**Targeting The Stress Response Kinase GCN2 To Restore Immunity In The Tumor Microenvironment**

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**Background**

The tumor microenvironment (TME) is characterized by deficiencies in oxygen and key nutrients, such as glucose and amino acids. Stromal cells and myeloid-derived suppressor cells (MDSC) within the tumor create a nutrient-poor environment that inhibits immune function and supports tumor growth. GCN2 (general control non-derepressible 2), a stress response kinase, plays a key role in sensing and modulating the response to amino acid deprivation. GCN2 activation leads to an induction of the integrated stress response pathway in T cells leading to T cell energy and apoptosis. Here, we demonstrate that the pharmacologic inhibition of GCN2 restores the T cell proliferation and effector function in amino-acid deficient media and in MDSC-induced T cell suppression.

Mouse and human T cell viability, proliferation and function were assessed in vitro under amino-acid deprived conditions and in a co-culture with MDSCs. Pharmacodynamic markers including phospho-GCN2, phospho-EIF2α, and ATF4 were measured via western blot. Cell proliferation (CFSE dye dilution) and effector markers (IFNγ and Granzyme B) were measured by flow cytometry. Our selective, sub-μM GCN2 inhibitor (GCN2i) was used to examine the role of GCN2 in T cell and MDSC function.

**GCN2 is an integral part of the integrated stress response**

- A) Amino acid limitation
- B) GCN2
- C) EIF2α
- D) Translation initiation
- E) Translation elongation
- F) Translation termination
- G) Cell cycle

![Image](https://example.com/image1)

A) GCN2 is a stress response kinase detecting amino acid starvation. Activation of GCN2 leads to T cell anergy, apoptosis and enhanced Treg suppression. B) MDSC/TAMs use multiple mechanisms such as amino-acid deprivation and oxidative stress to suppress CD8+ T effector cells. This may make GCN2 act as a critical convergence point downstream of tumor myeloid suppressor cells.

**Potent GCN2 antagonist inhibits p-EIF2α**

<table>
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<th>Parameter</th>
<th>FLX-GCN2i</th>
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<th>10000</th>
<th>1000</th>
<th>500</th>
<th>100</th>
<th>50</th>
<th>32</th>
<th>1800</th>
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<td>120</td>
<td>130</td>
<td>135</td>
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<td>145</td>
<td>150</td>
<td>155</td>
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A) Potency and selectivity parameters for FLX-GCN2i. B) FLX-GCN2i was pre-incubated with recombinant human GCN2 and p-EIF2α substrate. Enzymatic activity was measured by TR-FRET and used to demonstrate dose-dependent inhibition of p-EIF2α C) SKOV-3 cells were pre-incubated with FLX-GCN2I for 1hr and enzymatic activity measured by AlphaLisa.

**FLX-GCN2i restores human CD8+ T cell proliferation and function in tryptophan limited conditions**

A) CD8+ T cells were starved for 48hrs in tryptophan depleted media followed by the addition of GCN2i and scheduled spike-ins of low concentration of tryptophan for 96hrs. B) CD8+ T cell proliferation was measured by dye dilution and the effector functional markers were analyzed by flow cytometry. C) Western blot analysis of whole cell lysates from activated T cells starved for 24hrs with the GCN2i showed decrease in pGCN2, pEIF2alpha when compared to actin.

**FLX-GCN2i moderately restores CD8+ T cell proliferation in arginine-starved conditions**

A) Activated human CD8+ T cells were starved for 24hrs in arginine depleted media followed by the addition of GCN2i and a daily spike-in of arginine for four days. B) CD8+ T cell proliferation was assessed by dye dilution. C) Viability was assessed by cell tracer violet.

**Results and Conclusions**

- FLX Bio is developing potent and selective inhibitors of the stress response kinase GCN2 (GCN2i)
- FLX-GCN2i inhibited phosphorylation of GCN2 and EIF2α in human CD8+ T cells and human MDSC cultured in amino-acid starved conditions
- Inhibition of GCN2 increased human and mouse CD8+ T cell proliferation and effector functions when cultured in amino-acid deprived conditions
- FLX-GCN2i reversed both human and mouse tumor-derived MDSC-mediated suppression and effector functions of CD8+ T cells
- Treatment of human CD3+ MDSC alone with FLX-GCN2i reverses the suppressive function of MDSC on CD8+ T cells
- Inhibition of GCN2 is an attractive approach for relieving immune suppression and promotion of T cell activation

**FLX-GCN2i reverses suppressive function of mouse tumor-derived MDSC and restores CD8+ T cell effector functions**

A) mMDSC were isolated from C572 tumors; co-cultured 1:1 with labelled T cells (isolated from naive BALB/c mice) with DMSO or FLX-GCN2i. T cell proliferation and effector functions were measured by flow cytometry at 72hrs

**FLX-GCN2i reverses human MDSC suppressive function and increases effector functions of human CD8+ T Cells**

A) Schematic of the experimental design. B) FLX-GCN2i mediates complete reversal of CD8 T cells following activation with anti-CD3/CD28 and costimulated in a ratio of 1:1 with tumor-derived monocyte MDSC in the presence or absence of GCN2i/DMSO/Arg. Flow Cytometry was used to assess proliferation on CD8 T cells.

**Treatment of human CD33+ MDSC alone with FLX-GCN2i reverses their immuno-suppressive function**

A) CD33+ myeloid cells isolated from healthy donors (n=2) were expanded in the presence of GM-CSF and IL-4 for 7 days. MDSC were pre-starved in tryptophan-limited media with FLX-GCN2i for 4hrs leading to reduction in pGCN2 and pEIF2α. B) Pre-starved MDSC were co-cultured with activated CD8+ T cells and the FLX-GCN2. C) Proliferation and D) Effector functions were measured by flow cytometry.

**FNAR**

Unless otherwise indicated, significance tests and p-values refer to test compared to vehicle control group. P values represented as follows: 0.05 > ** 0.01 *** > 0.001 > **** > 0.0001