**Interrogating the Ikaros Axis and MM Heterogeneity in IMiD resistance**

Lorraine N. Davis1,2, Zachary J. Walker1,2, Brett M. Stevens1,2, Denis Ohlstrom3, Peter A. Forsberg1,2, Tomer M. Mark1,2, Daniel W. Sherbenou1,2

1Division of Hematology, University of Colorado Cancer Center, 2Department of Medicine, University of Colorado Anschutz Medical Campus, 3Emory University School of Medicine

**Imidazolone 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 eventually experiencing relapse during disease course. Anti-MM drug resistance also includes relapses, making it more difficult to treat patients using currently available agents. Our goal is to overcome drug resistance by assessing what existing 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 1A). 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 of these have not been investigated in patient samples. Our hypothesis is that IMiD treatment fails to downregulate the Ikaros axis in MM-resistant patient MM.

**Cytochemistry based approaches**

**Intracellular flow cytometry to measure IMiD-induced Ikaros axis degradation**

An intracellular flow cytometry staining assay was performed on MM cell lines and patient samples for IKZF1, IKZF3, IRF4, and MYC. MM cell lines or patient samples were treated with 10 μM Pom for 24 hours (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.

**Assessing ex vivo drug response in MM cell lines and patient samples**

MM cell lines were treated with 10μM Pom for 96 hours and relative cell viability was assessed by Cell TiterGlo. Mononuclear cells (MCNs) from patient bone marrow biopsies were Ficoll-separated and cryopreserved previously. Patient samples were treated with the indicated drug concentration for 96 hours and assessed for MM cell viability via flow cytometry using a panel of MM markers (CD38, CD138, CD46, BCMA, FCR5). Technical triplicates.

**Mass cytometry to interrogate Ikaros axis expression in MM subpopulations**

MCNs 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 (NH+).

**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 -IMiD mechanism of action intact in resistant cells

**Continuing to explore IMiD resistance**

-In progress:

-CTOF of IMiD resistant patients

-Expression of IKZF1/3 degradation specifically in patients

-Future directions:

-Determine the necessity of individual Ikaros axis proteins in resistant patient MM cells by siRNA

-Determine the necessity of individual Ikaros axis proteins in resistant patient MM cells by siRNA

**Acknowledgments**

We would like to thank Gwendolyn Tice for helping create the illustration for Figure 1

This work was supported by:

-OC139 T32 GM008745 (LND)

-RNA Bioscience Initiative Graduate Scholars Award (LND) K08CA222704 (DWS)

**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, IKZF3, IRF4, and MYC, MM cell line or patient samples were treated with 10 μM Pom for 24 hours (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.

**Figure 2.** Ikaros induced Ikara 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 patient (violet, blue) and a pom dose esacelated 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 small dot representing a patient sample across-genome. The smaller dots of the same shade are the corresponding technical replicates. Patient treats.

**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 (not red). Red = all live cells; defines high versus low expression. (C) Histogram of Ikaros axis protein log MSI in MM subpopulations.

**Figure 4.** Ex vivo Pom sensitivity in HTB-1389 MM subpopulations. (A) Representative plots of CD38 and CD138 in the cell lines that are either CD45+ or CD45- in an unmutated replicate. B) Relative viability of MM subpopulations from Fig. 4. Treated with 1μM MYCi975. (C) Waterfall plot showing the relative viability of IMiD resistant MM cell lines in patient samples treated with 1μM MYCi975. (D) Effects of MYCi975 on normal MM (normal) cells from the patient MM shown in (C). Unpaired T-tests.

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