MDSC Effectors of Ineffective Hematopoiesis

Regulation of Innate Immune Response

Somatic Mutations License the Inflammasome

Nlrp3 Inflammasome & Pyroptosis
Pattern Recognition Receptors (PRR) Central to Innate Immune Response

Classical Membrane Anchored PRRs (PAMPs, DAMPs)

Subgroup 1: IL1β

Subgroup 2: TLR

Subgroup 3: Membrane Anchored TLR-3, 7, 8, 9

Subgroup 4: Cytoplasmic NLRs

MYD88, myeloid differentiation primary response protein; TRIF, TIR-domain-containing adapter-inducing interferon-β; MAL (MyD88-adaptor-like protein).
MDS HSPCs are Primed for Response to Innate Immune Signals

MDSC Effectors of Ineffective Hematopoiesis

Regulation of Innate Immune Response

Nlrp3 Inflammasome & Pyroptosis

Somatic Mutations License the Inflammasome
Myeloid-Derived Suppressor Cells (MDSC)

- Immature myeloid cells (IMC)
  - Human: Lin⁻HLA-DR⁻CD33⁺;
  - Mouse: CD11b⁺Gr1⁺ (±B220, CD31)
- Expand with age, infection, chronic inflammation, and neoplasia.
- Induce tumor immune tolerance & T-reg expansion.
- Elaborate multiple soluble effectors: ROS, NO, and arginase; VEGF, TNFα, TGF-β, IFN, IL-6, IL-10 IL-1β & granzyme granules
- MDSC expansion and activation driven by TLR ligands (e.g., DAMP signals)

*DAMP: danger-associated molecular pattern.
MDSC Direct Ineffective Hematopoiesis in MDS

- Medullary MDSC are markedly expanded in LR-MDS and genetically distinct from the malignant clone.
- MDS MDSC suppress autologous hematopoiesis.
- The CD33-SIGLEC3 ITIM signaling receptor is over-expressed by MDS-MDSC & is indispensable for S100A9 induction of inflammatory cytokines.
- S100A9 is a Ca\(^{++}\) binding, proinflammatory myeloid-related protein that binds CD33 & heterodimerizes with S100A8 to engage TLR4 & CD33.
- S100A9 is overexpressed in MDS progenitors with high concentration in MDS BM plasma.
- S100A9-Tg mice develop trilineage dysplasia and pancytopenia that phenocopies human MDS.

*Immunoreceptor tyrosine-based inhibition motif (ITIM); Sialic Acid-binding Ig-Type Lectin*
S100A8/9-TLR4 Signaling Drives Mesenchymal Inflammation-induced Genotoxic Stress & Erythroid Death

Cell Extrinsic

Cell Intrinsic

Regulation of Innate Immune Response

Nlrp3 Inflammasome & Pyroptosis

MDSC Effectors of Ineffective Hematopoiesis

Somatic Mutations License the Inflammasome
Supramolecular Organizing Centers (SMOCs)

Innate Immune Signaling Modules

**MyDDosome**
- Cytokines
- TLR/IL1R
- MYD88
- IRAK4
- IRAK2
- TRAF6
- Regulated Cell Death
- Glycolysis

**Inflammasome**
- Cytokines
- NLRP3
- ASC
- Pyroptosis
- Autophagy
- Pro-caspase 1

Type I Interferon

**NLRP Inflammasomes**

- Nucleotide-binding & oligomerization domain (NOD)-like receptor proteins (NLRP) are cytosolic PRRs that respond to danger signals to trigger inflammasome (IFM) formation.
- NLRP3 (NALP3 or cryopyrin) forms IFM complex by associating with ASC adaptor, which recruits Pro-Caspase-1 through its CARD domain.
- Caspase-1 undergoes autocatalytic processing to yield two subunits that form the active caspase cleaving pro-IL-1 & -18, and gasdermin-D (pyroptosis).

