STUB1 regulates IFNGR1 expression and IFNG sensitivity in human tumor cell lines

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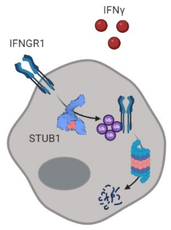
Background

Interferon gamma (IFNG) is a cytokine that is essential for anti-tumor immune activity in a variety of tumor models and settings. IFNG activity induces responses important for anti-tumor immunity, such as antigen processing and presentation molecules, but can also induce immunosuppressive molecules such as PD-L1. IFNG additionally exhibits direct growth inhibition or apoptosis-inducing potential in some cell settings. Regulation of IFNG sensing in tumor cells therefore represents a potential mechanism to enhance anti-tumor immunity. STUB1 is a ubiquitin ligase that has been reported to target the IFNG receptor IFNGR1 for degradation (1,2). Inactivation of STUB1 may therefore increase IFNGR1 expression and cellular sensitivity to IFNG. Here, we aimed to study the effects of genetic inactivation of STUB1 on IFNG responses in human tumor cell lines.



Methods

- Human tumor lines A-375 (melanoma), A549 (NSCLC) and NCI-H441 (NSCLC) were purchased from ATCC and maintained as recommended. STUB1 knockout (KO) cells were generated by nucleofection with CRISPR-Cas9 ribonucleoprotein complexes (RNPs). Successful modification and knockout (KO) were validated by Sanger sequencing and Western blotting.
- IFNGR1, PD-L1, and MHC-I (HLA-A/B/C) protein expression were measured by flow cytometry on an LSRFortessa X-20 cytometer (BD).
- RNA expression was measured by RT-qPCR using TaqMan gene probes (ThermoFisher).
- Chemokine secretion was measured by flow-cytometric bead array (BD).
- Cell viability was measured through the CellTiter-Glo 2.0 Cell Viability Assay (Promega) and a colony formation assay.
- Early apoptosis and secondary necrosis were measured via the RealTime-Glo Annexin V Apoptosis and Necrosis Assay (Promega).
- Recombinant human IFNG for cell experiments was purchased from R&D Systems.

Results

A549 STUB1 KO Cells Show Increased Baseline IFNGR1 Protein Expression

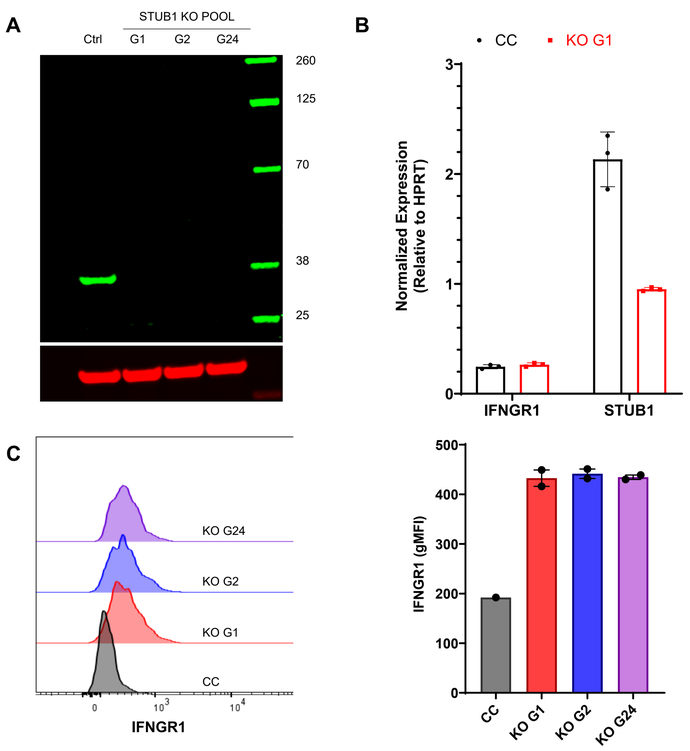


Figure 1: A) Western Blot validation of genetic inactivation of STUB1 in the A549 cell line using CRISPR-Cas9. Three pools of knockout (KO) cells were generated with Cas9-RNPs containing guides targeting different regions of the STUB1 gene (G1, G2, G24), while CRISPR-control (CC) cells were generated with Cas9 protein alone. STUB1 is shown in green and GAPDH is shown as the loading control in red. **B)** RT-qPCR analysis of RNA expression of IFNGR1 and STUB1 in A549 CC and KO G1 cells. RNA levels ($2^{-\Delta CT}$) were calculated relative to HPRT. **C)** ***Left***: Flow cytometry histogram plot of baseline IFNGR1 cell surface protein expression for CC and KO (G1, G2, G24) cells. Histogram shows all live cells. ***Right:*** Bar plot of geometric mean fluorescence intensity (gMFI) of IFNGR1 cell surface protein for A549 CC and KO cells. 10,000 cells were seeded in duplicates.

