Interrogating the Ikaros Axis and MM Heterogeneity in IMiD resistance

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IMiD resistance in Multiple Myeloma

Multiple myeloma (MM) is a plasma cell malignancy that afflicts more than 30,000 individuals each year and is the second most prevalent adult hematologic cancer (USA). Treatment options for MM have significantly improved over the past two decades, with the emergence of modern and potent anti-MM drugs. However, MM is a largely incurable disease, with nearly all patients enduring a relapse-remitting disease course. Drug resistance also increases with relapses, making it more difficult to treat patients using currently available agents. **Our goal is to overcome drug resistance by assessing what existent drugs patients will respond to and identifying resistance mechanisms that can be therapeutically targeted.**

Immunomodulatory drugs (IMiDs) are a cornerstone of MM therapy. IMiDs modulate the specificity of the E3 ubiquitin ligase receptor Cereblon; Upon IMID binding, Cereblon strongly binds the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) and tags them for proteasomal degradation (Figure 2). This leads to the downregulation of IKZF1 and IKZF3 downstream targets IRF4 and MYC (collectively termed the "Ikaros axis"). The overall downregulation of the Ikaros axis leads to MM cell cytotoxicity. Although resistance mechanisms driven by Cereblon abnormalities have been discovered in MM cell lines, the functional consequences have not been investigated in patient samples. **Our hypothesis is that IMiD treatment fails to downregulate the Ikaros axis in IMiD-resistant patient MM.**

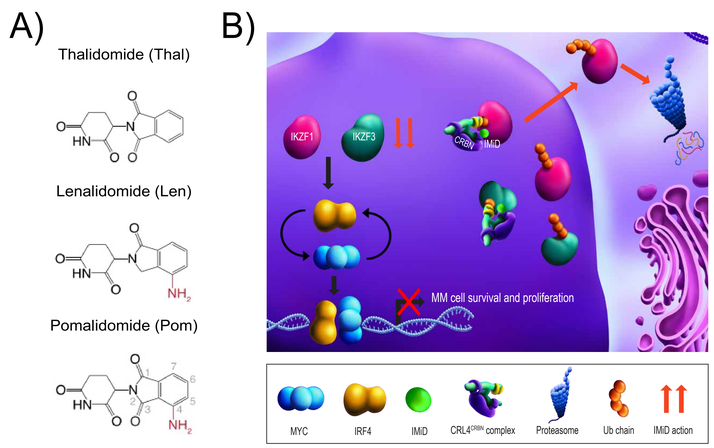
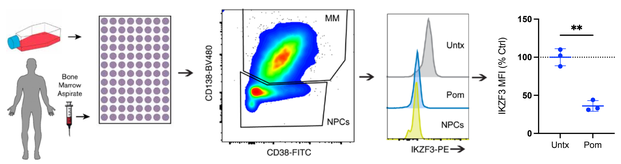


Figure 1. (A) Clinically available IMiDs. Thalidomide (Thal) is a first generation IMiD, lenalidomide (Len) is second generation, and pomalidomide (Pom) is the third generation (combined structure of Thal and Len). In terms of IKZF1 and IKZF3 degradation by Cereblon, pomalidomide is the most potent. (B) Schematic of IMiD mechanism targeting the Ikaros axis in a Cereblon-dependent manner. When bound to an IMiD, Cereblon binds and tags IKZF1 and IKZF3 for proteasomal degradation (right). IKZF1 and IKZF3 degradation leads to the gene downregulation of IRF4 and MYC in MM cells (left).

Cytometry based approaches

Intracellular flow cytometry to measure IMiD-induced Ikaros axis degradation
An intracellular flow cytrometry staining assay was performed on MM cell lines and patient samples for IKZF1, IKZF3, IRF4, and MYC. MM cell line or patient samples were treated with 10 μM Pom for 24 (IKZF1/IKZF3) or 48 hours (IRF4/MYC) before being fixed and permeabilized. The relative IKZF1, IKZF3, IRF4, and MYC protein levels in CD38+CD138+ MM cells were assessed by normalizing the geometric mean (gMFI) to untreated controls. *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001



Assessing ex vivo drug response in MM cell lines and patient samples
MM cell lines were treated with 10uM Pom for 96 hours and relative cell viability was assessed by Cell TiterGlo. Mononuclear cells (MNCs) from patient bone marrow biopsies were Ficoll-separated and cryopreserved previously. Patient samples were treated with the indicated drug concentration for 48hr and assessed for MM cell viability via flow cytometry using a panel of MM markers (CD38, CD138, CD46, BCMA, FCRL5). Technical triplicates.

