Interrogating the Ikaros Pathway in Multiple Myeloma IMiD Drug Resistance

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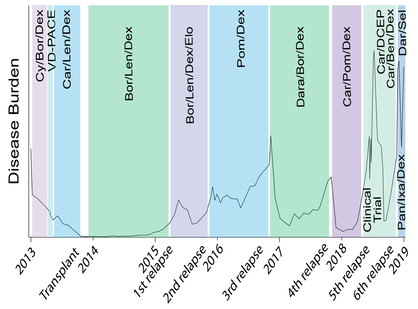
Drug Resistance in Multiple Myeloma

- Multiple myeloma (MM or myeloma) is a malignant plasma cell neoplasm
  - Second most prevalent adult hematologic malignancy[1]
- MM remains an incurable disease, as nearly all patients will eventually relapse and develop drug or multidrug resistance[2]
- Acquired drug resistance, leading to relapse/refractory disease, is the root cause of treatment failure and remains the greatest obstacle to successfully curing MM
- The IMiDs lenalidomide (Len) and pomalidomide (Pom) are frequently included in MM treatment regimens

- Patients exposed to IMiDs eventually become resistant, and the mechanisms of acquired resistance remain largely unknown
- IMiDs exert direct anti-myeloma effects by promoting the proteasomal degradation of critical MM transcription factors Ikaros (IKZF1) and Aiolos (IKZF3)[3]

Based on the known mechanism of action in IMiDs, we have begun to investigate whether the Ikaros pathway shows a differential response to IMiD treatment in patients with acquired IMiD resistance.



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

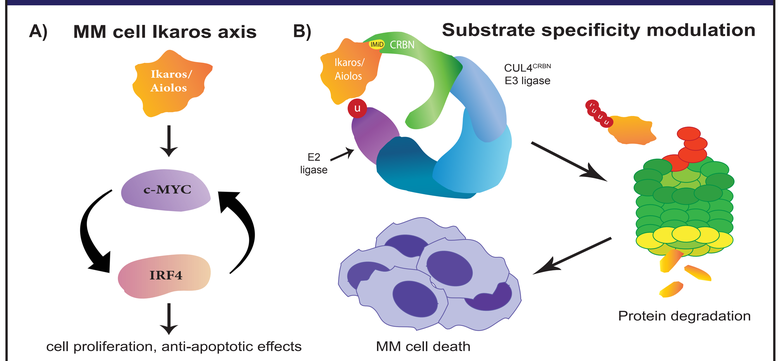


Figure 2. Myeloma Ikaros axis and IMiD mechanism of action. (A) Ikaros (IKZF1) and Aiolos (IKZF3) promote multiple myeloma survival and proliferation through inducing the upregulation of a c-Myc/IRF4 autoregulatory positive feedback loop. (B) IMiDs mediate and promote the degradation of IKZF1 and IKZF3 by modulating the conformation of the substrate specificity receptor, Cereblon, 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

Methods

Myeloma cell line and primary multiple myeloma sample drug treatment
Mononuclear cells from patient bone marrow aspirates were Ficoll-separated and cryopreserved. Patients are shown by identification number. All samples were treated (Tx) with 10 μM IMiD (Len or Pom) for 24 hours *ex vivo* unless otherwise indicated. IMiD sensitivity was classified by *ex vivo* Myeloma Drug Sensitivity test (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 staining assay was performed on MM cell lines and patient samples for IKZF1, IKZF3, IRF4, and c-MYC on the BD FacsCelesta. Samples were fixed and permeabilized with FoxP3 transcription factor kit. Patient samples included CD38 and CD138 as myeloma markers. IKZF pathway protein levels were analyzed in FlowJo by geometric mean (MFI) and normalized to untreated. P values were determined using 2-way ANOVA multiple comparisons in Graphpad Prism 8 (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns = not significant).

Mass cytometry
Patient 1389.2 bone marrow was thawed and treated with 10 μM Len for 24 hrs. Cells were stained for a panel of myeloma and immune cell markers, IKZF, proliferative, and apoptotic intracellular markers. Prepared CyTOF sample was sent to University of Rochester Medical Center. Data was analyzed by viSNE in Cytobank and CyTOF median in FlowJo.

