiation. HSCs divide into myeloid or lymphoid progenitor cells. Dendritic cells and a number of precursor states have been ommitted.

1.3 Clinical MM

1.3.1 Epidemiology

Multiple myeloma accounts for 1-2% of all cancers and has the second highest incidence of hematological malignancies, after non-Hodgkin's lymphoma[1]. MM is rare in individuals under the age of 40, with the average age at time of diagnosis centering around 70[2, 3]. MM is more prevalent in males than females and is around twice as common in black populations than in Caucasian or Asian populations[4]. The average incidence rate is approximately 1-6 cases per 100000 individuals[2, 3, 5], with the highest age-standardised incidence rates in the regions of Australasia, North America, and Western Europe[6]. Five-year survival rate of MM patients is approximately 49%, whilst approximately a third of MM patients survive ten years or greater[7, 8].

1.3.2 Presentation

Precursor states

All cases of MM are preceded by asymptomatic precursor states, monoclonal gammopathy of unknown significance (MGUS) and smoldering multiple myeloma (SMM). However, only some patients with SMM or MGUS progress to active MM.

MGUS is a pre-malignant condition where patients have the presence of monoclonal immunoglobulins in their blood or urine, <10% clonal plasma cells in their bone marrow, but lack any myeloma-related end-organ damage[9]. Patients with SMM have between 10 and 60% clonal plasma cells in their bone marrow, serum monoclonal immunoglobulin of ≥3 g/dL, and like MGUS, have no signs of end-organ damage[10]. Progression risk of MGUS into symptomatic MM is about 1% per year, whilst progression risk of SMM to MM is higher, at around 10% per year for the first 5 years, after which it decreases[11, 12].

Active MM

There are multiple classifications of active MM. The International Myeloma Working Group's definition[13] is as follows: Greater than 10% clonal plasma cells located in the bone marrow and one or more myeloma-defining event or biomarker of malignancy. Myeloma defining events consist of evidence of end-organ damage that can be attributed to the surplus of M protein and clonal plasma cells, namely the CRAB features:

- Hypercalcemia
  - Serum calcium > 1 mg/dL higher than the upper limit of normal, or
  - Serum calcium > 11 mg/dL
- Renal insufficiency
  - Creatinine clearance < 40 mL per min, or
  - Serum creatine > 2 mg/dL
- Anemia
  - Hemoglobin value of > 20 g/L below the lower limit of normal, or
  - Hemoglobin value < 100 g/L
- Bone lesions

– One or more osteolytic lesions on skeletal radiography, CT or PET-CT

Biomarkers of malignancy include greater than or equalt to 60% clonal plasma cells in the bone marrow, an involved:uninvolved serum free light chain ratio greater than or equal to 100, and more than one focal lesion on an MRI study[13].

It is currently unclear what causes the malignant transformation between precursor states and active MM. However certain factors have been identified as risk factors, including point mutations, a large array of up-regulated transcription factors, and numerous immune events.

1.3.3 Treatment of multiple myeloma

Multiple myeloma may be an incurable disease, however it is treatable. In fact, in the last decade median survival time for newly diagnosed MM patients has almost doubled[14]. Novel therapeutic advances have contributed to this improvement (Table1.1).

