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Hyperactivated Interferon-gamma Pathways in Perianal Fistulizing Crohn's Disease by Single-Cell and Spatial Multi-omics

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Perianal fistulizing Crohn's disease (PCD) is a common and debilitating complication with elusive pathophysiology. To define actionable immunologic targets in PCD, we recruited patients with PCD (n = 24), CD without perianal disease (NPCD, n = 10), and idiopathic/cryptoglandular perianal fistulas (IPF, n = 29). Biopsies from fistula tracts, fistula opening, and rectal mucosa were analyzed using single-cell RNA-sequencing (scRNA-seq), mass cytometry (CyTOF), and spatial transcriptomics (ST). Global hyperactivation of IFN-g pathways distinguished PCD from idiopathic perianal fistulas and CD without perianal disease in the fistula tracts and/or intestinal mucosa. IFN-g and TNF-a signaling directly induced genes involved in epithelial-to-mesenchymal transition in PCD rectal epithelial cells. Enhanced IFN-g signaling in PCD was driven by pathogenic Th17 (pTh17) cells, which were recruited and activated by myeloid cells overexpressing LPS signature (LPS_myeloid). pTh17 and LPS_myeloid cells co-localized adjacent to PCD fistula tracts on ST and drove local IFN-g signaling. Anti-TNFs facilitated fistula healing by downregulating T and myeloid cell signatures, while promoting mucosal barrier repair and immunoregulatory processes. Key single-cell findings were validated by bulk RNA-seq data of an independent CD cohort. To summarize, we identified IFN-g-driven mechanisms contributing to pathogenesis and highlighted its blockade as a therapeutic strategy for PCD.

Hyperactivated Interferon-γ Pathways in Perianal Fistulizing Crohn's Disease by Single-Cell and Spatial Multi-omics

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Conflict-of-interest statement

No conflict-of-interest is related to this study.

To the Editor: Perianal fistulizing Crohn's disease (PCD) occurs in up to 40% of CD patients and with substantial treatment-resistance and high morbidity. The etiology of PCD remains poorly understood, hindering the development of preclinical models and effective therapies (1). Here, we utilized a multi-omics approach including single cell transcriptomics (scRNA-seq), CyTOF, and spatial transcriptomics (ST) to characterize PCD, CD without perianal disease (NPCD), and idiopathic/cryptoglandular perianal fistula (IPF).

Fistula tract biopsies of 9 individuals with PCD and 6 with IPF (**Supplemental Table 1**) were analyzed using scRNA-seq which generated 56,560 high-quality cells (**Figures 1A; Supplemental Figure 1A**). Single-cell pathway analysis (SCPA) identified IFN-γ and TNF-α signaling as top activated pathways in PCD vs. IPF (**Figure 1B**). Hyperactivated IFN-γ-responsive genes including JAKs and STAT1 were present in major cell populations from PCD fistulas (**Figure 1C; Supplemental Figure 1B**). The transcriptomic data was validated by IHC, which showed increased IFN-γ and phosphorylated-STAT1 in PCD (**Figure 1D**). By re-analyzing published scRNA-seq datasets of rectum (2) (active PCD vs. inactive/healed PCD; n = 6/group) and colon and terminal ileum (3) (PCD vs. NPCD; n = 8 for PCD, n = 39 for NPCD), we showed upregulated IFN-γ and TNF-α pathways in all the three locations in PCD intestine compared to NPCD (**Figure 1E**). Re-clustering of the rectal cells (2) uncovered similar induction of IFN-γ-responsive genes in multiple epithelial and immune cells from PCD vs. PCD-healed (**Supplemental Figure 1C**). Thus, PCD is characterized by heightened IFN-γ response in both fistula tracts and intestinal mucosa. Analysis of rectal epithelial cells (2) also identified enriched clusters 9/12 with elevated IFN-γ, TNF-α, and EMT pathways in PCD compared to CD without perianal disease. IFN-γ and TNF-α responses were significantly correlated with EMT (**Figure 1F)**. Compared to IPF, PCD fistulas exhibited increased extracellular matrix organization by stromal cells and cell adhesions by endothelial cells (**Supplemental Figure 1D**).

Unbiased ligand-receptor analysis revealed PCD-enriched pairs (**Supplemental Figure 2A**) known to drive inflammation and EMT. Moreover, IFN-γ senders Th17 and Tregs were significantly enriched in PCD (**Figure 1G**). IFN-γ-producing Th17 cells represent a pathogenic antigen-induced state (4) (pathogenic Th17; pTh17), which may underlie the activated IFN-γ response. PCD myeloid cells overexpressed NLRP3, AREG, and IFN-γ-induced CXCL9/10 (**Supplemental Figure 2B**). Signaling by bacterial molecule LPS was upregulated in NLRP3+AREG+ cells (or LPS_myeloid cells) and the entire myeloid compartment in PCD (**Supplemental Figure 2C**). Ligand-receptor analysis further revealed multiple myeloid-derived chemokines/cytokines that activate Th17 cells (**Supplemental Figure 2D**). IFN-γ-producing cells may also be upregulated by higher IL15 in PCD (**Supplemental Figure 2E**). Other elevated pathways in PCD myeloid cells include NOD2-RIPK2 and TL1A-DR3 (**Supplemental Figure 2G-I**). Additionally, our CyTOF data revealed features of IFN-γ signaling and established LPS_myeloid cells as a hallmark of PCD microenvironment across fistula tracts, fistula opening, and rectum (**Supplemental Figure 2, J-L**).

