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

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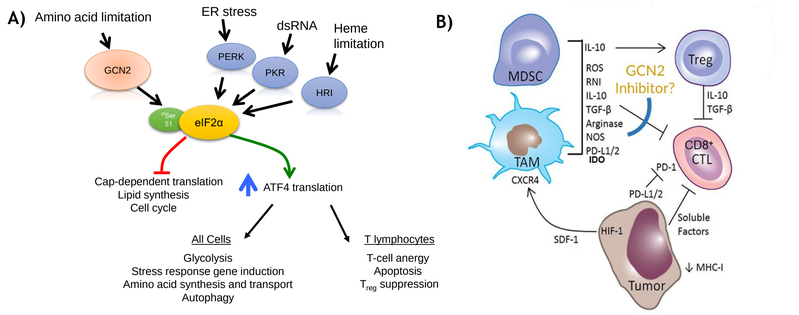
Background

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 nonderepressible 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 anergy 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

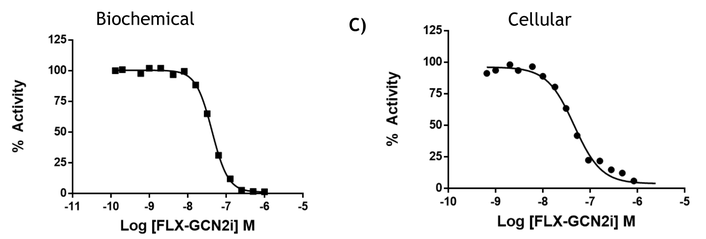


A) GCN2 is a stress response kinase detecting amino acid starvation. Activation of GCN2 leads to T cell anergy, apoptosis and enhanced T_{reg} suppression. B) MDSC/TAMs use multiple mechanisms such as amino-acid deprivation and oxidative stress to suppress CD8⁺ T effector cells. This may make GCN2 act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

Potent GCN2 antagonist inhibits p-EIF2α

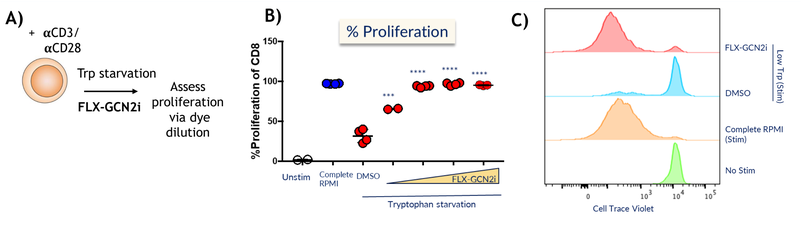
A)

	GCN2 Biochemic IC_{50} (nM)	PERK Biochemical IC_{50} (nM)	PKR Biochemical IC_{50} (nM)	HRI Biochemical IC_{50} (nM)	SKOV-3 Cellular IC_{50} (nM)	SKOV-3 Cellular Tox IC_{50} (nM)	Bioavailability (Rodent)
FLX-GCN2i	40	50000	1000	500	32	18000	>20%



