Efzofitimod Promotes Macrophages with Anti-inflammatory Profile via Neuropilin-2 Receptor

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Introduction

Aminocyl-tRNA synthetases, primarily known for their role in protein synthesis, have been demonstrated to be the source of tissue-specific naturally occurring splice variants (SVs) thought to confer regulatory functions[1]. Efzofitimod is a therapeutic candidate[2] derived from a SV of human histidyl-tRNA synthetase (HARS) fused to human IgG1 Fc. This SV of HARS, enriched in human lung, is upregulated by inflammatory cytokines in lung epithelial and immune cells. Efzofitimod exhibits highly specific and selective binding to the cell surface receptor neuropilin-2 (NRP2) that plays an important role in regulating immune responses. Our previous findings showed that NRP2 expression is induced by differentiation and/or activation of immune cells, particularly myeloid cells such as macrophages. We hypothesize that efzofitimod plays a novel role in regulating macrophage function via NRP2 that may be exploited therapeutically for inflammatory diseases.

Here we examined the role of the efzofitimod-NRP2 axis in macrophage differentiation and function. We show that NRP2 is strongly upregulated in primary monocyte-derived macrophages (PMDM) during differentiation. Efzofitimod enhanced the percentage of PMDM with a round-shaped morphology that exhibit an anti-inflammatory gene expression pattern. These observations were consistent across cells from multiple donors. High-throughput RNAseq and pathway analysis demonstrated the down-regulation of immunoregulatory gene sets for inflammatory and mTOR signaling pathways. Notably, efzofitimod reduced cell surface receptors that trigger inflammation (e.g. CD14, CCR2) and pro-inflammatory cytokines such as MCP-1, TNF-α and IL-6.

These results reveal novel immunomodulation of myeloid cells by efzofitimod, and promotion of macrophage differentiation towards a unique, anti-inflammatory profile. Leveraging this novel mechanism in macrophage biology, efzofitimod may represent a breakthrough in the treatment of macrophage-mediated inflammatory diseases such as ILDs.

tRNA synthetase drug discovery platform

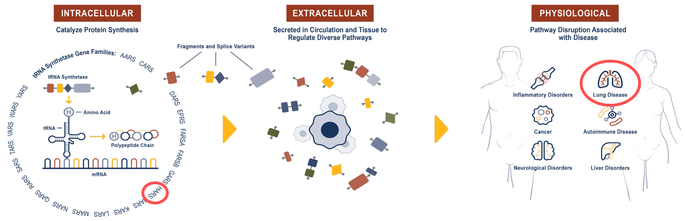


Figure 1. Splice variants and proteolytic fragments of tRNA synthetases have been identified in extracellular spaces, where they have novel functions. aTyr Pharma is engaged in the discovery and development of potential first-in-class medicines based on newly discovered pathways effected by extracellular tRNA synthetases.

Efzofitimod, a novel ligand for Neuropilin-2 (NRP2) receptor

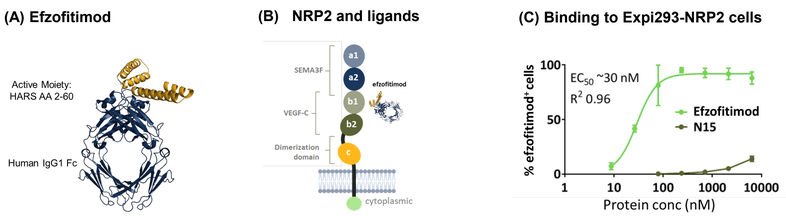


Figure 2. Efzofitimod binds to NRP2 receptor. (**A**) Efzofitimod is a fusion protein consisting of amino acids 2-60 of Histidyl-tRNA synthetase (HARS) and human IgG1 Fc. (**B**) Schematic of NRP2 domains and ligands. (**C**) Efzofitimod binds to Expi293F-NRP2 cells.

NRP2 is highly expressed on activated myeloid cells from healthy donors

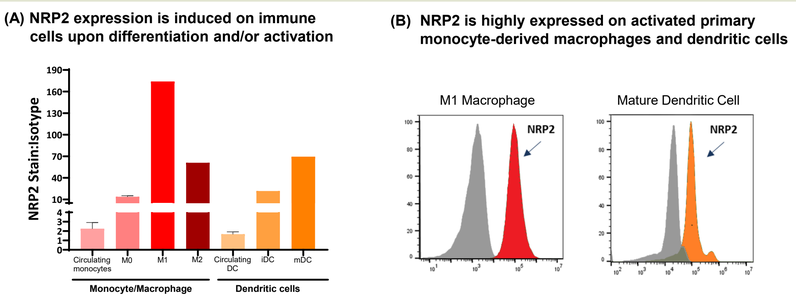


Figure 3. For circulating monocytes and dendritic cells (DC), PBMC were evaluated for NRP2 expression by flow cytometry on monocytes ($CD14^+$) and DC ($CD14^-CD11c^+$). M0, M1 and M2 macrophages or immature DC (iDC) and mature DC (mDC) were generated *in vitro* from primary monocytes isolated from PBMC, and NRP2 expression was determined by flow cytometry.