1.3.4 Proteasome inhibitors

The ubiquitin-proteasome system

1.4 Drug resistance in multiple myeloma

1.5 Transcriptomics, Epigenomics

1.6

Year	Treatment	Usage	Ref
1958	Melphalan	The alkylating agent melphalan was first used in plasma cell myeloma in 1958.	[15]
1960s	Corticosteroids	Placebo-controlled double-blind trial of prednisone in multiple myeloma. Combinations of prednisone and melphalan showed an increased survival over melphalan alone. Dexamethasone and prednisone have become a cornerstone in the treatment of multiple myeloma.	[16, 17]
1980s	Stem-cell transplantations	Numerous successful allogenic and autologous bone marrow transplantations in patients with multiple myeloma	[18–21]
2003	Proteasome inhibitors	Bortezomib, a first-in-class proteasome inhibitor, was first approved by the FDA for use in relapsed and refractory multiple myeloma. In 2008 it was approved for patients with no prior treatment. Carfilzomib was approved in 2012 for advanced MM and later in 2015 for treatment of relapsed MM. The oral proteasome inhibitor, ixazomib, was approved as a combination treatment with lenalidomide and dexamethasone in 2016 for people who have received at least one previous treatment.	[22–24]
2006	IMiDs	The antitumour activity of thalidomide was demonstrated in 1999, this led to the development of lenalidomide, the first approved immunomodulatory imide drug (IMiD) for use in multiple myeloma. Currently, thalidomide, lenalidomide and pomalidomide are approved for use in multiple myeloma	[25–27]
2015	Monoclonal antibodies	In 2015, daratumumab, an anti-CD38 monoclonal antibody and elotuzumab, an anti-SLAMF7 monoclonal antibody, were approved for MM treatment.	[28, 29]

Table 1.1: Timeline of treatment options for multiple myeloma. Listed by first usage or FDA approval for MM.

2
Background

2.1 Drug resistance in MM

2.1.1 Genomic changes in drug resistant MM

2.1.2 Epigenetic changes in drug resistant MM

3
Methods

3.1 Cell culture

3.1.1 AMO-1 cells

AMO-1 cells, plasma cells from a 64-year old female myeloma patient, were used as a model cell-line for multiple myeloma. Bortezomib and carfilzomib resistant AMO-1 cells were generated and kindly gifted by the Driessen lab (1). Bortezomib, carfilzomib, pomolidimide and bortezomib plus pomolidimide resistant AMO-1 cells were also generated by Dr James Dunford by continual and escalating drug exposure. (WHERE did we get AMO-1 WT cells from, kindly gifted by MARIA???) AMO-1 cells were cultivated in RPMI-1640 medium (WHERE we get it from), supplemented with 10% fetal bovine serum (FBS), 100μg/ml streptomycin and 100 U/ml penicillin (P/S) and 2mM L-glutamine (Invitrogen, UK). Cells were passaged at approximately 1.5-2 million cells per ml (IS THIS THE RIGHT MEASURE??). Media was replaced twice a week.

3.2 Bulk RNA-seq

3.2.1 RNA extraction

RNA was extracted from AMO-1 cells using the Direct-Zol RNA MiniPrep kit (Zymo), following the manufacturer's protocol. The RNA was quantified using a NanoDrop ND-1000 Spectrophotometer, and normalised to 100ng of RNA per sample.

3.2.2 RNA library preparation

NEBNext® UltraTM RNA library prep kit for Illumina® with TruSeq indexes was used to prepare RNA libraries, following the manufacturer's protocol. In short... The molarities of the libraries were determined by electrophoresis on a TapeStation (Agilent).

3.3 Single-cell RNA-seq

3.3.1 Cell encapsulation

Nadia instrument etc...

3.3.2 Library preparation

kit ...

3.4 ATAC maybe

3.4.1 ATAC stuff

3.5 ChIP maybe

3.5.1 ChIP stuff

3.6 Sequencing

Sequencing of the resultant libraries was performed on the NextSeq 500 (Illumina, USA) platform using a paired-end run, according to the manufacturer's instructions.

3.7 Phosphoproteomics

3.7.1 Cell lysis

Approximately 20 million cells for each condition in triplicate were pelleted and stored in 500μl of PBS at -80°C. 300μl of fresh lysis buffer (10ml RIPA buffer, 3μl benzonase, 1 tablet phos stop) was added to each pellet, vortexed and left

for 10 minutes on ice and then sonicated. The supernatant was transferred to a fresh (WHAT TYPE) tube.

3.7.2 Protein quantification

Protein concentrations were determined by BCA protein assay (Thermofisher, UK). 400μg of protein was taken from each sample. Samples were made up to a volume of 200μl with MilliQ-H_2O.