Pyroptosis: Caspase-1 Dependent Inflammatory Cell Death

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Apoptosis</th>
<th>Pyroptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysis</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cation channel activation</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Nuclear condensation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PS externalization</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inflammasome assembly</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Caspase-1 activation</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Caspase-3 activation</td>
<td>+</td>
<td>late</td>
</tr>
<tr>
<td>Inflammatory cytokines</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

PS denotes Phosphatidylserine
**NLRP3 Inflammasome (IFM) Priming & Activation**

**Signal 1: Priming**
- S100A9
- TLR4/S100A9 & DAMPs
- IL-1R1/IL-1β
- TNFR/TNFα
- IFNAR/IFN-α, IFN-β

**Signal 2: Activation**
- Nlrp3 (CIAS1)
- IL-1β
- ASC (PYCARD)
- Caspase-1 (CASP-1)

Functional Priming by PTM
- phosphorylation
- deubiquitylation
- desumoylation

**Signal sensing by NLRP3**
(Ox-mtDNA, NEK7, PKR, TXNIP, cathepsin-B)

**Cation/Ca2+ influx**
(vimentin digestion → cytoskeletal collapse & osmotic swelling)

**NLRP3 anchorage to mitochondrial MAVS**

**NLRP3 IFM assembly & caspase-1 activation**

**Caspase-1 substrate cleavage**
(IL1β, IL-18, gasdermin D, GATA1)

**Inflammatory cytokine release**

**Pore formation & pyroptotic rupture**
Primary MDS Bone Marrow Progenitors Display NLRP3 Inflammasome Activation


Plasma ASC Specks are a Pyroptosis Biomarker in MDS \([n=249]\)

Log10 \% ASC Specks Normalized to Glucose

\[P = 7.70 \times 10^{-35}\]

\[P = 4.81 \times 10^{-39}\]

\[P = 1.21 \times 10^{-14}\]

ROC/AUC = 0.918
Specificity 0.89, Sensitivity 0.87 at 0.93 cutoff.


ROC: receiver operating characteristic (ROC), AUC, area under the curve.
Flow Cytometric Assessment of Pyroptotic Versus Apoptotic Cell Death

Heparinized BM Aspirate
- MDS
- Normal donors

Ficoll-Hypaque Plus Gradient Centrifugation

Lineage Gating:
- MSC (CD45-CD105+)
- Endothelial cells (CD31+)
- Osteoblasts (CD34-OCN+)

Annexin-V+

Plasma
- MNC
- Ficoll
- RBC/PMN

Apoptotic Pyroptosis execution

Early

a-Caspase-1

a-Caspase-3
Functional Dependence on Pyroptosis vs. Apoptosis in MDS

**Mean % Pyroptotic Cells**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean percentage of pyroptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td><strong>9.0</strong> ± 59.2</td>
</tr>
<tr>
<td>MDS</td>
<td><strong>64.1</strong> ± 44.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean percentage of pyroptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+CD38-</td>
<td>10.9 ± 45.3</td>
</tr>
<tr>
<td>CD34+CD38+</td>
<td>9.6 ± 46.7</td>
</tr>
<tr>
<td>CD33+</td>
<td>9.0 ± 59.2</td>
</tr>
<tr>
<td>CD71+</td>
<td>64.1 ± 44.5</td>
</tr>
</tbody>
</table>

**Pyroptotic vs. Apoptotic Fraction**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean percentage of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+CD38-</td>
<td>45.3 ± 3.1</td>
</tr>
<tr>
<td>CD34+CD38+</td>
<td>46.7 ± 4.8</td>
</tr>
<tr>
<td>CD33+</td>
<td>59.2 ± 2.7</td>
</tr>
<tr>
<td>CD71+</td>
<td>44.5 ± 3.7</td>
</tr>
</tbody>
</table>