Ikaros Pathway Degradation in IMiD-sensitive and -resistant Myeloma

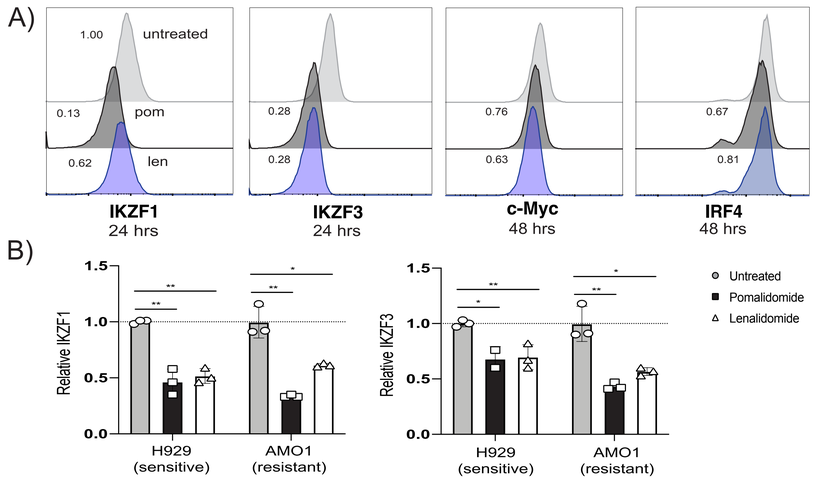


Figure 3. Detection of Ikaros pathway response to IMiDs in MM cell lines. (A) Histograms of optimal Ikaros response detection timepoint in Amo-1 cells. All numbers indicate fraction abundance compared to untreated control (untreated = 1.00). (B) H929 (IMiD-sen) versus Amo-1 (IMiD-res) IKZF1 and IKZF3 at 24 hr post-Tx.

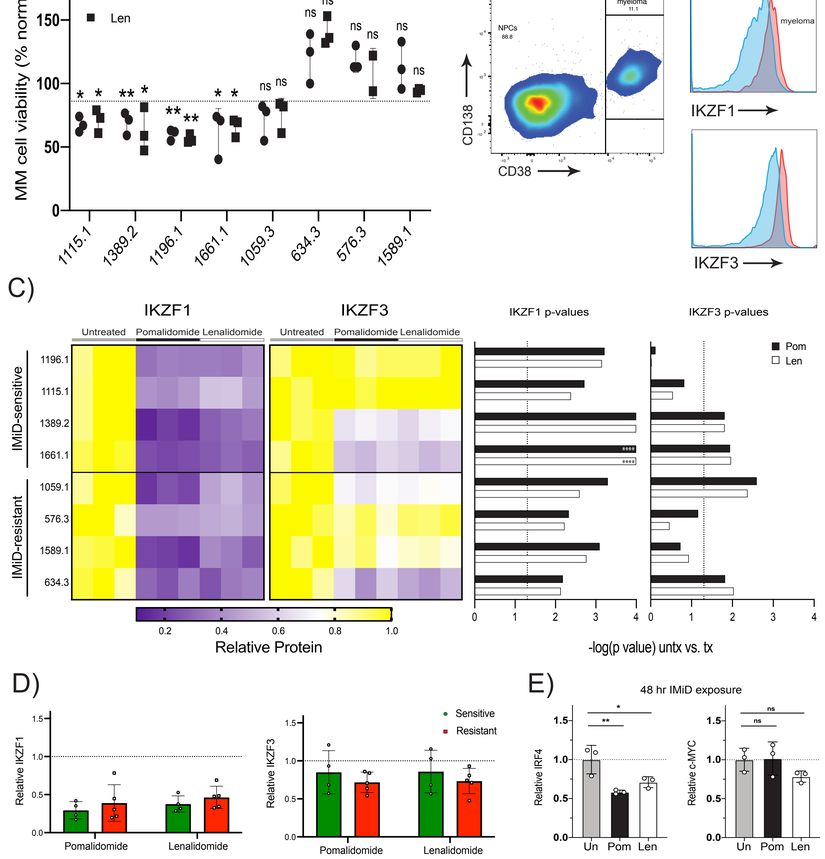


Figure 4. Ikaros pathway response to IMiDs in primary MM patients. (A) *Ex vivo* IMiD sensitivity of patient samples relative to respective untreated controls (untreated = 100%). Samples with a significant decrease in MM cell viability were classified as IMiD-sensitive and no significant viability decrease were IMiD-resistant. (B) Gating strategy of myeloma cells from mononuclear cells in primary samples. NPC= non-plasma cells. Representative sample is patient 576.3. (C) Left: Relative IKZF1 and IKZF3 protein in 8 patients post-IMiD Tx. Right: Statistical significance of IKZF1/3 decrease. -log(p values) to right of dashed line are significant. ****-log(p value) > 4. (D) Samples from (C) were grouped by IMiD sensitivity. (E) Relative IRF4 and c-MYC protein in patient 634.3 (IMiD-resistant) at 48 hrs post-IMiD Tx.