Methods and Results

NRP2 is highly expressed on circulating monocytes and tissue macrophages in patients with chronic inflammatory diseases

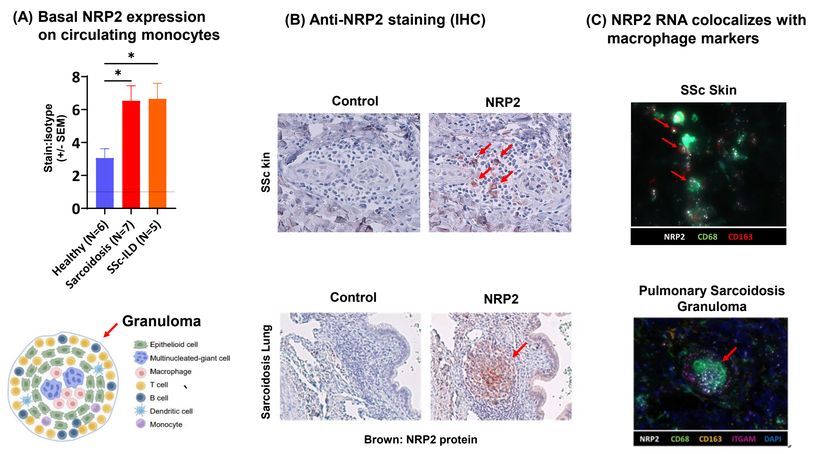


Figure 4. (**A**) NRP2 cell surface expression in circulating $CD14^+$ monocytes from healthy donors, pulmonary sarcoidosis patients, and Scleroderma-associated ILD (SSc-ILD) patients (**B**) NRP2 (brown) immunohistochemical staining in skin from Scleroderma (SSc) patients (top) and lung granulomas from pulmonary sarcoidosis patients (bottom). (**C**) NRP2 RNA (white dots) colocalizes with macrophage markers CD68 (green) and CD163 (red or orange) in skin from SSc patients and lung granulomas from pulmonary sarcoidosis patients.

Efzofitimod promotes morphology change of primary monocyte-derived macrophages in concordance with NRP2 upregulation during differentiation

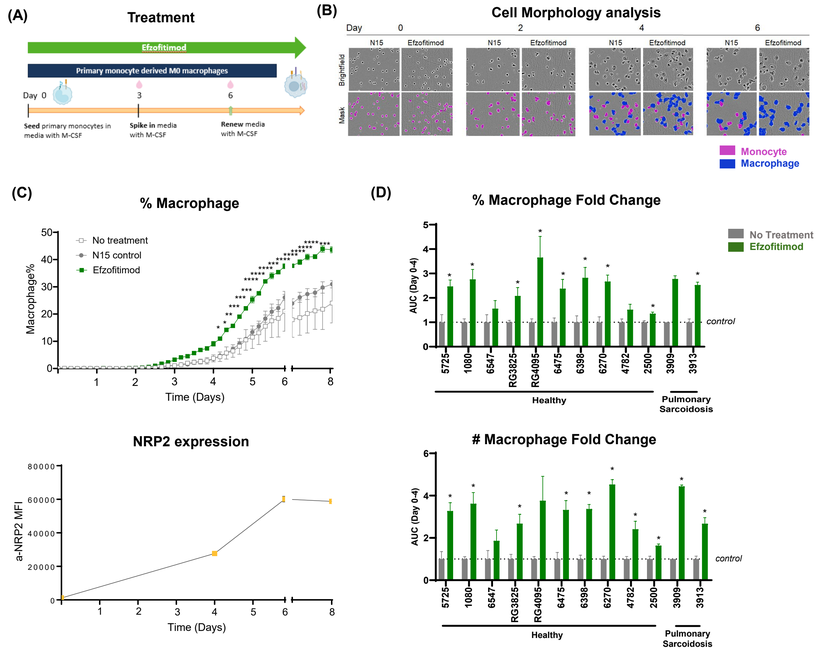
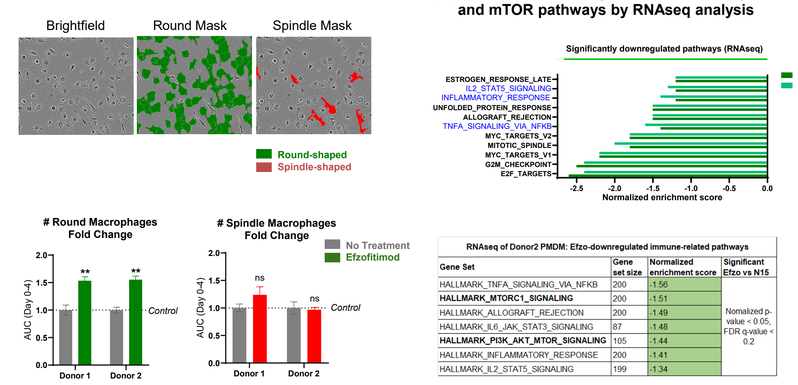


Figure 5. (**A**) Schematic representation for generation of primary monocyte-derived macrophages (PMDM). (**B**) Images of PMDM generated in the presence of efzofitimod or N15 control. Pink mask: monocyte morphology and blue mask: macrophage morphology by IncuCyte live cell imaging and analysis. (**C**) Percentage of cells with macrophage morphology over 8 days (top). NRP2 expression on monocyte-derived macrophages by flow cytometry analysis over the same time frame (bottom). (**D**) Fold change of efzofitimod over N15 control in area under the curve from days 0-4 in PMDM from healthy donors and pulmonary sarcoidosis patients.