Apoptotic: cleaved Caspase-3+/Annexin V+

**CASP-1, NLRP3 vs. CASP-3 shRNA**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean percentage of pyroptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP3</td>
<td><strong>1.2</strong> ± 0.6</td>
</tr>
<tr>
<td>CASP1</td>
<td><strong>1.0</strong> ± 0.5</td>
</tr>
<tr>
<td>CASP3</td>
<td><strong>0.8</strong> ± 0.3</td>
</tr>
</tbody>
</table>

Scrambled vs. Target Gene

S100A9 Neutralization Suppresses Pyroptosis & Improves CFC in LR-MDS BM Specimens

**aCaspase-1 MFI**
Normalized to Plasma Treated Control

**IL-1β MFI**

**Colony-Forming Capacity**

**Erythroid** (BFU-E/CFU-E)

<table>
<thead>
<tr>
<th></th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.0</td>
</tr>
<tr>
<td>0.1 μg CD33-IgG</td>
<td>2.9</td>
</tr>
<tr>
<td>0.5 μg CD33-IgG</td>
<td>3.5</td>
</tr>
<tr>
<td>1 μg CD33-IgG</td>
<td>4.2</td>
</tr>
</tbody>
</table>

**CFU-GM**

<table>
<thead>
<tr>
<th></th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.0</td>
</tr>
<tr>
<td>0.1 μg CD33-IgG</td>
<td>1.8</td>
</tr>
<tr>
<td>0.5 μg CD33-IgG</td>
<td>2.0</td>
</tr>
<tr>
<td>1 μg CD33-IgG</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**CFU-GEMM**

<table>
<thead>
<tr>
<th></th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.0</td>
</tr>
<tr>
<td>0.1 μg CD33-IgG</td>
<td>1.2</td>
</tr>
<tr>
<td>0.5 μg CD33-IgG</td>
<td>1.7</td>
</tr>
<tr>
<td>1 μg CD33-IgG</td>
<td>1.2</td>
</tr>
</tbody>
</table>

NLRP3 IFM Inhibition Improves Hematopoiesis in LR-Risk MDS & S100A9-Tg Mice

Cation Channel Activation Triggers Cell Swelling & NLRP3 Inflammasome Assembly

**ROS-sensitive Ion Channels Promote Cation Influx & Cell Volume Expansion in MDS Precursors**

**Cell Size**

- Normal: Mean Cell Area = 919
- LR: Mean Cell Area = 1,813
- HR: Mean Cell Area = 1,271

*p = 0.0001
*p = 6.2 x 10^{-14}

**Cell Size by Hematopoietic Lineage**

- Ungated
- CD34+CD38-
- CD34+CD38+
- CD33+
- CD71+

Ethidium Bromide Update

- MDS
- Normal

ROS & Nuclear β-Catenin Expression is Increased in LR MDS & Induced by S100A9

MDSC Effectors of Ineffective Hematopoiesis

Regulation of Innate Immune Response

Nlrp3 Inflammasome & Pyroptosis

Somatic Mutations License the Inflammasome
**U2AF1 Splicing Gene Mutations Induce Nuclear β-Catenin Localization via NOX-generated ROS**

**U2AF1** Mutant Cells Display Increased Pyroptosis & Cation Channel Activation

### Mean % Pyroptotic Cells

<table>
<thead>
<tr>
<th></th>
<th>Mean % Pyroptotic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.0</td>
</tr>
<tr>
<td>Q157R</td>
<td>8.5</td>
</tr>
<tr>
<td>S34F</td>
<td>7.3</td>
</tr>
</tbody>
</table>

