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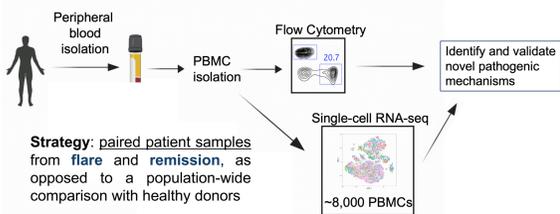
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## BACKGROUND

- Idiopathic multicentric Castleman disease (iMCD) is a rare and deadly hematologic illness involving episodic disease flares with systemic inflammation, polyclonal lymphoproliferation, as well as multiple organ system dysfunction<sup>1</sup>.
- iMCD involves multiple regions of **lymphadenopathy** as well as **vital organ dysfunction** due to a cytokine storm that often includes interleukin-6.
- Despite approximately 1,500 cases of iMCD diagnosed per year in the US, the **etiology, cell types, and pathological mechanisms remain largely unknown**<sup>2</sup>.
- iMCD is further subtyped to define patients with thrombocytopenia, anasarca, fever, myelofibrosis, renal dysfunction, and organomegaly as **iMCD-TAFRO**, which is the focus of this study<sup>3</sup>.

## OBJECTIVES AND METHODS

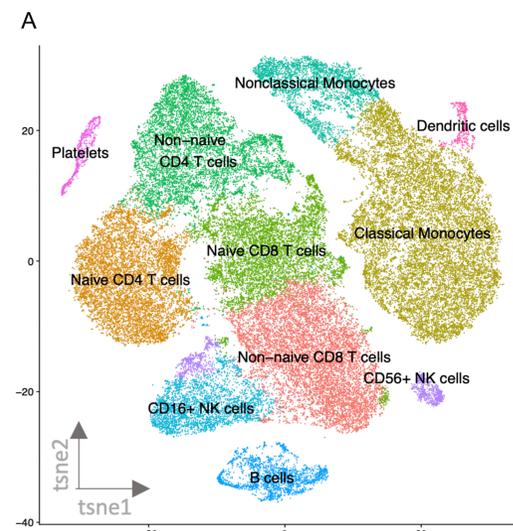
- To identify potential pathophysiological mechanisms and cellular drivers of iMCD-TAFRO, we utilized 10X Genomics single-cell RNA sequencing (scRNAseq) to characterize gene expression in peripheral blood mononuclear cells (PBMCs).
  - Samples:** PBMCs from three iMCD-TAFRO patients, obtained during active disease (**flare**) and later after resolution of flare (**remission**).
  - 3/3 patients were diagnosed with iMCD-TAFRO, with related diseases excluded per diagnostic criteria.
- Following identification of potential novel pathogenic mechanisms, we performed functional experiments and **phospho-flow cytometry**, using additional iMCD-TAFRO patient samples from remission (n=8).



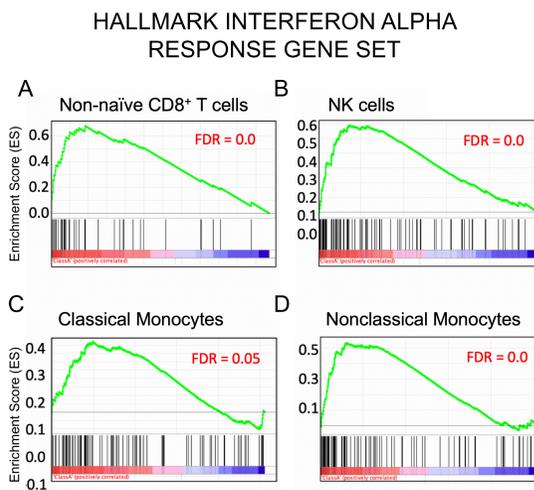
**Circulating immune cell populations are reliably identified within clusters following scRNAseq.** Immune cell populations within clusters in the scRNAseq dataset were identified using known lineage genes and further validated using gene sets unique to each immune cell population. These cell populations were then extracted for downstream analyses (Fig. 1).

**An elevated Type I Interferon (IFN-I) response gene signature during flare is correlated with mTOR activation in classical monocytes.** GSEA analysis of the 50 Hallmark gene sets showed a statistically significant enrichment of the Interferon Alpha Response (IFN-I) Gene Set in flare compared to paired remission across all three iMCD-TAFRO patients (Fig. 2). GSEA also identified enrichment of the mTORC1 signaling gene set during flare in classical and nonclassical monocytes (Fig. 3A-B). We also observed a strong positive and significant correlation between the average expression of IFN-I response genes and mTORC1 signaling genes selectively in classical monocytes from iMCD-TAFRO flare (Fig. 3C-D).

**IFN-I stimulation induces greater mTOR activation in T cells and monocytes from iMCD patients in remission and can be abrogated through JAK inhibition.** We stimulated PBMCs from iMCD patients in remission and healthy donors with IFN $\beta$  and assessed phosphorylation of S6 protein, a readout of mTOR activation, using flow cytometry. We observed significantly greater mTOR activation in response to IFN-I in both monocytes and T cells from iMCD patients in remission compared to healthy donors (Fig. 4A). Finally, we found that treatment of patient PBMCs with the JAK inhibitor, ruxolitinib, was sufficient to abrogate IFN-I-mediated mTOR activation (Fig 4B).

