The Role of Innate Immunity in MDS Pathogenesis

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Tampa, FL
MDSC Effectors of Ineffective Hematopoiesis

Regulation of Innate Immune Response

Nlrp3 Inflammasome & Pyroptosis

Somatic Mutations License the Inflammasome
Pattern Recognition Receptors (PRR) Central to Innate Immune Response

**Classical Membrane Anchored PRRs**

Subgroup 1: IL1β

Subgroup 2: PAMPs, DAMPs

Subgroup 3: Membrane Anchored

Subgroup 4: Cytoplasmic

NLRs

IL1R

TLR-1,2,4,5,6

TLR-3,7,8,9

MAL

MYD88

TRIF

IRA4

IRA1

TRAF6

TAK1

NF-κB signaling

MAPK signaling

inflammasome assembly

caspase-1 activation

IL1β maturation, pyroptosis

MYD88, myeloid differentiation primary response protein; TRIF, TIR-domain-containing adapter-inducing interferon-β; MAL (MyD88-adaptor-like protein).
Supramolecular Organizing Centers (SMOCs)

Innate Immune Signaling Modules

MyDDosome

Cytokines

Regulated Cell Death

Glycolysis

Type I Interferon

Inflammasome

Cytokines

Pyroptosis

Autoptagy

Type I Interferon

MDS HSPCs are Primed for Response to Innate Immune Signals

Excess TLR Ligands (S100A8/9, DAMPs)

Haplodeficiency Induction of DAMPs (Del5q)

RPS14 HSPA9 CSNK1a1

Haplodeficiency of Negative Regulators (Del5q)

Overexpression & IRAK & TRAF6 Activation

MDSC Expansion (TLR4, CD33)

TLR Overexpression (Cell Death & Cytopenias)

Pyroptosis & Ineffective Hematopoiesis

Regulation of Innate Immune Response

Nlrp3 Inflammasome & Pyroptosis

MDSC Effectors of Ineffective Hematopoiesis

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

- Immature myeloid cells (IMC)
  - Human: Lin⁻HLA-DR⁻CD33⁺
  - Mouse: CD11b⁺Gr-1⁺ (±B220, CD31)
- Expand with age, infection, 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.
BM-MDSC are Markedly Expanded in MDS & Genetically Distinct from the Malignant Clone


* MDSC lack both cytogenetic abnormalities & gene mutations intrinsic to the MDS clone
MDS-MDSC Suppress Autologous Hematopoiesis

The CD33-SIGLEC3 ITIM Signaling Receptor is Over-expressed in MDS-MDSC

MDSC CD33 Surface Density

*Immunoreceptor tyrosine-based inhibition motif (ITIM);
Sialic Acid-binding Ig-Type Lectin

Promotes Myeloid Differentiation & Maturation

Blocks Differentiation & Maturation

ITAM Signaling

ITIM Signaling

Activatory signal

Inhibitory signal
S100A9 is the Native Ligand for CD33

CD33-IgG₁ Fc Fusion

CD33 Binds S100A9


Primary BM Specimens
MDS Normal
Human S100A9

- S100-Calcium binding protein A9, also known as migration inhibitory factor-related protein 14 (MRP14) or calgranulin B
- A calcium & zinc binding protein with key role in regulation of inflammation & innate immune response
- Predominant protein in myeloid cells that promotes the membrane assembly & activation of NADPH oxidase
- S100A9 is essential for S100A8 protein (MRP8) stabilization\(^{^\wedge}\) & forms homo- & hetero-dimers with S100A8 (calprotectin)
- S100A9 & calprotectin function as alarmins & ligands for TLR4 & RAGE
- S100A9/8 increases with inflammation, aging in parallel with MDSCs and promotes insulin resistance & atherosclerosis\(^*\).