### Cation Channel Activation over Time

- **Mean % EB⁺ Cells**
  - WT
  - S34F

### Cell Area

- WT
- S34F

---


**Splicing mutations**
- U2AF1
- SF3B1
- SRSF2

**Chromatin remodeling**
- ASXL1

**DNA methylation**
- TET2

**Ribosomopathy**
- RPS14⁺⁻
Pyroptotic Fraction Increases with Somatic Mutant Clone Size & Complexity

% Pyroptotic Erythroids vs. Splicing Mutation VAF


Plasma ASC Specks by Somatic Mutation Number

# Genetic Priming of TLR-Signaling in MDS

<table>
<thead>
<tr>
<th>Genetic Abnormality</th>
<th>Gene Class</th>
<th>Mutant Gene or Chromosome Alteration</th>
<th>Innate Immune Signaling Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Somatic Mutations</strong></td>
<td>Epigenetic Modifiers</td>
<td><em>TET2</em></td>
<td>↓HDAC2 recruitment; ↑IL-6, NF-κB, ↑IL-1β</td>
<td>23, 25, 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>DNMT3A</em></td>
<td>↑HDAC9; ↑Type 1 IFN</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Spliceosomal</td>
<td><em>ASXL1</em></td>
<td>↑NADPH oxidase ROS; ↑TLR4, TICAM2</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>EZH2</em></td>
<td>↑ S100A8/A9</td>
<td>20-21</td>
</tr>
<tr>
<td><strong>Spliceosomal</strong></td>
<td></td>
<td><em>SF3B1</em></td>
<td>↑ Degradation of TLR negative regulator MyD88S</td>
<td>26, 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>SRSF2</em></td>
<td>↑ S100A8/9, DNA-RNA hybrids</td>
<td>20-21; 34, 35</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>U2AF1</em></td>
<td>↑ DNA-RNA hybrids, ATG7 alternate splicing impairing autophagy; IRAK4-L mydosome activation</td>
<td>34-37</td>
</tr>
<tr>
<td><strong>Chromosomal Abnormality</strong></td>
<td>N/A</td>
<td>Deletion 5q</td>
<td>Allelic deletion <em>RPS14</em>: ↑S100A8/A9; <em>miR-145/146</em>: ↑TIFAB; ↑TRAF6, IRAK1</td>
<td>8-10; 19</td>
</tr>
</tbody>
</table>

Sallman & List. BLOOD 2019.
Targeting the IL-1β Signaling Axis in MDS

• IL-1β is the principal inflammatory cytokine generated by the Nlrrp3 inflammasome that has broad biological activities:
  – TLR/myddosome signaling induces inflammatory cytokines (S100A9, TNFα, IL-6), PDL-1, SPY1 (PU.1) and Nlrrp3 inflammasome activation
  – activates β-catenin to induce MYC and MDSC expansion, and chromatin remodeling
  – active caspase 1 cleaves GATA1 to raise the Spi1/GATA1 ratio favoring myeloid commitment, maturation arrest & anemia
  – directs myeloid skewing & immuno-senescence, suppresses late stage erythropoiesis, and decreases release of erythropoietin

• Canakinumab is a fully humanized monoclonal antibody of the IgG1/k isotype that neutralizes IL-1β

• FDA approved & effective in autoinflammatory disorders including CAPS syndromes with activating NLRP3 mutations

Phase Ib/II Study of Canakinumab with Darbepoetin in Patients with LR-MDS who Failed ESAs

**Eligibility:** VL, LR, IR- IPSS-R; ≥1unit RBC x8 wks prior to randomization, ESA failure

**Exclusions:** prior HMA or allo-HCT

**Design:** Phase 1b: 3+3 dose escalation; Phase 2: Simon’s two-stage (Stage 1: enroll 10 at MTD, if > 2 achieve HI-E, Stage 2: enroll additional 19 pts). >6 HI-E, merits further study.

**Primary end-point:** Phase 1b – MTD & RP2D; Phase 2 – IWG 2006 HI-E.
Acknowledgements

Collaborators:
Drs. David Sallman & Rami Komrokji
Dr. Kathy McGraw & Amy McLemore
Drs. Seishi Ogawa & Masashi Sanada
Drs. Benjamin Ebert & Esther Obeng
Dr. Omar Abdel-Wahab

Contact:
Email: dr.alanlist@gmail.com
Cell: 813-521-4630

Specialized Center for Research Program [SCOR]

The Henry & Marilyn Taub Foundation