Mass cytometry to interrogate Ikaros axis expression in MM subpopulations
MNCs from patient BM biopsies were thawed and cells were stained for a panel of MM and immune cell surface markers, as well as light chains (κ/λ), IKZF1/3, IRF4, MYC, proliferative and apoptotic markers, and live/dead cell stain. The prepared mass cytometry samples were run on the Helios mass cytometer and analyzed in R (N=4).

IMiD resistant cell lines lose Ikaros axis downregulation, but the IMiD mechanism remains intact in resistant patients

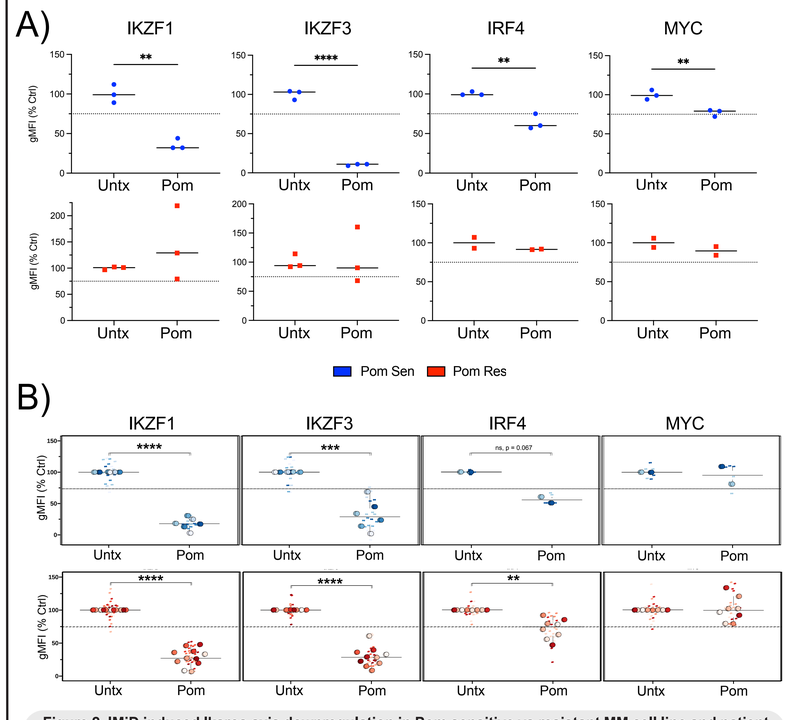


Figure 2. IMiD induced Ikaros axis downregulation in Pom sensitive vs resistant MM cell line and patient samples. (A) Geometric mean fluorescence intensity (gMFI) of IKZF1, IKZF3, IRF4, and MYC in parental (sensitive, blue) and a pom dose escalated MM1S cells (resistant, red) relative to the untreated controls. Each dot is a technical replicate. Unpaired t-test. (B) Superplots showing the same flow assay as for (A), but in patient samples gated on CD38+CD138+ MM cells. Each larger circle with a border is the mean of a patient sample, with each individual shade of blue or red corresponding to a patient sample across graphs. The smaller dots of the same shade are the corresponding techincal replicates. Paired t-tests.

Ikaros axis proteins are heterogeneously expressed between different MM

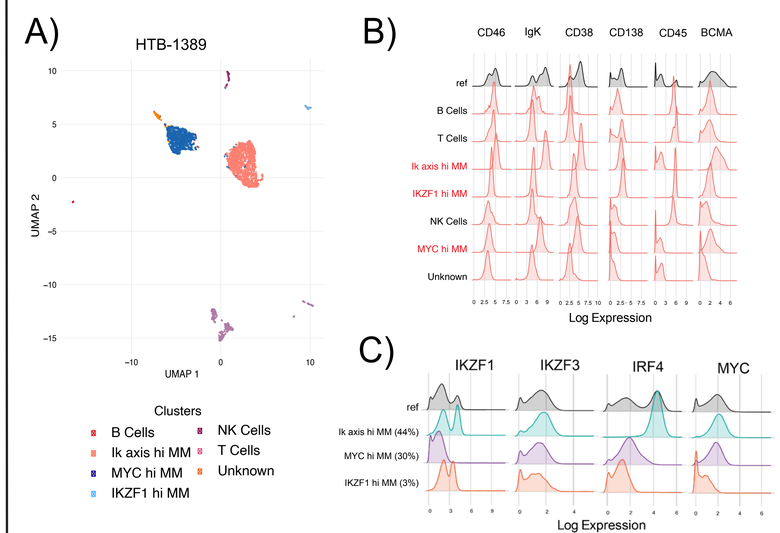


Figure 3. Mass cytometry of Ikaros axis in a patient sample. (A) UMAP of patient sample HTB-1389 showing three MM populations, as indicated by cluster color legend. (B) Histograms of every cluster showing the log mean signal intensity (MSI) of MM markers used to classify MM populations (red text). Ref = all live cells; defines high versus low expression. (C) Histograms of Ikaros axis protein log MSI in MM subpopulations.