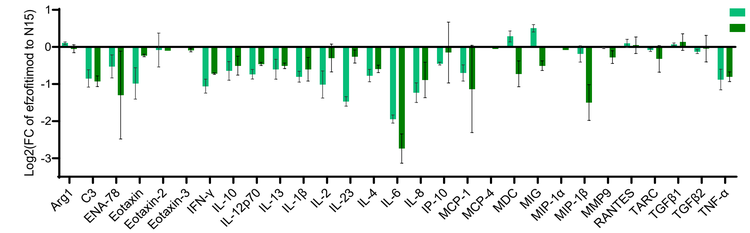
Characterization

Efzofitimod promotes macrophages with an anti-inflammatory profile



RNAseq of Donor2 PMDM: Efzo-downregulated immune-related pathways			
Gene Set	**Gene set size**	**Normalized enrichment score**	**Significant Efzo vs N15**
HALLMARK_TNFA_SIGNALING_VIA_NFKB	200	-1.56	Nomalized p-value < 0.05, FDR q-value < 0.2
HALLMARK_MTORC1_SIGNALING	200	-1.51	
HALLMARK_ALLOGRAFT_REJECTION	200	-1.49	
HALLMARK_IL6_JAK_STAT3_SIGNALING	87	-1.48	
HALLMARK_PI3K_AKT_MTOR_SIGNALING	105	-1.44	
HALLMARK_INFLAMMATORY_RESPONSE	200	-1.41	
HALLMARK_IL2_STAT5_SIGNALING	199	-1.34	

(C) Efzofitimod reduces secretion of cytokines including MCP-1, TNF-α and IL-6



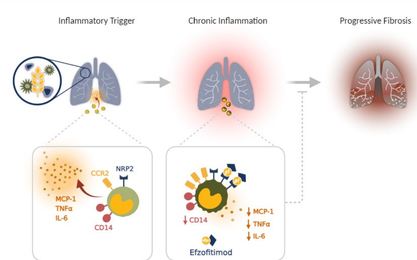
(D) Efzofitimod reduces inflammatory receptors and cytokines in primary monocyte-derived macrophages

		Clinical serum biomarkers	DE RNA-seq	DE qPCR	Cell surface receptors	Secreted proteins
Cytokines	CCL2 (MCP-1)	Down	Down	Down		Down
	TNF	Down	Down	Down		Down
	IL-6	Down	n.d.	n.d.		Down
	IL1B		Down			Down
	CXCL9 (MIG)		n.d.	n.d.		Down
Receptors	CD14		Down	Down	Down	Down
	CCR2		n.d.	n.d.	Down	
	CCR7		n.d.	Down	Down	
	CXCR4		-	Down	Down	
	MRC1 (CD206)		-	-	Down	
	CD28		Down	Down		
	TLR2		Down	Down		
	TLR4		-	Down		

Figure 6. (**A**) Images of monocyte-derived macrophages in brightfield. Green mask: macrophages with round-shaped morphology and red mask: macrophages with spindle-shaped morphology (top). Fold change of efzofitimod over N15 control in area under the curve from days 0-4 in monocyte-derived macrophages from two healthy donors (bottom). (**B**) Gene sets downregulated by efzofitimod in monocyte-derived macrophages by Gene Set Enrichment Analysis (GESA) of high-throughput RNAseq data of PMDM (day 8) from two donors. (**C**) Fold change in cytokine secretion from monocyte-derived macrophages with efzofitimod treatment compared to N15 control by MSD cytokine assay of culture supernatants. (**D**) Summary of key inflammatory receptor and cytokine changes in monocyte-derived macrophages treated with efzofitimod compared to control. Down: down-regulation and n.d. not detected.

Conclusions

- NRP2, the sole binding partner to efzofitimod, is highly expressed on circulating monocytes and tissue macrophages in patients with chronic inflammatory diseases.
- Efzofitimod promotes differentiation of primary monocyte-derived macrophages with an anti-inflammatory profile.
- Efzofitimod may represent a novel therapy in treatment of macrophage-mediated inflammatory diseases such as ILDs.



Disclosures:
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References:
[1]Lo WS et al. Human tRNA synthetase catalytic nulls with diverse functions Science. 345(6194):328-32. (2014)
[2]Baughman RP et al. Efzofitimod: A novel anti-inflammatory agent for sarcoidosis. *Sarcoidosis, Vasculitis and Diffuse Lung Diseases*. 40,1:e2023011 (2023)