Results

A549 STUB1 KO Cells Show Increased Levels of Growth Inhibition in Response to IFNG Treatment

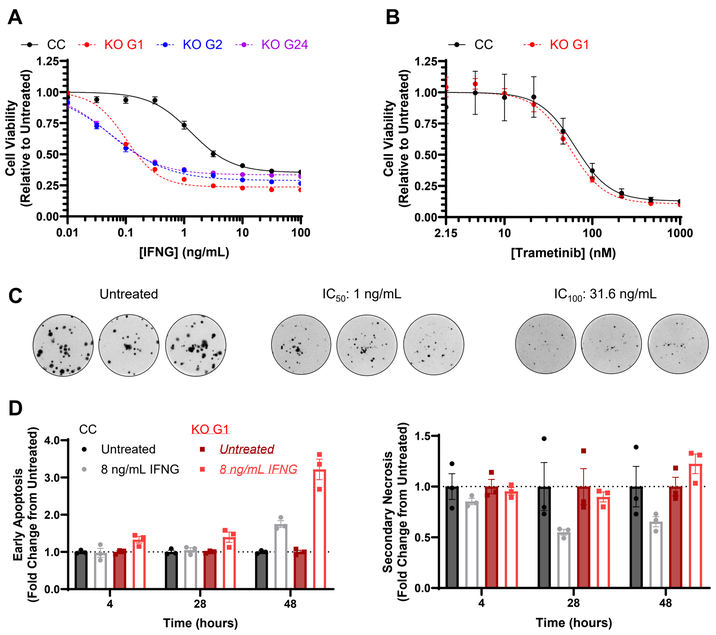


Figure 2: A) Cell viability of A549 cells in response to treatment with IFNG. 5,000 cells were seeded in triplicate and treated for 4 days. Relative luminescence units (RLU) were normalized to untreated cells on a per-cell line-basis. **B)** Cell viability of A549 cells in response to treatment with Trametinib. 5,000 cells were seeded in triplicate and treated for 4 days. Relative luminescence units (RLU) were normalized to untreated cells on a per-cell line-basis. **C)** A549 CC cell line colony formation assay showing cell viability of cell colonies after no treatment and continuous IFNG treatment. Treatment was refreshed every 3 to 4 days and colonies were stained with a 6% glutaraldehyde, 0.5% crystal violet mixture at 11 days. **D)** ***Left:*** Early apoptosis was detected through luminescence by an annexin V luciferase fusion protein. 10,000 cells were seeded in triplicate. Fold change from untreated was determined by normalizing RLU to untreated cells on a per-cell line-basis. ***Right:*** Secondary necrosis was detected through fluorescence by a cell membrane impermeable DNA-binding dye. Fold change from untreated was determined by normalizing relative fluorescence units (RFU) to untreated cells on a per-cell line-basis.

Enhanced Upregulation of Surface MHC-I and PD-L1 Protein in A549 STUB1 KO Cells in Response to IFNG

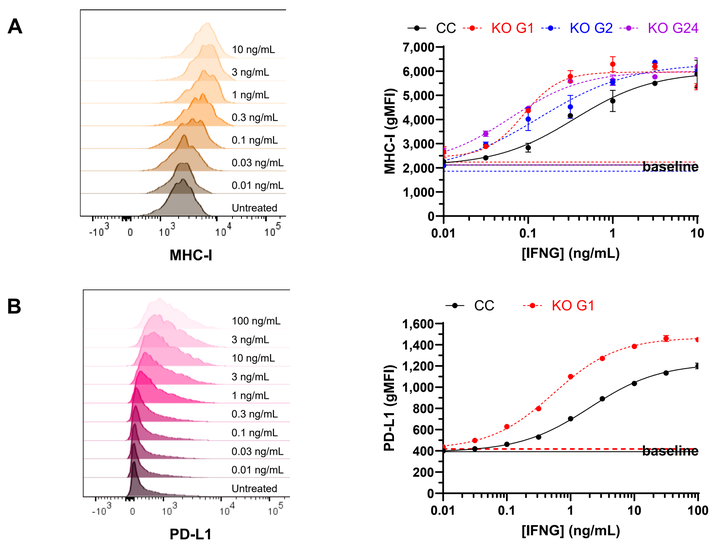


Figure 3: A) Left: Flow cytometry analysis of MHC-I expression in A549 CC cells following IFNG treatment. 10,000 cells were seeded in duplicates and treated for 16 hours. Histograms show all live cells. **Right:** Flow cytometry analysis of MHC-I expression in A549 cells following IFNG treatment. Baseline (no IFNG treatment) levels are indicated by solid or dashed lines. **B) Left:** Flow cytometry analysis of PD-L1 expression in A549 CC cells following IFNG treatment. 20,000 cells were seeded in duplicates and treated for 20 hours. Histograms show all live cells. **Right:** Flow cytometry analysis of PD-L1 expression A549 cells following IFNG treatment. Baseline (no IFNG treatment) levels are indicated by solid or dashed lines.