3.7.3 Protein Digestion

Kessler lab protocols were followed (`https://www.tdi.ox.ac.uk/research/research/tdi-mass-spectrometry-laboratory/mass-spectrometry/protocols-and-tools`). The lysed samples were reduced with 5%μl of 200 mM DTT in 0.1 M Tris buffer and incubated for 40 minutes at room temperature. The reduced samples were alkylated with 20μl of 200mM iodoacetamide in 0.1M Tris buffer, vortexed and then incubated for 45 minutes in the dark at room temperature. The protein was precipitated using methanol/chloroform extraction. The alkylated samples were transferred to 2ml eppendorfs. 600μl of methanol was added to each sample, followed by 150μl of chloroform and then vortexed gently. 450μl of MilliQ-H_2O was then added and vortexed gently. The samples were centrifuged at maximum speed on a table top centrifuge for one minute. The upper aqueous phase was removed, without disturbing the precipitate at the interface. 450μl of methanol was added to each sample, without disturbing the disc and centrifuged for two minutes. Protein pellets were resuspended, one sample at a time: the supernatant was removed and 100μl of 6M urea in 0.1M Tris buffer was added. The samples were vortexed and then sonicated (???). Samples were diluted with 500μl MilliQ-H_2O, to ensure the final urea concentration was below 1M. Porcine trypsin (Sequencing Grade Modified Trypsin; Promega) was added in a 1:50 ratio of enzyme:total protein content of sample, such that 40μl of trypsin solution containing 8μg trypsin in 0.1M Tris buffer was added to each sample. Samples were left to digest overnight at 37°C in an incubator shaker.

3.7.4 Peptide purification

The following day, the reaction was stopped, acidifying samples to 1% Trifluoroacetic acid (TFA). Samples were desalted and concentrated using 1ml C-18 Sep-Pak (Waters) cartridges. Two reagents were used: solution A (98% MilliQ-H_2O, 2% Acetonitrile (CH_3CN) and 0.1% TFA) for washing and solution B (65% Acetonitrile, 35% MilliQ-H_2O and 0.1% TFA) for activation and elution. The columns were flushed with 1ml of solution B and then washed with 1ml of solution A. The digested samples were added to the columns and vacuumed through slowly. Two 1ml washes with solution A were performed. Fresh, labelled eppendorfs were placed beneath the columns and peptides were eluted with 500μl of solution B. For phosphopeptide-enrichment, 90% of the peptides were removed for Immobilized Metal Affinity Chromatography (IMAC) on a Bravo Automated Liquid Handling Platform (Agilent). 10% of the peptides were used for total proteome analysis. Eluted peptides were dried using a vacuum concentrator (Speedvac, Eppendorf) and stored at -20°C until analysis by mass spectrometry (MS). Prior to MS analysis, dried peptides were resuspended in solution A.

3.8 Ubiquitinomics

3.8.1 Cell lysis

Approximately 100 million cells for each condition in triplicate were pelleted and stored in 500μl of PBS at -80°C. PMTScan Ubiquitin Remnant Motif Kit (K-ε-GG; Cell signalling) was used, following the manufacturer's protocol (REF). Pellets were solubilized and denatured in 4ml urea lysis buffer (20mM HEPES, pH 8.0, 9M urea, 1mM sodium orthovanadate, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate). The lysates were sonicated on ice, with two bursts of 15 seconds with a one minute break in-between.

3.8.2 Protein quantification

Protein concentrations were determined by BCA protein assay (Thermofisher, UK). All samples were found to contain between 10mg and 20mg of protein, so all of the available protein was used, with no normalisation.

3.8.3 Protein Digestion

Lysates were reduced using dithiothreitol (DTT) at a final concentration of 4.5 mM for 30 minutes at room temperature. The reduced samples were alkylated using iodoacetamide (100mM final) for 15 minutes in the dark at room temperature. The alkylated samples were diluted four-fold with 20mM HEPES (pH 8.0) and digested with 400μl trypsin solution, containing 1mg/ml trypsin-TPCK (Worthington, LS003744) in 1mM HCl. Samples were left to digest overnight at room temperature on a rotator.

3.8.4 Peptide purification

The following day, the reaction was stopped, acidifying samples to 1% Trifluoroacetic acid (TFA). Samples were desalted and concentrated using 10ml C-18 Sep-Pak (Waters) cartridges. The columns were activated using 5ml of solution B, washed with 10ml of solution A. The samples were added to the columns and ran through slowly. The peptides were washed with 10ml of solution A. The cartridges were then removed from the vacuum and the peptides were eluted into fresh falcon tubes with 6ml of solution B, using the plunger of the syringes. 20μg of digested protein was removed from each sample for matching total proteome analysis. The eluate was kept at -80°C overnight. The frozen peptide solutions were lyophilized for two days and then stored at -80°C.