CD33 is Indispensable for S100A9 Inflammatory Cytokine Induction

Normal donor BM-MNC’s RAGE, TLR4, CD33, or the combination were blocked prior to culturing cells +/- 1 μg S100A9 x 48 H followed by assessment of IL-10 mRNA & protein expression (qPCR – top, ELISA on the bottom).
S100A9 is Increased in Lower Risk MDS BM-MNC & BM Plasma

BM Plasma Concentration by IPSS

S100A9-Tg Mice Develop Trilineage Cytological Dysplasia Phenocopying MDS

A. Hypercellular marrow with megakaryocytic hyperplasia

B. Dysplastic megakaryocytes with single or hypolobation & increased micromegakaryocytes (dwarf megakaryocytes)

C. Hypogranulated and hyposegmented PMNs (pseudo-Pelger-Huet changes) and nuclear budding in erythroid precursors. (All cells are partially degenerated)

D. PAS stain highlights erythroid predominance

S100A8/9-TLR4 Signaling Drives Mesenchymal Inflammation-induced Genotoxic Stress & Erythroid Death

Cell Extrinsic

Cell Intrinsic

MDSC Effectors of Ineffective Hematopoiesis

Regulation of Innate Immune Response

Nlrp3 Inflammasome & Pyroptosis

Somatic Mutations License the Inflammasome
# 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.
NLRP Inflammasomes

- Nucleotide-binding domain & oligomerization domain (NOD)-like receptor proteins (NLRP)
- Family of cytosolic pattern recognition receptors responding to danger signals triggering 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-interleukin-1 & -18

NLRP3 Inflammasome (IFM) Priming & Activation

Signal 1: Priming
- S100A9
- TLR4/S100A9 & DAMPs
- IL-1R1/IL-1β
- TNFR/TNFα
- IFNAR/IFN-α, -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
Osmotic Swelling in S100A9 Treated U937 Cells
Spacial Dynamics of Inflammasome Nucleation

- Mitochondrial (mt) assembly of the IFM complex is initiated at priming.
- TLR-induced ROS externalizes cardiolipin to the MOM which anchors Nlrp3 & casp-1.
- The myddosome induces CMPK2, the rate-limiting enzyme in mtDNA synthesis.
- Newly synthesized mtDNA is oxidized by ROS and liberated.
- Cytosolic & ER Nlrp3 transitions along microtubules to the perinuclear space associating with MAVS bridging to TGN.

MAVS, mitochondrial antiviral signaling protein; CMPK2, Cytidine/Uridine Monophosphate Kinase 2.
Primary MDS Bone Marrow Progenitors Display NLRP3 Inflammasome Activation


Plasma ASC Specks are a Pyroptosis Biomarker in MDS \([n=249]\)
Flow Cytometric Assessment of Pyroptotic Versus Apoptotic Cell Death

**Heparinized BM Aspirate**
- MDS
- Normal donors

**Ficoll-Hypaque Plus Gradient Centrifugation**
- Plasma
- MNC
- Ficoll
- RBC/PMN

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

**Annexin-V+**

**a-Caspase-3**

**a-Caspase-1**

**Apoptotic execution**

**Early**
Functional Dependence on Pyroptosis vs. Apoptosis in MDS

Mean % Pyroptotic Cells

<table>
<thead>
<tr>
<th>CD34+CD38-</th>
<th>CD34+CD38+</th>
<th>CD33+</th>
<th>CD71+</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.94±6.3</td>
<td>9.64±6.7</td>
<td>9.06±9.2</td>
<td>6.1±4.5</td>
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</tbody>
</table>


CASP-1, NLRP3 vs. CASP-3 shRNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Scrambled</th>
<th>Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP3</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>CASP1</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>CASP3</td>
<td>ns</td>
<td></td>
</tr>
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</table>

Pyroptotic vs. Apoptotic Fraction

<table>
<thead>
<tr>
<th>CD34+CD38-</th>
<th>CD34+CD38+</th>
<th>CD33+</th>
<th>CD71+</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.3±3.1</td>
<td>46.7±4.8</td>
<td>59.2±2.7</td>
<td>44.5±3.7</td>
</tr>
</tbody>
</table>
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)**

