ATYR1923 Reduces Neutrophil Infiltration in an Acute Lipopolysaccharide (LPS) Lung Injury Model

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Abstract

A number of aminoacyl tRNA-synthetases have evolved non-canonical functions including the tRNA synthetase for histidine, HARS. HARS downregulates immune responses via its N-terminal domain, which we have termed the iMod (immunomodulatory) domain. The iMod domain was fused to human IgG1 Fc to generate ATYR1923, which is currently in clinical evaluation for pulmonary sarcoidosis. ATYR1923 binds to neuropilin-2 (NRP-2), a pleiotropic co-receptor participating in several pathways including class III semaphorins/plexins and VEGF-C/VEGFR3. To date, little is known about the role of NRP-2 in immune regulation, although growing evidence indicates that NRP-2 influences myeloid cell biology such as activation and recruitment to inflammatory sites. For instance, NRP-2 expression on alveolar macrophages regulates airway inflammatory responses to inhaled LPS (Immormino *et al*. 2018). To determine whether ATYR1923 was able to influence myeloid cell migration, ATYR1923 or a pentameric iMod construct, iMod-COMP, were administered intravenously to C57BL/6 mice 24h prior to LPS challenge, either by intraperitoneal (IP) injection to induce a systemic inflammatory response or by airway administration to generate acute lung inflammation. Multi-color flow cytometry was used for immunophenotyping analysis and detection of NRP-2 levels on surfaces of various immune cell populations. *In vitro,* mouse bone-marrow derived macrophages (BMDM), human THP-1 monocytic cells, and primary human dendritic cells (DCs) were used to confirm NRP-2 cell surface expression. Results indicated that LPS stimulation *in vitro* or *in vivo* upregulated NRP-2 on a variety of myeloid cells including macrophages (splenic & alveolar), DCs and neutrophils. Notably, prophylactic administration of ATYR1923 or iMod.COMP led to a significant and dose-dependent reduction in LPS-induced neutrophil infiltration into the bronchoalveolar space. This finding appeared to be specific to neutrophil trafficking, since the number of monocytes, alveolar macrophages, or other myeloid cells was not altered. Altogether, these results suggest that the activity of ATYR1923 includes inhibition of neutrophil migration to inhibit lung inflammation.

Introduction

- A number of non-canonical functions of proteins generated from tRNA synthetase genes have been reported, demonstrating diverse roles for these proteins outside of protein synthesis (Wakasugi & Schimmel, 1999; Park et al., 2008; Arif et al., 2017).
- Proteins derived from the histidyl-tRNA synthetase (HARS) gene are found extracellularly and are thought to play a role in regulating key cells in the immune system to ensure appropriate control of immune responses.
- ATYR1923 is a clinical stage immunomodulatory protein.
- ATYR1923 comprises the iMod domain of HARS fused to Human IgG Fc (Fig 1.) to extend plasma half-life.
- NRP-2 was identified to be a binding partner of ATY1923.
- ATYR1923 exerts some of its immunomodulatory functions by affecting T cell activation & cytokine release (data presented at AAI 2018 by E. Mertcshing).
- NRP-2 was shown to play a role in airway inflammatory responses to inhaled LPS (Immormino et *al.* 2018)

Fig 1. Generation of HARS-Derived Proteins

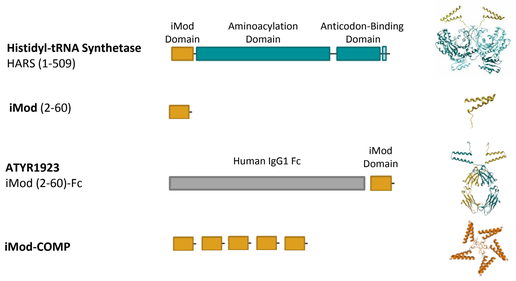


Fig. 1 Schematic representation of HARS, iMod domain, ATYR1923 & iMod-COMP

In vivo Experimental Procedure

Group	Test Article (TA)	Dose (mg/kg)	TA Route	LPS Treatment (µg)	Time Point (hrs)
1	NA				24
2	Vehicle	0	IV	0	24
3	Vehicle	0	IV	10	24
4	ATYR1923	1	IV	10	24
5	ATYR1923	3	IV	10	24
6	ATYR1923	10	IV	10	24
7	iMod-comp	3	IV	10	24