Anti-TNFs currently have the best evidence for PCD treatment with unclear mechanisms (1). We subdivided our scRNA-seq PCD cohort into patients on anti-TNFs or anti-TNF-naïve groups based on treatment at sampling (n = 4/group; all patients on anti-TNFs later responded to therapy). Weighted gene co-expression network analysis identified two gene modules significantly correlated with anti-TNFs: the “lightpink1” module suppressed by anti-TNFs associated with immune cell activation and IFN response, while the “royalblue2” module induced by anti-TNFs supported cell proliferation and wound healing (**Supplemental Figure 2, M-O**).

ST of IPF and PCD fistula tracts (n = 3/group) generated 6 spatially correlated clusters (C0-5; **Supplemental Figure 3A**). Epithelial cells lining the fistula tract in C1 primarily mapped to IFNGR+TNFR+, IFNG/TNF-responsive colonocytes (**Supplemental Figure 3B**), further supporting the roles of IFN-γ and TNF-α signaling in fistulization. LPS_myeloid cells were

ubiquitously present at tract-adjacent spots, suggesting their interactions with microbial elements in the fistula tract (**Supplemental Figure 3C**), while pTh17 closely co-localized with LPS_myeloid cells (**Supplemental Figure 3D**). Notably, these cells were present in all PCD samples but were scarce in IPF (**Supplemental Figure 3E**).

Finally, we validated our single-cell findings using intestinal bulk RNA-seq data of intestinal sample from patients with active PCD (n = 12), inactive PCD (n = 23), CD without perianal disease (n = 84) in an independent SPARC-IBD cohort. The analysis demonstrates hyperactivation of IFN-γ response (e.g., STAT1, IRF1; **Figure 1H**), EMT/tissue remodeling, inflammation, and endoplasmic reticulum stress in PCD tissues (**Supplemental Figure 3F**). Current PCD treatments (e.g., anti-TNFs and upadacitinib) are mainly approved through registrational trials in luminal CD; they have moderate efficacy and cannot heal the fistulas in most cases (1, 5). The therapeutic potential of IFN-γ antagonists warrants investigation using physiologically relevant PCD models.

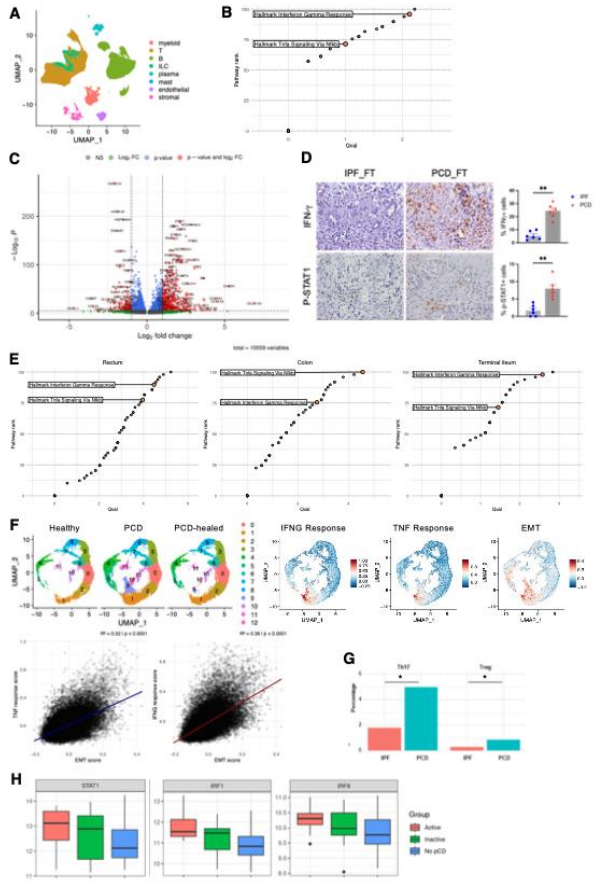

Figure 1. Hyperactivated IFN-γ signaling is a distinguishing feature of PCD in both fistula tracts and intestinal mucosa. (**A**) UMAP of major cell compartments in perianal fistulas. (**B**) Top upregulated pathways in PCD vs. IPF fistulas by SCPA. (**C**) Altered gene expression in monocytes/macrophages/dendritic cells in PCD vs. IPF fistulas. (**D**) Representative IHC image and quantification in fistula tracts (FT). scale bar = 50um. ** p<0.01. (**E**) Top upregulated pathways in rectum (PCD vs. PCD-healed) and colon/terminal ileum (PCD vs. NPCD) by SCPA. (**F**) Left: UMAP of rectal epithelial cells; Middle: Single-cell module scores; Right: correlation between TNF or IFNG response and EMT scores. (**G**) IFN-γ senders in PCD and IPF. (**H**) IFN-γ downstream genes in bulk RNA-seq of CD intestinal samples. P values generated by Dunn post-hoc test: STAT1 – 0.019 (Active vs. No PCD); IRF1 – 0.00024 (Active vs. No PCD); 0.012 (Inactive vs. No PCD); IRF8 – 0.014 (Active vs. No PCD).

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