**CFU-GM**

**CFU-GEH**

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

**CFU-GEMM**

<table>
<thead>
<tr>
<th>Fold Change CFC</th>
<th>Plasma (µM)</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC950</td>
<td></td>
<td>10</td>
<td>5.1</td>
<td>4.6</td>
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</table>

**BFU-E**

<table>
<thead>
<tr>
<th>Fold Change CFC</th>
<th>Plasma (µM)</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC950</td>
<td></td>
<td>10</td>
<td>3.7</td>
<td>4.2</td>
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</table>

**CFU-GM**

<table>
<thead>
<tr>
<th>Fold Change CFC</th>
<th>Plasma (µM)</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC950</td>
<td></td>
<td>10</td>
<td>1.5</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**S100A9-Tg Mice**

- **Hemoglobin**
  - WT: [Graph showing HGB (g/dL) vs Week of treatment]
  - Tg: [Graph showing HGB (g/dL) vs Week of treatment]
  - ICTA: [Graph showing HGB (g/dL) vs Week of treatment]

- **WBC**
  - WT: [Graph showing WBC (10^3/µL) vs Week of treatment]
  - Tg: [Graph showing WBC (10^3/µL) vs Week of treatment]
  - ICTA: [Graph showing WBC (10^3/µL) vs Week of treatment]

- **Platelets**
  - WT: [Graph showing Platelets (10^3/µL) vs Week of treatment]
  - Tg: [Graph showing Platelets (10^3/µL) vs Week of treatment]
  - ICTA: [Graph showing Platelets (10^3/µL) vs Week of treatment]

Cation Channel Activation Triggers Cell Swelling & NLRP3 Inflammasome Assembly

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

Cell Size by Hematopoietic Lineage

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Normal Mean Cell Area</th>
<th>MDS Mean Cell Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR</td>
<td>1,813</td>
<td>1,813</td>
</tr>
<tr>
<td>HR</td>
<td>1,271</td>
<td>1,271</td>
</tr>
</tbody>
</table>

Cell Size

- Mean Cell Area: Normal: 919, LR: 1,813, HR: 1,271
- p = 0.0001
- p = 6.2 x 10^{-14}

Ethidium Bromide Update

- Mean % EB+ Cells: Normal: 10-60, MDS: 10-60
- p = 0.0001
- p = 6.2 x 10^{-14}

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

### % Pyroptotic

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Q157R</th>
<th>S34F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean % Pyroptotic Cells</td>
<td>1.6</td>
<td>8.5</td>
<td>7.3</td>
</tr>
</tbody>
</table>

### Cation Channel Activation over Time

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>S34F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean % EB+ Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minutes</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

### 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>Somatic Mutations</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></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>Spliceosomal</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>Chromosomal Abnormality</td>
<td>N/A</td>
<td>Deletion 5q</td>
<td>Allelic deletion <em>RPS14</em>: ↑S100A8/A9; <em>mir-145/146</em>:+ <em>TIFAB</em>: ↑TRAF6, IRAK1</td>
<td>8-10; 19</td>
</tr>
</tbody>
</table>

Sallman & List. BLOOD 2019.
Interferon Signaling is the Leading Pathway Deregulated in MDS CD34\(^+\) Cells

**Microarray GEP**

<table>
<thead>
<tr>
<th>Inguinity canonical pathway</th>
<th>P-value</th>
<th>Up-regulated molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon signaling</td>
<td>2.57E-06</td>
<td>IFIT3, IFIT1, IFITM1, MX1, IRF9, STAT1</td>
</tr>
<tr>
<td>Thrombopoietin signaling</td>
<td>2.09E-06</td>
<td>PIK3R3, RRAS2, MPL (includes EG:4352), SOS1, STAT1, PRKCZ, PRKCA</td>
</tr>
<tr>
<td>IL-3 signaling</td>
<td>0.00010</td>
<td>PIK3R3, PTPN6, RRAS2, SOS1, STAT1, PRKCZ, PRKCA</td>
</tr>
<tr>
<td>Natural killer cell signaling</td>
<td>0.00019</td>
<td>PIK3R3, FCGR3B, PTPN6, RRAS2, TYROBP, SOS1, PRKCZ, PRKCA</td>
</tr>
<tr>
<td>Function of pattern recognition receptors in recognition of bacteria and viruses</td>
<td>0.00021</td>
<td>PIK3R3, CLEC7A, DDX58, TLR8 (includes EG:51311), C1QC, C1QA, CCL5</td>
</tr>
</tbody>
</table>