Table 1. In vivo study design

- Day -1: Body weights (BW) for TA administration were recorded & animals were dosed with TA at 5ml/kg according to the table above
- Day 0: Mice in grps 3-7 were anesthetized at 2-4% Isoflurane (1L/min.) & dosed OP (oropharyngeally) with 10 ug LPS in 50 µL PBS. Grp 2 received 50 µL PBS only. Grp 1 mice were naïve to induction & treatment.
- Day 1: BWs were recorded and mice were euthanized with lethal ketamine/xylazine cocktail (~300/30 mg/kg) at 24 hours post LPS induction. Blood was collected from the abdominal vein, processed for serum for pathway analysis (cytokine, ATYR1923 &/or NRP2 levels). The lung and trachea were exposed & perfused for BALF (bronchoalveolar lavage) collection using 0.8 ml PBS through cannulated trachea. Collected BALF was placed on ice and volume recorded. BALF cells were collected by centrifugation and supernatant was retained for potential measurement of pathway analysis (HARS pathway proteins, cytokines and/or ATYR1923 etc.). RBC lysis was applied to BALF cells & stained for flow cytometry analysis. Results from flow analysis were analyzed using FlowJo & statistical analysis performed using Prism.

Fig 2. Flow Cytometry Gating Strategy

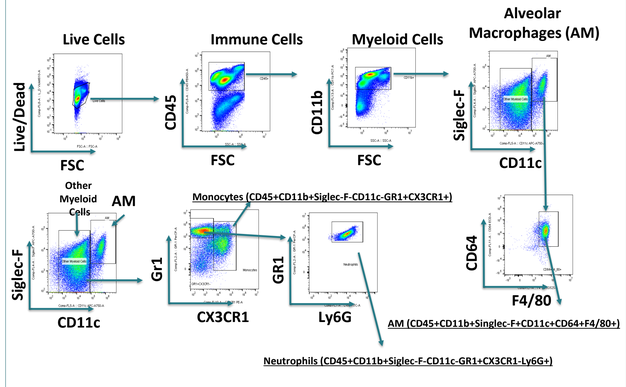


Fig. 2 Flow cytometry dot plots demonstrating the gating strategy utilized to identify immune cells, myeloid cells, alveolar macrophages (AM), monocytes and neutrophils.

In vitro Experimental Procedure

Fig. 3 Generation of mouse BMDM

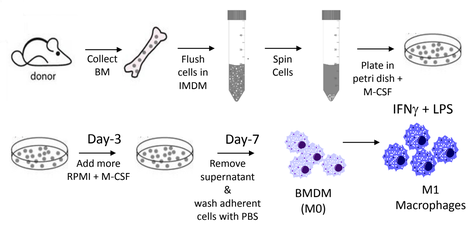


Fig 4. Generation of Human Macrophages from THP1 Cell Line

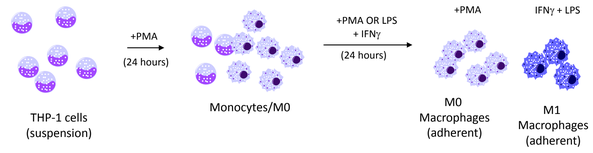


Fig 5. Generation of Human Primary Macrophages

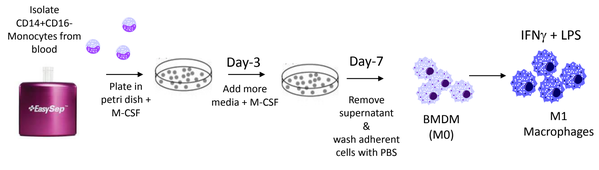


Fig 6. Generation of Human Primary Dendritic Cells

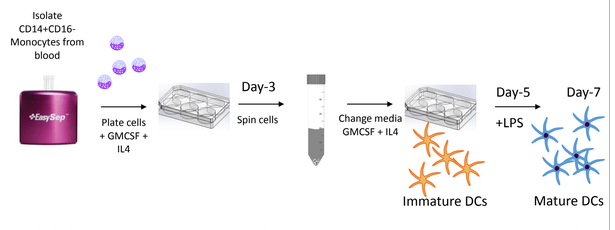
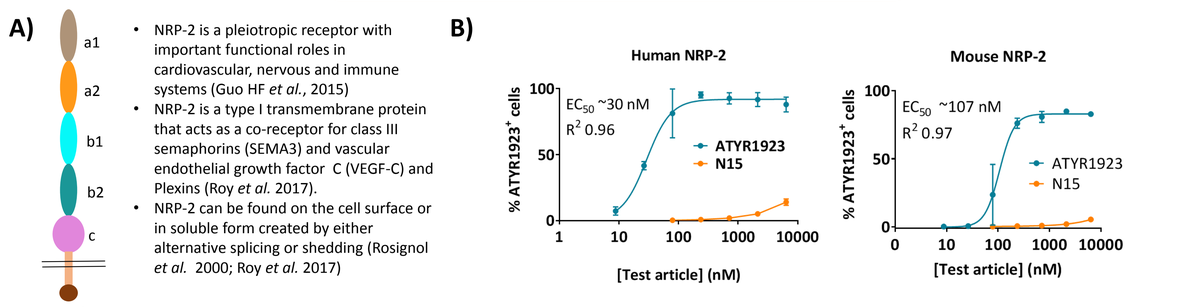