3.8.5 Immunoaffinity purification

10X immunoaffinity purification (IAP) buffer provided with PTMScan Kit was diluted to 1X concentration with MilliQ-H_2O. Resuspended purified peptides pellets in 1.4ml of IAP buffer by pipetting up and down and transferred to 1.7ml eppendorfs.

The samples were centrifuged at 4°C for 5 minutes at 10000xg and kept on ice whilst preparing antibody beads. The anti-body bead slurry was centrifuged (30 seconds at 2000xg) and 1ml of PBS was added and then centrifuged. The supernatant was removed and the antibody beads were washed a further four times with PBS and resuspended in 40μl of PBS. The peptide solution was transferred to the antibody vial and the solution was incubated on a rotator for two hours at 4°C. The samples were centrifuged, put on ice and the supernatant was removed. The beads were washed twice with 1ml IAP, followed by three washes with 1ml chilled HLPC water. Immunoprecipitated material was eluted at room temperature in 55μl and 50μl 0.15% TFA in water, letting the sample stand for 10 minutes after each elution, with gentle mixing every two-three minutes. The eluates were centrifuged and the supernatant was transferred to new tubes. Peptide material was desalted and concentrated using 1ml C-18 Sep-Pak cartridges as above. Prior to mass spectrometry analysis, purified GlyGly-modified peptide eluates and matching proteome material were dried by vacuum centrifugation, and re-suspended in solution A.

3.9 Liquid-chromatography-tandem mass spectrometry

Liquid-chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using a Dionex Ultimate 3000 nano-ultra high pressure reverse-phase chromatography coupled on-line to an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) (REF: adan's 3-5 dropbox). In brief, samples were separated on an EASY-Spray PepMap RSLC C18 column (500 mm × 75μm, 2μm particle size; Thermo Scientific) over a 60 min (120 min in the case of the matching proteome) gradient of 2–35% acetonitrile in 5% dimethyl sulfoxide (DMSO), 0.1% formic acid at 250 nl/min. MS1 scans were acquired at a resolution of 60000 at m/z 200 and the top 12 most abundant precursor ions were selected for high collision dissociation (HCD) fragmentation.

3.10 Data Processing

3.10.1 Bulk RNA-Seq

Fasta files were processed using a CGAT-flow (REF) pipeline, workflow can be found at: `https://github.com/cgat-developers/cgat-flow/blob/master/cgatpipelines/tools/pipeline_rnaseqdiffexpression.py`. Pseudo-alignment tool, Kallisto (REF), was implemented to pseudo-align reads to the reference human genome sequence (GRCH38 (hg38) assembly) and to construct a counts matrix of samples against transcripts (/GENES??). DESeq2 (REFERENCE) was used for differential expression analysis of the generated counts matrix (using negative binomial generalized linear models) within the R statistical framework (v3.5.1).

3.10.2 Single-cell RNA-Seq

Pipeline etc...

3.10.3 LC-MS/MS

Mass-spectrometry raw data were searched against the UniProtKB human sequence data base and label-free quantitation (LFQ) was performed using MaxQuant Software (v1.5.5.1). Digestion was set to trypsin/P. Search parameters were set to include carbamidomethyl (C) as a fixed modification, oxidation (M), deamidation (NQ), and phosphorylation (STY) as variable modifications. A maximum of 2 missed cleavages were allowed for phosphoproteome analysis and 3 for the GlyGly peptidome analysis, with matching between runs. LFQ quantitation was performed using unique peptides only. Label-free interaction data analysis was performed using Perseus (v1.6.0.2). Results were exported to Microsoft Office Excel Sheets and imported into the R statistical framework (v3.5.1) for further analysis.

3.11 CyTOF

Get data off ADAM

3.11.1 CyTOF stuff

Appendices

A
Epigenetic compound screen

A compound screen consisting of approximately 140 epigenetic inhibitors was performed for AMO-1 cells.

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