Interrogating the Ikaros Axis and MM Heterogeneity in IMiD resistance

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Drug Resistance in Multiple Myeloma

Multiple myeloma (MM or myeloma) is a malignant plasma cell neoplasm that afflicts more than 30,000 Americans each year and is the second most prevalent adult hematologic malignancy[1]. Over the last decade its incidences have continued to increase, with an estimated 12,960 deaths occurring this year[2]. MM is characterized by the infiltration and rampant clonal proliferation of plasma cells and the overproduction of immunoglobulin (Ig)-like M-proteins in the bone marrow. Treatment options for MM have significantly improved over the past two decades, with the emergence of modern and potent anti-myeloma drugs increasing the average life expectancy to 7-8 years. However, this remains an largely incurable disease, as nearly all patients will eventually relapse and develop drug or multidrug resistance[3]. This acquired drug resistance, leading to relapse/refractory disease, is the root cause of treatment failure and remains the greatest obstacle to successfully curing MM.

Immunomodulatory drugs (IMiDs) are a cornerstone of MM therapy. IMiDs are a pleiotropic drug class with several mechanisms of action. Most importantly, IMiDs possess direct anti-tumor effects by promoting the proteasomal degradation of critical MM transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) (Figure 2)[4,5]. Although most patients initially respond, the majority of patients exposed will become resistant or cross-resistant to IMiDs[3], and the mechanisms of acquired IMiD resistance remain largely unknown. **Based on the known mechanism of action in IMiDs, we have investigated whether the Ikaros axis shows a differential response to IMiD treatment in patients with IMiD resistance.**

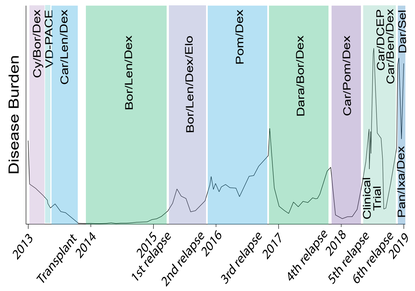

Figure 1. Disease course of Patient 614. Bottom axis is time and vertical axis is free light chain (FLC), indicative of disease burden. Colored boxes are labeled with treatment for the corresponding period of time. Revlimid (R)=lenalidomide (len), Pomalyst (Pom)=pomalidomide, Cy=cyclophosphamide, Bor (V)=bortezomib, D=dexamethasone, PACE=chemo, Elo=elotuzumab, Dara/Dar=daratumumab, Car=carfilzomib, Cis=cisplatin, DCEP=chemo+Dex, Pan=panobinostat, Sel=selinexor.

IMiD Mechanism of Action

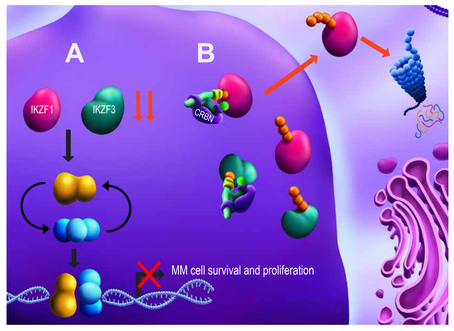

Figure 2. Myeloma Ikaros axis and IMiD mechanism of action. (A) Ikaros (IKZF1) and Aiolos (IKZF3) promote multiple myeloma survival and proliferation through inducing the upregulation of IRF4, which in turn upregulated c-MYC (MYC) to form a positive autoregulatory feedback loop. In IMiD-sensitive cells, this axis is necessary for MM proliferation and survival. (B) IMiDs mediate and promote the degradation of IKZF1 and IKZF3 by modulating the conformation of the substrate specificity receptor Cereblon (CRBN) in the $CUL4^{CRBN}$ E3-ubiquintin ligase.This in cooperation with an E2-ubiquitin ligase results in efficient proteasomal degradation of IKZF1 and IKZF3 and subsequent downregulation of IRF4 and MYC, leading to MM cell death.

Methods

MM cell line and primary MM sample drug treatment
For cell line viability, MM cell lines were treated with the indicated IMiD concentrations for 96 hours and relative live cell count with assess by flow cytometry. Mononuclear cells (MNCs) from patient bone marrow biopsies were Ficoll-separated and cryopreserved previously. Patients are shown by identification number. All samples were treated (Tx) with 10 μM IMiD (Len or Pom) for 24 (IKZF1/3) or 48 hours (IRF4/MYC) *ex vivo*. IMiD sensitivity was classified by *ex vivo* Myeloma Drug Sensitivity test[3] on $CD38^{+}CD138^{-/+}$ cells (My-DST, Sherbenou lab). Cell lines were plated at $9x10^5$ cells/well and primaries at $25x10^5$ cells/well. All conditions were performed in triplicate.

Measuring Ikaros pathway via intracellular flow cytometry
An intracellular flow cytrometry staining assay was performed on MM cell lines and patient samples for IKZF1, IKZF3, IRF4, and MYC. Samples were fixed and permeabilized with FoxP3 transcription factor kit. Patient samples were also stained for CD38, CD138 and κ or λ light chain (LC). Ikaros axis and MYC protein levels were analyzed in FlowJo by geometric mean (MFI) and normalized to untreated. P values were determined using unpaired t-test or one-way ANOVA multiple comparisons in Graphpad Prism 8.