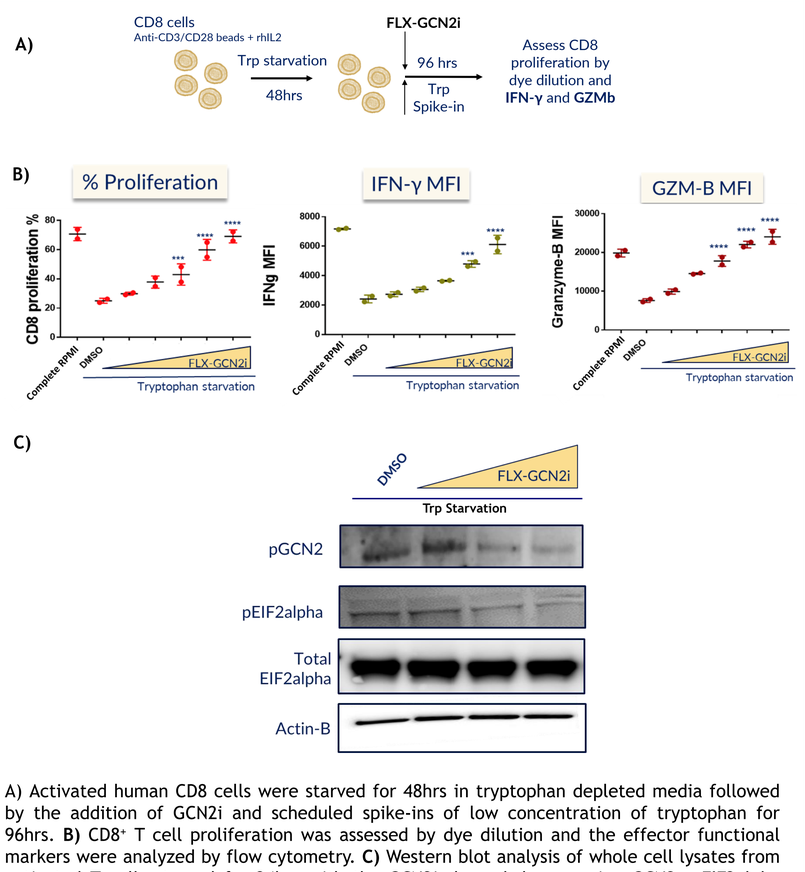
A) Potency and selectivity parameters for FLX-GCN2i. B) FLX-GCN2i was preincubated with recombinant human GCN2 and pEIF2α substrate. Enzymatic activity was measured by TR-FRET and used to demonstrates dose-dependent inhibition of p-EIF2α C) SKOV-3 cells were preincubated with FLX-GCN2i for 1hr and enzymatic activity measured by AlphaLisa

FLX-GCN2 inhibitor restores proliferation of mouse CD8⁺ T cell in amino acid starved conditions



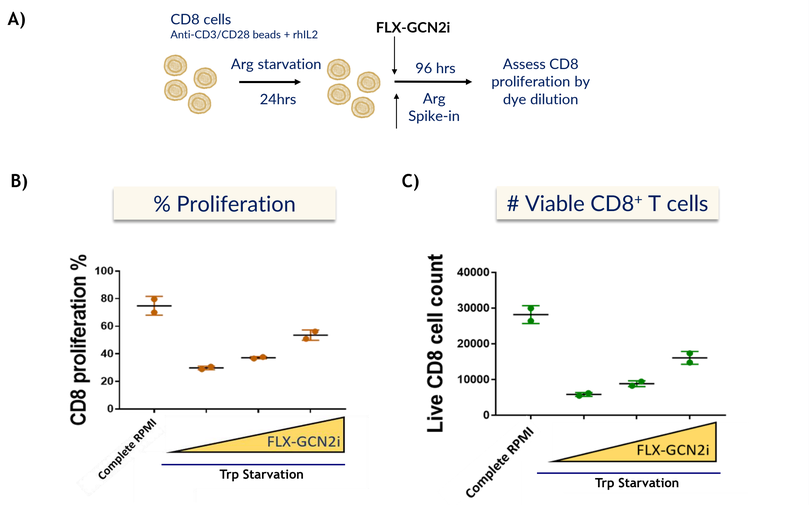
A) Activated mouse CD8⁺ T cells were starved under tryptophan limiting conditions.
B) Assessed for proliferation via flow cytometry in the presence or absence of FLX-GCN2i.
C) Represents proliferation of CD8⁺ T cell (offset histogram) of the quantified data (B)

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



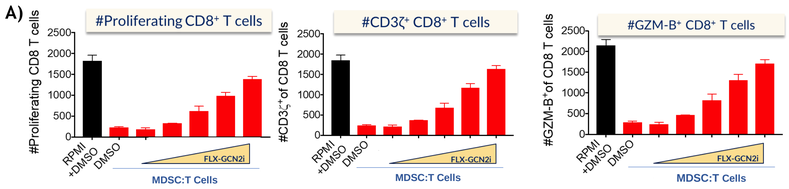
A) Activated human CD8 cells were starved for 48hrs in tryptophan depleted media followed by the addition of GCN2i and scheduled spike-ins of low concentration of tryptophan for 96hrs. **B)** CD8⁺ T cell proliferation was assessed by dye dilution and the effector functional markers were analyzed by flow 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