- IFN-stimulated genes (ISGs) were the most highly expressed genes in MDS with 15 of 22 known ISGs highly up regulated in CD34\(^+\) cells.
- Analogous GEP in RNASeq datasets of mutant-\textit{U2AF1} [GSE66793] and \textit{Tet2}\(^-\) mice [GSE27816]
dsDNA Sensors Direct Type I Interferon Response

Cytosolic Sensors
- cGAS, IFI16
- DAI, DDX41
- DHX36, DHX9, AIM2

RIG-I like helicases (RLH)

CpG-rich DNA

Endosomal

TLR9

Inflammatory Cytokines
(IL-6, TNFα)

Interferon-stimulated Genes (ISG)

Autoimmune diseases

Nlrp3 inflammasome activation

Candidate Cytosolic DNA DAMPs
- DNA:RNA hybrids
- Micronuclei
- Mitochondrial DNA

Courtesy of Dr. J. Ting, 2018.
Cells were cultured in β-estradiol free media for 24 hours prior to treatment with RU.521.
cGAS Inhibition Suppresses Pyroptosis & Induces Terminal Differentiation in Primary MDS Specimens

CD11b Expression*

<table>
<thead>
<tr>
<th>CD11b frequency of live</th>
<th>Vehicle</th>
<th>1 µM RU.521</th>
<th>10 µM RU.521</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td></td>
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</tbody>
</table>

* Case 1, MDS-RS, ETV6, SF3B1, & TET2 mutations; 2., MDS-MLD, DNMT3 & TET2 mutations; 3., MDS-EB, no NGS data.

Erythroid Differentiation

<table>
<thead>
<tr>
<th>GATA1 fold change</th>
<th>Vehicle</th>
<th>1 µM RU.521</th>
<th>10 µM RU.521</th>
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</thead>
<tbody>
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P=0.05

MDS BM-MNC [n=3]

CD71 Fraction

<table>
<thead>
<tr>
<th>CDA Frequency</th>
<th>Vehicle</th>
<th>1 µM RU.521</th>
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</table>

GATA1 CD71 Fraction

IRF3 Phosphorylation

<table>
<thead>
<tr>
<th>IRF3 Phosphorylation</th>
<th>Vehicle</th>
<th>0.1 µM RU.521</th>
<th>1 µM RU.521</th>
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P=0.05

MDS BM-MNC [n=3]

Caspase-1 Cleavage

<table>
<thead>
<tr>
<th>Caspase-1 Glo-assay</th>
<th>Vehicle</th>
<th>1 µM RU.521</th>
<th>10 µM RU.521</th>
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</thead>
<tbody>
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</table>

* Caspase-1 Glo-assay
Summary: Nlrp3 Inflammasome in MDS

NLRP3 Inflammasome
- ASC speck formation
- Cation influx
- NOX ROS liberation
- Caspase-1 activation
- IL1, IL-18 maturation
- Gasdermin-D cleavage
- Pyroptosis

MDSC

DAMPs
S100A9

Phenotype
- Disease biomarker (IF, FC)
- Cell swelling, macrocytosis
- Nuclear β-catenin, self-renewal
- GATA1 cleavage → anemia
- Inflammatory cytokines
- Membrane pore formation
- Cell lysis

Somatic Gene Mutation

IF, immunoflourescence; FC, flow cytometry.
Strategies for Therapeutic Intervention

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David Sallman, MD

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Drs. Benjamin Ebert & Esther Obeng
Dr. Omar Abdel-Wahab

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