Targeting the Stress Response Kinase GCN2 to Restore Immunity in the Tumor Microenvironment

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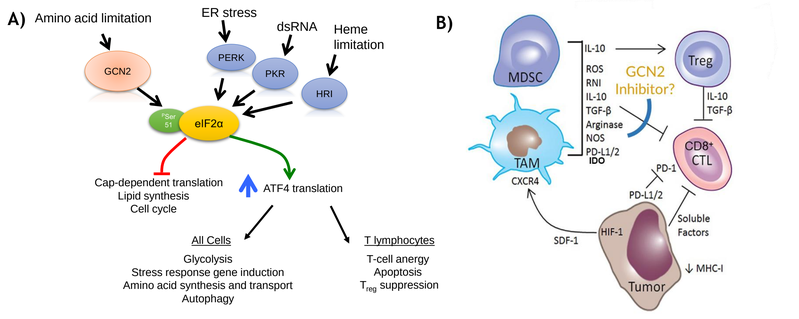
Background

The tumor microenvironment (TME) is characterized by deficiencies in oxygen and key nutrients, such as glucose and amino acids, resulting in an overall immune suppressive environment. 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 in T cells leads to an induction of the integrated stress response pathway and subsequently 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, glucose-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 (CVT 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 pathway



Michael S, Trends Endo. Metabol. 20(9), 2009

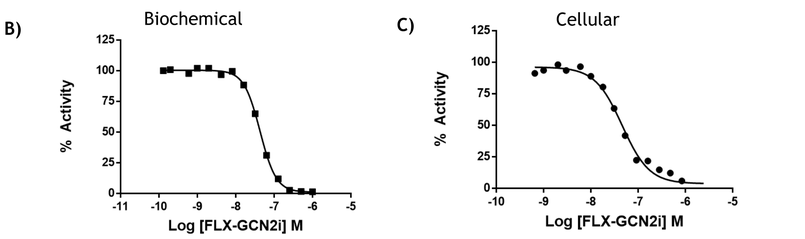
Adapted from Vatner, et al. Frontiers in Immunology 2014

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.

FLX-GCN2i potently reduces EIF2α phosphorylation

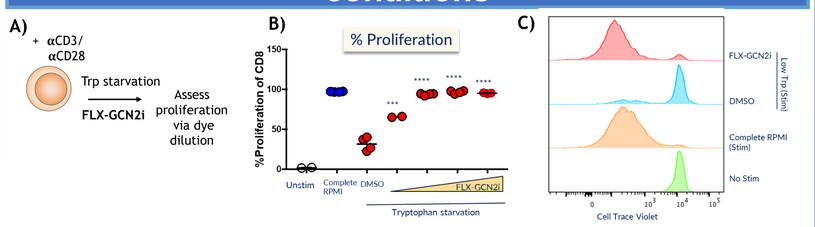
A)