Phenotyping and Ikaros Detection via CyTOF

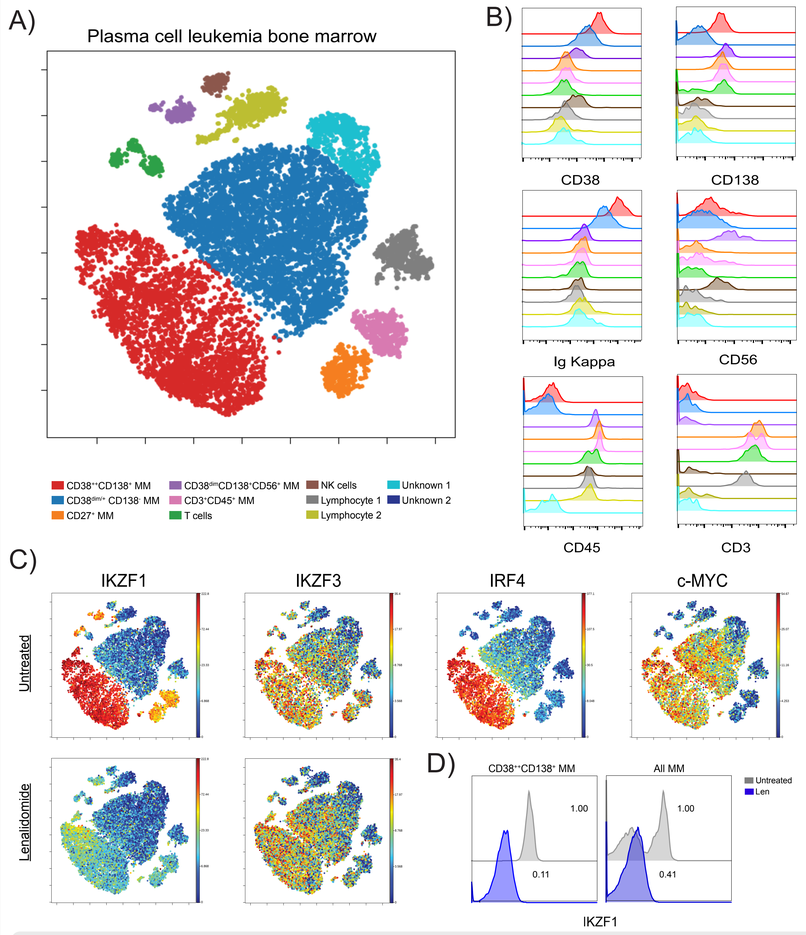


Figure 5. CyTOF analysis of patient 1389.2 (plasma cell leukemia - IMiD-sensitive) bone marrow. (A) Overlaid viSNE plot of cell populations present in sample. (B) Histograms of surface markers used to characterize cell populations. Populations are given by colored labels in (A). (C) Ikaros pathway expression viSNE plot heatmaps of IKZF1 and IKZF3 in untreated (top) vs Len-treated (bottom), as well as IRF4 and c-MYC in untreated. IKZF marker expression is highest in CD38^{++}CD138^{++} myeloma population. (D) Histograms of relative IKZF1 decrease in CD38^{++}CD138^{+} MM cluster (red) and in all MM clusters (red, blue, orange, purple, pink). Histograms normalized to mode.

Conclusions and Future Directions

- IKZF1/3 degradation can be detected via cytometry-based methods in MM cell lines & patient samples
- IKZF1 degradation is retained in IMiD-resistant patients and IKZF3 degradation does not differ between sensitive and resistant patients
  - major mechanisms of resistance likely occur downstream of IKZF1/3 or through alternate pathways
- In a patient with acquired IMiD resistance, IRF4 was decreased with IMiD Tx while c-MYC remained unchanged - significance of this finding is not yet clear
- CyTOF analysis revealed extensive MM heterogenity, and Ikaros pathway expression and IKZF1/3 degradation is primarily isolated to the more differentiated CD38^{++}CD138^{+} myeloma population
  - could suggest only a subpopulation of MM utilizes or relies on this canonical Ikaros pathway

Ongoing Work & Future Directions:

- ☐ Validate cytometry assays for Ikaros pathway proteins with immunoblot
- ☐ Further assess IRF4 and c-MYC degradation/downregulation in IMiD-sensitive and -resitant patients
- ☐ Investigate Ikaros pathway IMiD response by mass cytometry in IMiD-resistant patients
- ☐ Perform RNA-seq on IMiD-resistant patients to investigate differential transcriptional programs

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