IKZF1 low MM cells are associated with IMiD resistance

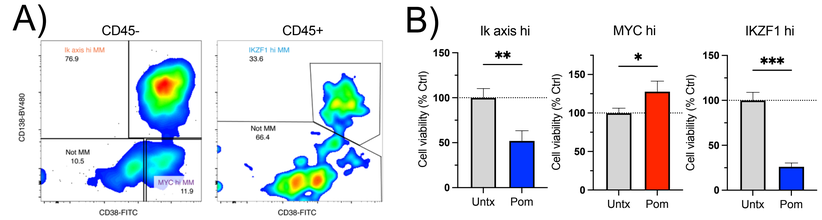


Figure 4. Ex vivo Pom sensitivity in HTB-1389 MM subpopulations. (A) Representative biaxial plots of CD38 and CD138 in live cells that are either CD45+ or CD45- in an untreated replicate. Based on mass cytometry data, Ik axis hi is CD38+CD138+, MYC hi is CD45-CD38+, and IKZF1 hi is CD45+CD38+CD138+. This gating strategy was used to obtain (B). (B) The sensitivity of MM populations to 10uM Pom treatment relative to untreated control. Unpaired T-tests.

IMiD resistant MM is sensitive to MYC inhibition

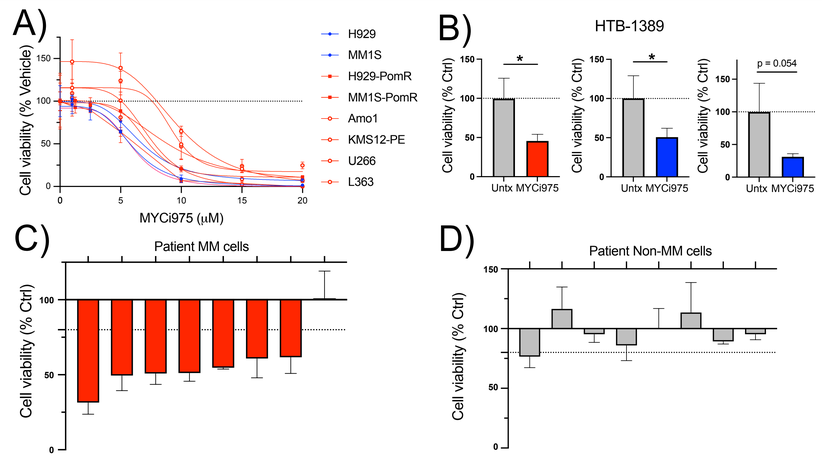


Figure 5. Ex vivo MYC inhibitor sensitivity of IMiD resistant MM. (A) Dose response curve of IMiD sensitive and resistant cell lines to MYCi975. (B) Relative viability of MM subpopulations from Fig. 4 treated with 1uM MYCi975. (C) Waterfall plot showing the relative viability of IMiD resistant MM cells in patient samples treated with 1 uM MYCi975. (D) Effects of MYCi975 on non-MM (normal) cells from the patient MM shown in (C). Unpaired T-tests.

Conclusions

-While Pom resistant cell lines lose the ability to downregulate the Ikaros axis, this resistance mechanism has not been observed in our patient cohort
  -IMiD mechanism of action intact in resistant cells

-Ikaros axis proteins are heterogeneously expressed in MM intratumoral subpopulations, and low IKZF1 expression may be associated with resistance

-IMiD resistant MM subpopulations still express MYC, and IMiD resistant MM is sensitive to MYC inhibition

Continuing to explore IMiD resistance

In progress:
-CyTOF of IMiD resistant patients
-Assess IKZF1/3 degradation specifically in patients

Future directions:
-Determine the necessity of individual Ikaros axis proteins in resistant patient MM cells by siRNA
-Synergy of IMiDs and MYCi975 - Re-sensitize to IMiDs?
-Single-cell RNA-seq of resistant patient samples - gene expression programs driving MYC and phenotypes of resistant MM subpopulations

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