	GCN2 Biochemical 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%



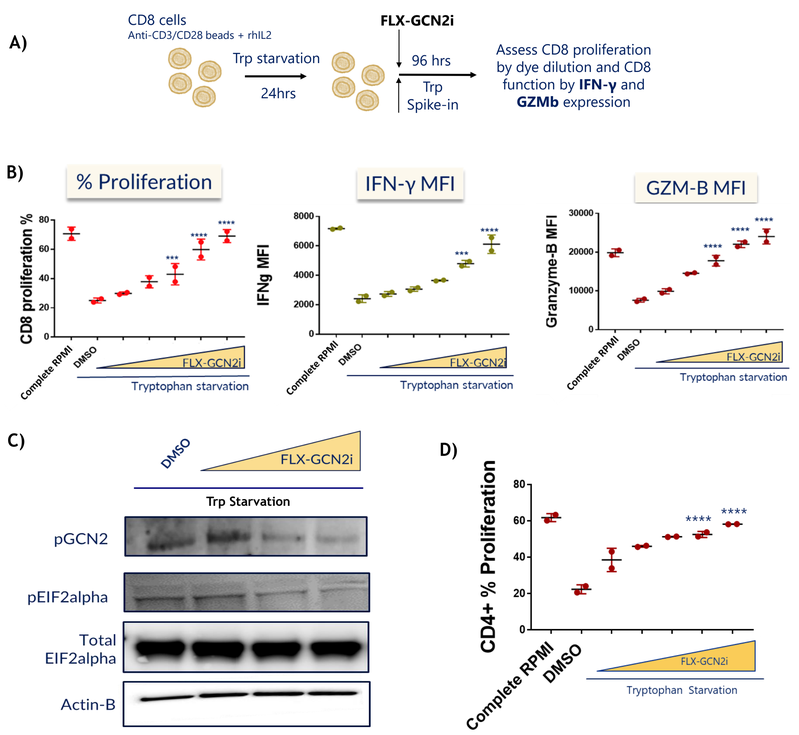
A) Potency and selectivity parameters for FLX-GCN2i. B) FLX-GCN2i was preincubated with recombinant human GCN2 and pEIF2α substrate. Enzymatic activity was measured by TR-FRET and used to demonstrates dose-dependent inhibition of p-EIF2α C) SKOV-3 cells were preincubated with FLX-GCN2i for 1hr and enzymatic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

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



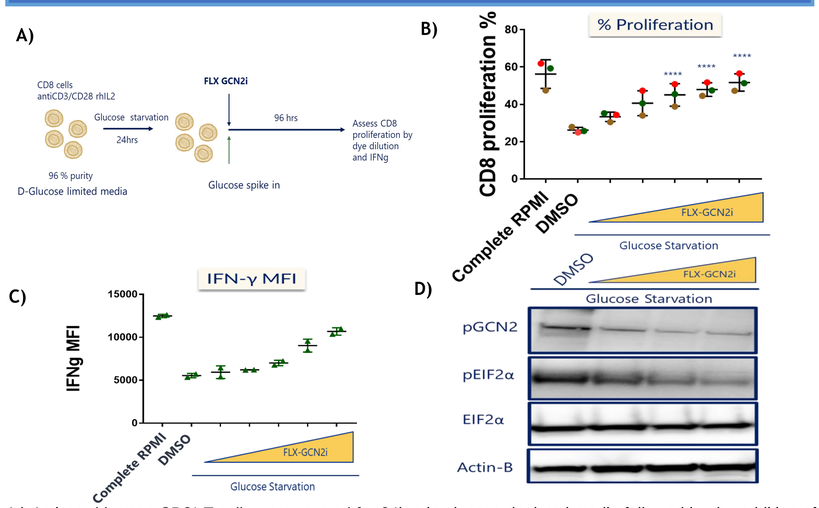
A) Activated mouse CD8⁺ T cells were starved under tryptophan limiting conditions.
B) Assessed for proliferation via flow cytometry in the presence or absence of FLX-GCN2i.
C) Representative proliferation of CD8⁺ T cell (offset histogram) of the quantified data (B)

FLX-GCN2i restores human CD8⁺ and CD4⁺ T cell proliferation and function in tryptophan limited conditions



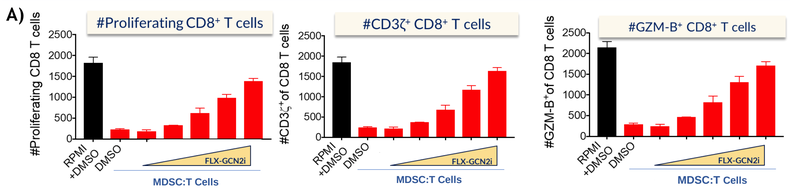
A) Activated human CD8⁺ T cells were starved for 24hrs in tryptophan-depleted media followed by the addition of GCN2i and scheduled spike-ins of low concentration of tryptophan for 96hrs.
B) CD8⁺ T cell proliferation was assessed by dye dilution and the effector functional markers were analyzed by flow 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
D) CD4⁺ T cell proliferation was assessed by dye dilution.