Mass cytometry
MNCs from patient BM biopsies were thawed and cells were stained for a panel of MM and immune cell surface markers, as well as LCs, IKZF1/3, IRF4, MYC, proliferative, and apoptotic intracellular markers. Prepared CyTOF sample were run on the Helios mass cytometer. Data was analyzed by viSNE in Cytobank and median signal intensity histograms were generated in FlowJo.v9.

IMiD cross-resistance is prevalent in MM cell lines

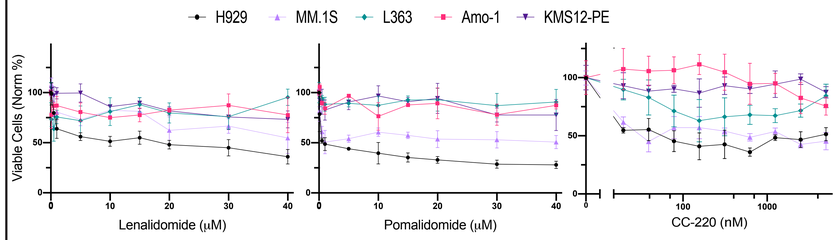

Figure 3. IMiD sensitivity in MM cell lines. Five MM cell lines were treated with increasing doses of three different IMiDs for 96 hours. MM.1S and H929 were laregly sensitive to all IMiDs, while Amo1, L363, and KMS12-PE were largely insensitive to all IMiDs tested.

Alterations in the Ikaros axis that affect the IMiD MOA are not found in IMiD-resistant MM

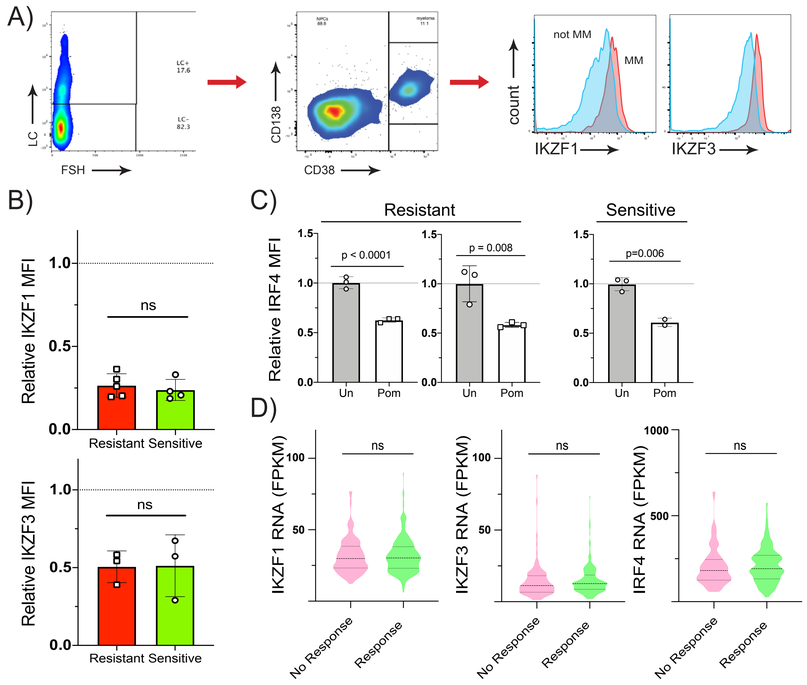

Figure 4. Ikaros axis IMiD response and expression in primary MM patients. (A) Representative gating scheme of live cells primary samples. (B) Relative protein expression of IKZF1 or IKZF3 following 24hr 10 μM Pom treatment. Each dot represents an individual patient sample. Each is relative to its own respective untreated control. (C) Relative IRF4 expression in primary samples after 48hr 10 μM Pom treatment. Each dot represents a technical replicate, and relative MFI is calculated compared to respective untreated controls. Resistant = HTB-646.1, HTB-634.3. Sensitive = HTB-656.1 (D) IKZF1, IKZF3, and IRF4 normalized RNA expression data from bulk RNA-sequencing in patients enrolled in the MMRF CoMMpass research study. No response was classified as a patient who received either Len or Pom and had progressive disease or relapsed, whereas a responsive patient completed their regimen succesfully. RNA-sequencing data was processed by MMRF Research Gateway consortium using Cufflinks in R.