Fig.3-6 Schematic representations showing the experimental procedures utilized to generate: mouse Bone Marrow Derived Macrophages (Fig 3); human macrophages from THP1 cell line (Fig 4); human primary macrophages from monocytes (Fig 5); human primary dendritic cells (Fig 6).

Results

Fig. 7 ATYR1923 Binds Human and Mouse NRP-2



- NRP-2 is a pleiotropic receptor with important functional roles in cardiovascular, nervous and immune systems (Guo HF *et al.*, 2015)
- NRP-2 is a type I transmembrane protein that acts as a co-receptor for class III semaphorins (SEMA3) and vascular endothelial growth factor C (VEGF-C) and Plexins (Roy *et al.* 2017).
- NRP-2 can be found on the cell surface or in soluble form created by either alternative splicing or shedding (Rosignol *et al.* 2000; Roy *et al.* 2017)

Fig 7. A) Schematic representation of NRP-2 showing various domains (a1, a2, b1, b2 and c). B) EC50 curves of ATYR1923 binding to human NRP-2 or mouse NRP-2 in a HEK293 overexpressing system; N15 is an Fc control for ATYR1923. %ATYR1923+ cells was identified by flow cytometry based binding assay.

Fig. 8 In Vitro NRP-2 Expression

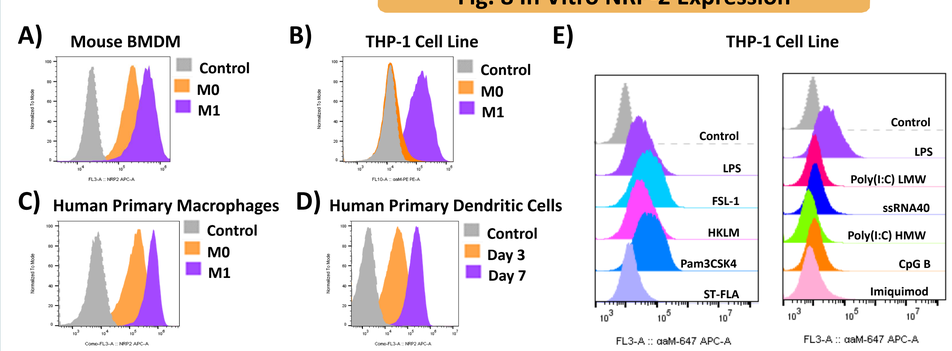


Fig 8. Histogram representation of NRP-2 expression detected on the cell surface of various in vitro generated myeloid cells. A) M0 and M1 macrophages generated from mouse bone marrow cells. B) M0 and M1 macrophages generated from human THP-1 cell line. C) M0 and M1 macrophages generated from primary human monocytes. D) Day 3 and Day7 of human dendritic cells differentiated from primary monocytes. E) M0 Human THP-1 cells stimulated with various TLR ligands as indicated.