FLX-GCN2i restores CD8⁺ T cell proliferation and function in glucose-starved conditions



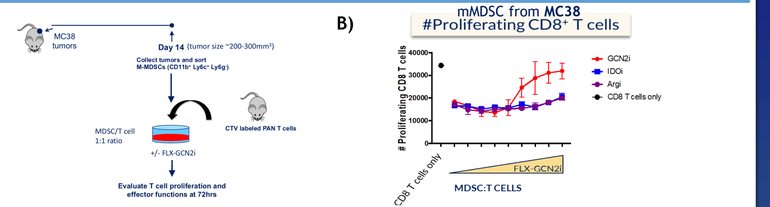
A) Activated human CD8⁺ T cells were starved for 24hrs in glucose-depleted media followed by the addition of GCN2i and scheduled spike-ins of glucose for four days B) CD8⁺ T cell proliferation was assessed by dye dilution and C) effector functional marker IFN-γ was analyzed by flow cytometry. D) Western 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 reverses suppressive function of mouse tumor-derived mMDSC and restores CD8⁺ T cell effector functions



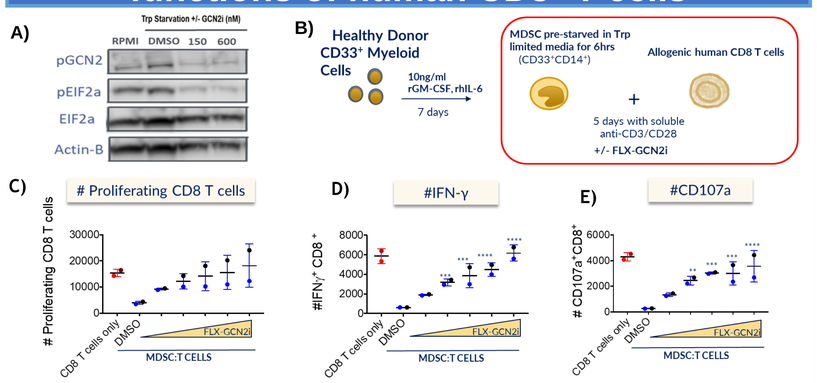
A) mMDSC were isolated from CT26 tumors; co-cultured 1:1 with labelled T cells (isolated from naïve BALB/C mice) with DMSO or FLX-GCN2i. CD8⁺ T cell proliferation and effector functions were measured by flow cytometry at 72hrs.

Differentiation of FLX-GCN2i from IDOi and ARGi on tumor-derived mouse mMDSC



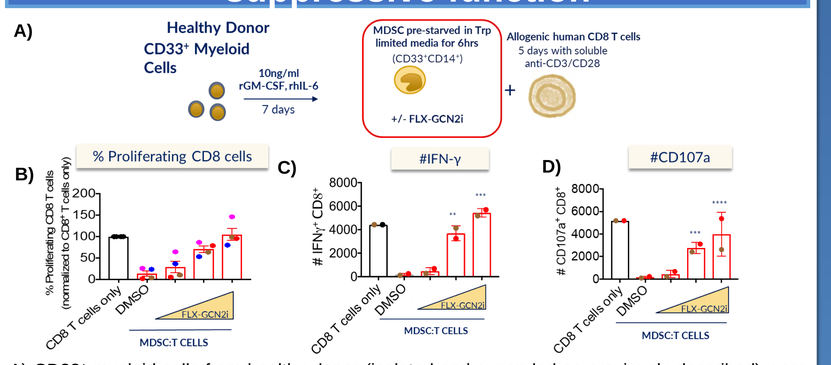
A) Schematic of the experimental design. B) FLX-GCN2i mediates complete reversal of CD8⁺ T cells following activation with anti-CD3/CD28 and cocultured in a ratio of 1:1 with tumor-derived mMDSC in the presence or absence of GCN2i/IDOi/Argi. Flow Cytometry was used to assess proliferation on CD8⁺ T cells.

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 and pretreated 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 with FLX-GCN2i alone 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 and pretreated with GCN2i for 6hrs and then washed. Pre-starved and pretreated MDSC were co-cultured in complete media 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 and glucose 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 mediated 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