MYC expression is maintained in IMiD-resistant MM cell lines and primary samples

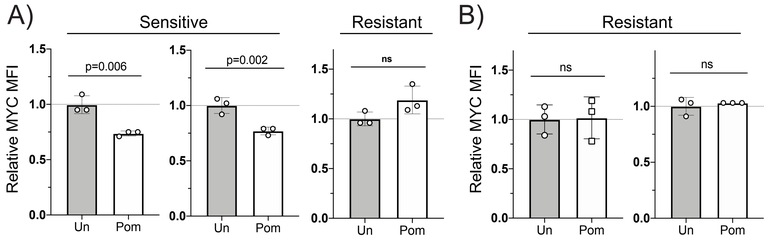

Figure 5. MYC protein is decreased in response to IMiDs in IMiD-sensitive MM cell lines but not IMiD-resistant cell lines or primary samples. Cell lines (A) or primary samples (B) were treated with 10 μM Pom for 48hr. Relative MYC protein was determined by flow cytometry and normalized to untreated control. Each point represents a technical replicate. (A) Sensitive = MM.1S, OPM2. Resistant = Amo-1. (B) Resistant = HTB-646.1 and HTB-634.3.

The Ikaros Axis is expressed in more PC-like MM cells, while MYC is expressed in all MM cells

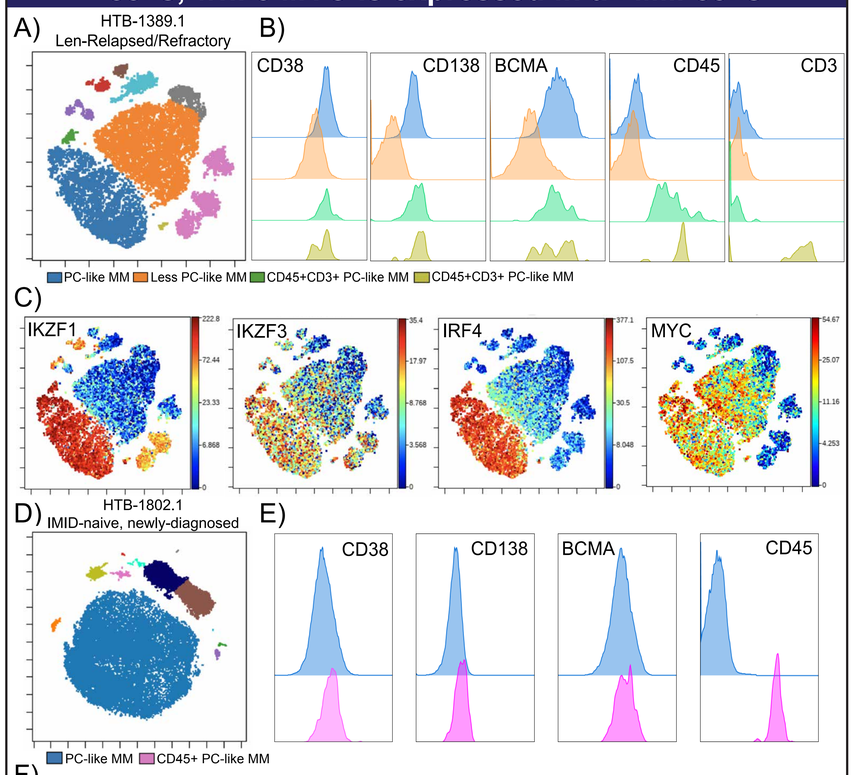

Figure 6. CyTOF analysis of two plasma cell leukemia (PCL) patients. (A) Overlaid viSNE of all live MNCs in Len-relapsed/refractory PCL patient with MM clusters denoted below. (B) Histograms of MM clusters in (A) to illustrate how populations were classified. (C) Heatmap of IKZF1, IKZF3, IRF4, and MYC in different clusters with scales indicated on the right. (D, E, F) Same as (A, B, C) but for an IMiD-naive, newly diagnosed PCL patient.

Conclusions and Future Directions

- IKZF1 degradation in response to IMiDs is retained in IMiD-resistant patients and IKZF3 response does not differ between sensitive and resistant patients.
- IRF4 is also equivalently downregulated between IMiD-sensitive and -resistant cell lines and patient samples
  - Collectively suggesting that the major mechanisms of resistance occur downstream of these proteins or through alternate pathways.
- Greater heterogeneity is present in a relapsed/refractory len-treated PCL patient (HTB-1389.1) compared to a treatment naive PCL patient (HTB-1802.1).
- Within the Len R/R PCL patient, a less plasma cell-like MM population is predominant, and interestingly this corresponds to the loss of the Ikaros axis expression while maintaining MYC expression.
  - Suggesting that *less PC-like MM subpopulations* may emerge in IMiD-exposed patients and be responsible for IMiD resistance through losing dependence on PC-related transcriptional pathways like the Ikaros axis.

Ongoing Work & Future Directions:

- ☐ Mass cytometry of IMiD-resistant patients
- ☐ Determine necessity of Ikaros axis and PC genes in IMiD-resistant primary samples
- ☐ Single-cell RNA-sequencing of IMiD-resistant primary samples
- ☐ *in vivo* models of acquired IMiD resistance for longitudinal studies

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1. Kumar. *Nat Rev Dis Primers* (2017). 2. Siegel et al. *CA Cancer J Clin* (2020) 3. Walker, VanWyngarden et al. *Blood Adv* (2020) 4. Kronke et al. *Science* (2014) 5. Lu et al. *Science* (2014)