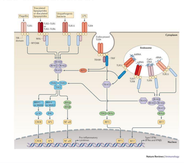


Fig. 9 ATYR1923 Reduces Neutrophil Infiltration in BALF

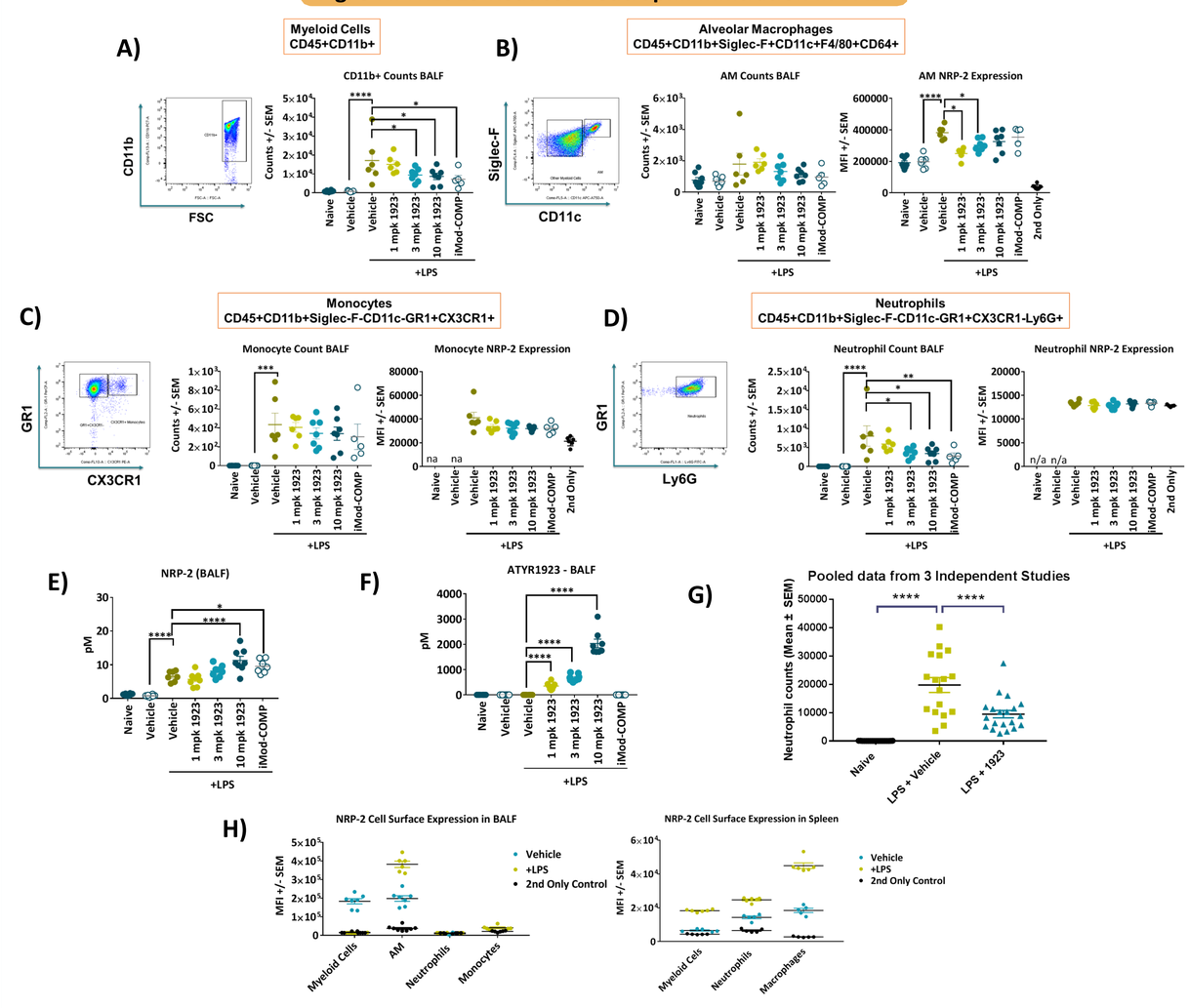


Fig 9. Results obtained from BALF Immunophenotyping A) Dot plot and immune cell count. B) Dot plot, Myeloid cell count and NRP-2 .C) Dot, plot, Monocyte cell count and NRP-2 expression. D) Dot plot, Neutrophil count and NRP-2 expression. E) Soluble NRP2 in BALF. F) Levels of ATYR1923 detected in BALF. G) Neutrophil counts across three independent studies H) NRP-2 expression across various Myeloid cells. Mean values +/- SEM. Statistical analysis: one-Way ANOVA; Dunnett's Multiple Comparison.

Conclusions

- **ATYR1923 binds to both human and mouse NRP-2.**
- **NRP-2 is detected on the cell surface of myeloid cells both *in vitro* and *in vivo.***
- **NRP-2 was induced following activation of TLR found on the cell surface (mainly TLR1, 2, 4, 5 & 6), but not endosomal TLR ligands (TLR3, 7/8, & 9).**
- **In an acute LPS lung injury model, a significant increase in immune cell infiltration and NRP-2 expression was observed in positive control group.**
- **ATYR1923 significantly decreased the CD11b+ population following LPS installation in the lung, which was ascribed to an inhibitory effect on neutrophil infiltration.**
- **These findings highlight the potential of ATYR1923 to regulate myeloid cell biology during lung inflammation.**

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