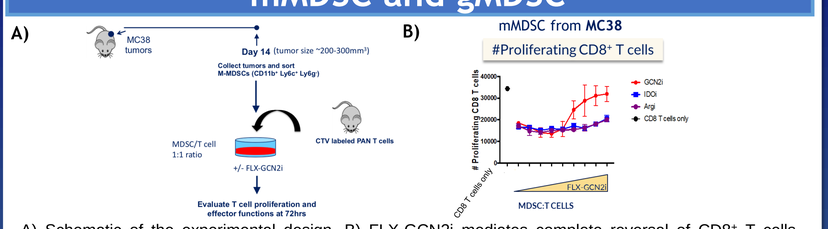
A) Activated human CD8 cells were starved for 24hrs in arginine depleted media followed by the addition of GCN2i and a daily spike-in of arginine for four days **B)** CD8 proliferation was assessed by dye dilution, C) viability was assessed by cell tracer violet

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



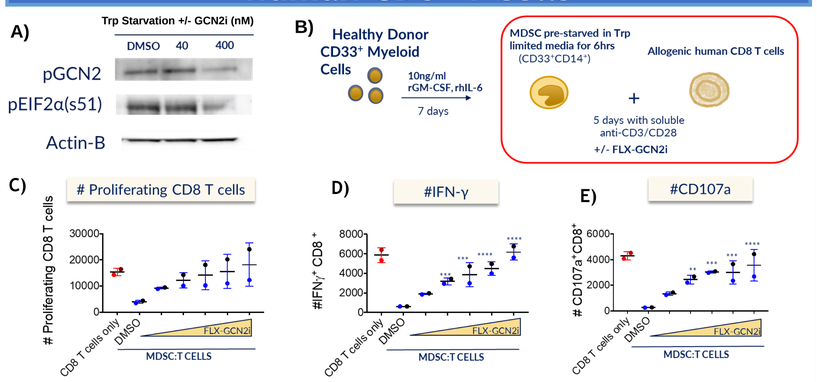
A) mMDSC were isolated from CT26 tumors; co-cultured 1:1 with labelled T cells (isolated from naïve BALB/C mice) with DMSO or FLX-GCN2i. T cell proliferation and effector functions were measured by flow cytometry at 72hrs

Differentiation of FLX-GCN2i from IDO and ARG inhibitors on tumor-derived mouse mMDSC and gMDSC



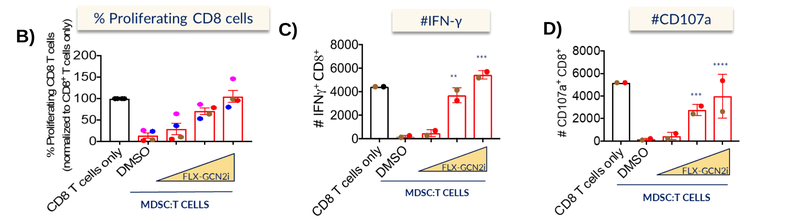
A) Schematic of the experimental design. B) FLX-GCN2i mediates complete reversal of CD8⁺ T cells following activation with anti-CD3/CD28 and cocultured in a ratio of 1:1 with tumor-derived monocytic MDSC in the presence or absence of GCN2i/IDOi/Argi. Flow Cytometry was used to assess proliferation on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

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



A) CD33⁺ myeloid cells isolated from healthy donors (n=2) were expanded in the presence of rGM-CSF and rIL-6 for 7 days. MDSC's were pre-starved in tryptophan-limited media with FLX-GCN2i for 6hrs leading to reduction in pGCN2 and pEIF2α. B) Pre-starved MDSC's were co-cultured with activated CD8⁺ T cells and the FLX-GCN2i. C) Proliferation and D-E) effector functions were measured by flow cytometry.

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



A) CD33⁺ myeloid cells from healthy donor (isolated and expanded as previously described) were pre-starved in tryptophan-limited media with GCN2i for 6hrs and then washed. Pre-starved MDSC were co-cultured with activated CD8⁺ T cells. B) Proliferation (n=4) and C-D) effector functions (n=2) were measured by flow cytometry

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 CD33⁺ 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 effector activation

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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