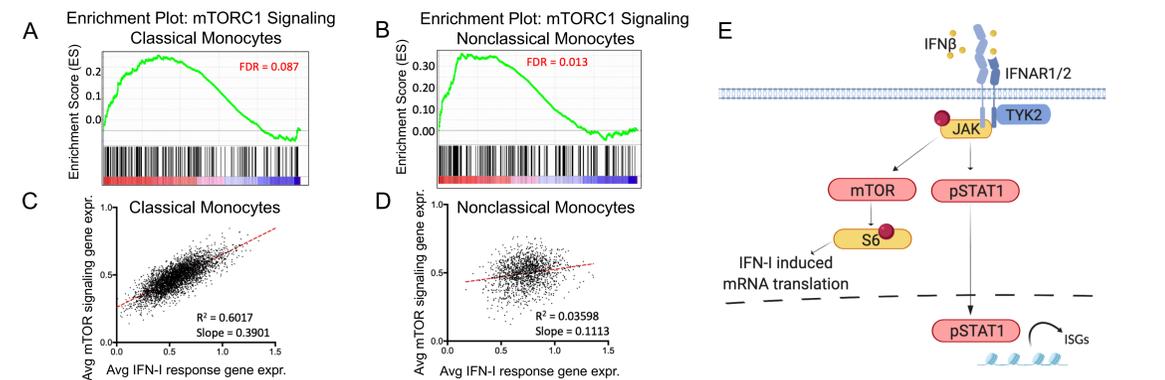


**Figure 1. Visualization of PBMCs using tSNE and identification of circulating immune cell subsets.** A. The top 20 canonical correlation vectors were used to perform clustering and visualization using a tSNE plot. Cell types were identified using common lineage genes and further validated using gene sets unique to individual immune cell populations. Immune cell populations were subsetted from this dataset for downstream analyses.

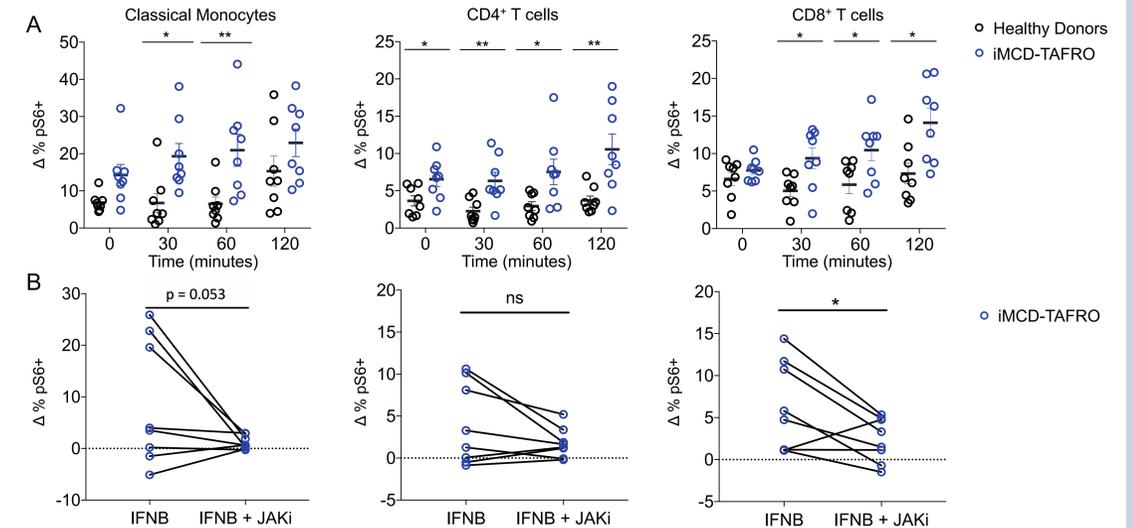


**Figure 2. The Type I Interferon Response gene set is enriched across multiple immune cell populations during iMCD flare.** GSEA comparing the ratio of gene expression between flare and paired remission across three iMCD patients. A-D. Enrichment plots for the Hallmark Interferon alpha response gene set in non-naïve CD8<sup>+</sup> T cells (A), NK cells (B), classical monocytes (C) and nonclassical monocytes (D).

## RESULTS



**Figure 3. mTORC1 signaling gene expression is correlated with IFN-I response gene expression in classical monocytes from iMCD flare.** A-B. Enrichment of mTORC1 signaling in classical monocytes (A) and nonclassical monocytes (B). C-D. Representative single-cell dot plot of the average expression of genes from the mTORC1 signaling and Interferon alpha (IFN-I) response gene sets within classical monocytes (C) and nonclassical monocytes (D). Schematic detailing IFN-I proximal signaling to JAK, activation of mTOR to phosphorylate S6, and phosphorylation of STAT1 leading to expression of interferon stimulated genes (ISGs).



**Figure 4. Phospho-flow cytometry identified increased JAK-dependent mTOR activation in response to IFN-I in iMCD.** A. Percent of healthy donor (black) and iMCD-TAFRO remission (blue) classical monocytes, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells expressing phosphorylated S6 (S235/S236) following stimulation with IFN $\beta$ . B. Comparison of the change in frequency of cells expressing phosphorylated S6 following treatment with either IFN $\beta$  alone or both IFN $\beta$  and the JAK inhibitor (JAKi), ruxolitinib.

## CONCLUSIONS

- Through scRNAseq, we observed a **IFN-I response gene signature** across CD8<sup>+</sup> T cells, NK cells, and monocytes during iMCD-TAFRO flare compared to paired remission.
- mTORC1 signaling is enriched within monocytes during flare compared to remission.
- Expression of mTOR signaling genes and IFN-I response genes is correlated within classical monocytes during iMCD-TAFRO flare.
- IFN $\beta$  induced mTOR activation** to a significantly greater extent within monocytes and T cells from iMCD remission cells compared to healthy donor cells.
- IFN-I-induced mTOR induction was abrogated following treatment of iMCD-TAFRO patient cells with the JAKi, ruxolitinib. Thus, **JAK may be a viable therapeutic target in iMCD.**