Enhanced RNA Expression of Interferon-Stimulated Genes in A549 KO Cells in Response to IFNG

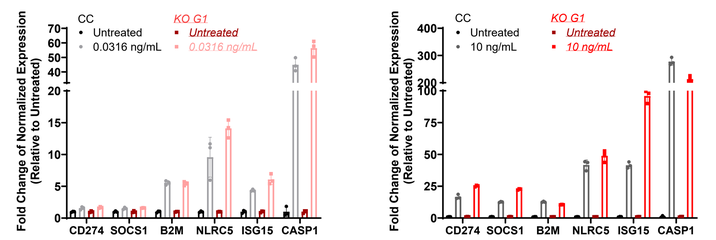


Figure 4: RT-qPCR analysis of RNA expression of genes of interest in the A549 CC and KO G1 cells with no IFNG and with IFNG treatment (**Left** – 0.0316 ng/mL, **Right** – 10 ng/mL). 150,000 cells were seeded and treated for 24 hours. RNA levels ($2^{-\Delta CT}$) were calculated relative to HPRT and fold change was determined by normalizing to untreated cells on a per-cell line-basis.

Results

A-375 STUB1 KO Cells Show Enhanced Sensitivity to IFNG Treatment

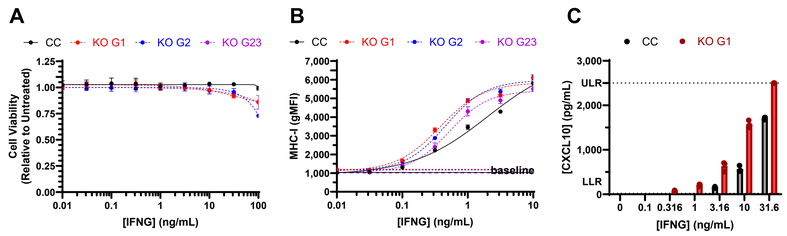


Figure 5: A) Cell viability of A-375 cells in response to treatment with IFNG. 2,000 cells were seeded in triplicate and treated for 4 days. RLU was normalized to untreated cells on a per-cell line-basis. **B)** Flow cytometry analysis of MHC-I protein expression in A-375 cells at baseline and with IFNG treatment. 20,000 cells were seeded in triplicate and treated for 22 hours. Data shows quantification of all live cells. **C)** Cytometric bead array measurements of CXCL10 levels in the media of A-375 cells in response to IFNG treatment. 100,000 cells were seeded in triplicate and treated for 48 hours. The assay linear range is 20-2,500 pg/mL.

H441 STUB1 KO Cells Show Enhanced Sensitivity to IFNG Treatment

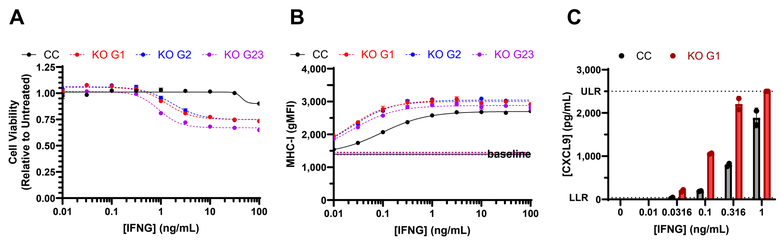
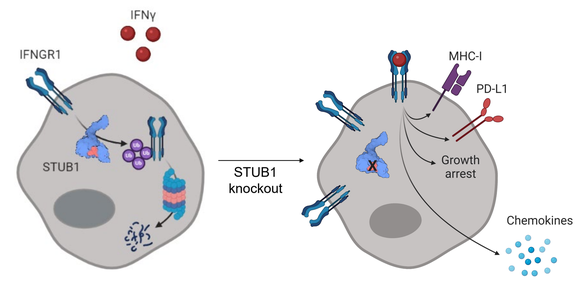


Figure 5: A) Cell viability of H441 cells in response to treatment with IFNG. 5,000 cells were seeded in triplicate and treated for 4 days. RLU was normalized to untreated cells on a per-cell line-basis. **B)** Flow cytometry analysis of MHC-I protein expression in H441 cells at baseline and with IFNG treatment. 20,000 cells were seeded in triplicates and treated for 24 hours. Data show quantification of all live cells. **C)** Cytometric bead array measurements of CXCL9 levels in the media of H441 cell lines in response to IFNG treatment. 100,000 cells were seeded in duplicate and treated for 48 hours. The assay linear range is 40-2,500 pg/mL.

Summary

- STUB1 inactivation increases IFNGR1 expression and increases cellular responsiveness to IFNG treatment.
- This increase is detected in pathways that are important for anti-tumor efficacy (e.g., growth inhibition, MHC-I expression) as well as pathways that contribute to immune evasion (e.g., PD-L1 expression).
- Further investigation of the role of STUB1 in promoting immune sensitivity is warranted and STUB1 inhibition may be attractive to enhance anti-tumor immune responses in appropriate settings.



References

1. Ng, S. et al. (2022). *Scientific reports* vol. 12,1 14087.
2. Apriamashvili, G. et al. (2022). *Nat Commun* 13, 1923.

Figures were prepared with BioRender.