A novel neuropilin-2 (NRP2) antibody for immunohistochemical staining of patient tissue samples

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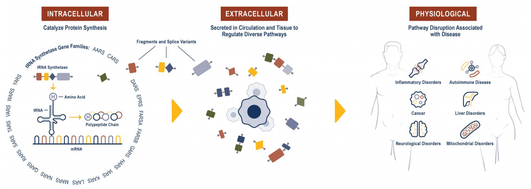
Abstract

Neuropilin-2 (NRP2) is a cell surface receptor that has been identified as the binding partner for efzofitimod, a novel immunomodulatory molecule in development as a potential treatment of pulmonary sarcoidosis and other interstitial lung diseases (ILDs). To date, the availability of reagents for immunohistochemical (IHC) detection of NRP2 protein in patient tissue samples has been less than ideal, as commercially available anti-NRP2 antibodies tend to display high non-specific staining. We aimed to generate and validate a specific and sensitive anti-NRP2 antibody for use in IHC staining of patient tissue samples. We have generated a panel of anti-human NRP2 monoclonal antibodies for a variety of research uses. A promising candidate for IHC was identified by Western blotting and validated by staining cells known to be positive and negative for NRP2 followed by specific blocking with NRP2 peptides. This novel antibody against human NRP2 was validated for use in IHC and was shown to be highly specific for NRP2, binding to all major splice variants while displaying negligible background staining and no cross-reactivity with neuropilin-1. Using this antibody, we demonstrated a high level of NRP2 protein expression in granulomas of sarcoidosis patient lung and skin biopsy samples. This antibody provides a specific and sensitive IHC detection method for NRP2 in patient tissue samples and could be a useful clinical tool (e.g. for facilitating patient selection or as a predictive biomarker) in sarcoidosis, other ILDs, oncology and other indications in which NRP2 plays a role.

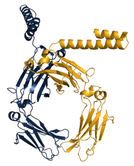
Introduction

Figure 1. Efzofitimod, a novel immunomodulatory molecule that binds to the NRP2 receptor

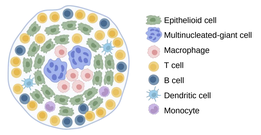
Novel functions of tRNA synthetases



Efzofitimod crystal structure



Sarcoid granuloma

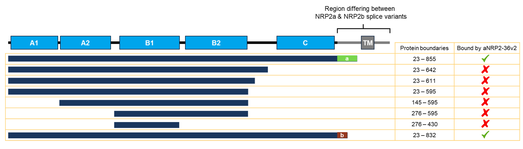


- The canonical role of tRNA synthetases is to catalyze protein synthesis within the cell. In addition to this, splice variants and proteolytic fragments of tRNA synthetases have been identified in extracellular spaces, where they have novel functions separate from their essential role in protein synthesis. 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 is a novel therapeutic molecule based on a histidyl-tRNA synthetase domain. It has been shown to have immunomodulatory activity and is currently in clinical development for treatment of pulmonary sarcoidosis and has potential in other ILDs. Treatment with efzofitimod has been shown to reduce pro-inflammatory and sarcoidosis disease biomarkers in pulmonary sarcoidosis patients.[1]
- Sarcoidosis is an inflammatory disorder of unknown etiology. It is characterized by formation of non-caseating epithelioid cell granulomas, which can result in decreased lung volume and trigger fibrosis in patients with pulmonary involvement. Granulomas consist of epithelioid and multinucleated-giant cells encircled by inflammatory cells such as macrophages, T cells, B cells and dendritic cells.
- Neuropilin-2 (NRP2) has been identified as the sole binding partner of efzofitimod.[2] NRP2 is a cell surface receptor that is upregulated on key immune cells within sarcoid granulomas.[3]
- We have generated a panel of anti-human NRP2 monoclonal antibodies for a variety of uses. An important use case in which a suitable antibody was not readily available is immunohistochemical detection of NRP2 in patient tissue samples.

Results

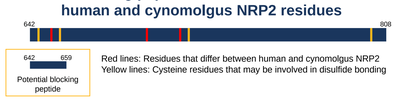
Figure 2. Identification and characterization of an anti-NRP2 antibody

Binding to recombinant NRP2 proteins by ELISA

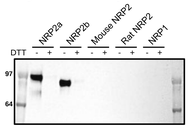


Protein boundaries	Bound by aNRP2-36v2
23 – 855	✓
23 – 642	✗
23 – 611	✗
23 – 595	✗
145 – 595	✗
276 – 595	✗
276 – 430	✗
23 – 832	✓

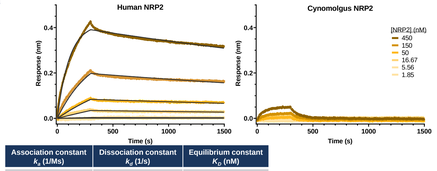
NRP2 blocking peptide based on non-conserved human and cynomolgus NRP2 residues



Specificity for NRP2 by Western Blot

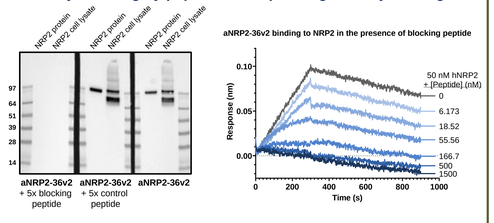


Affinity and specificity for NRP2 by BLI



Association constant K_a (1/Ms)	Dissociation constant K_d (1/s)	Equilibrium constant K_D (nM)
1.56×10^4	2.11×10^{-4}	13.6

Antibody blocking by peptide corresponding to likely binding site

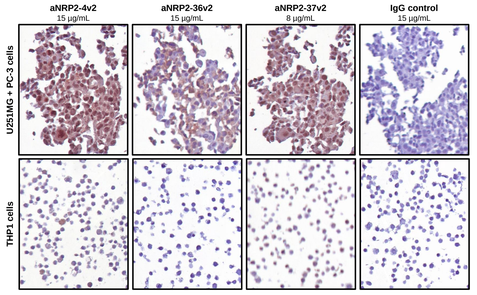


- We generated a panel of NRP2 antibodies from mouse immunizations. While antibodies suitable for flow cytometry staining of NRP2 performed poorly when tested in IHC, antibodies strongly reactive to denatured protein via Western blot showed more promise. One of the antibodies, designated aNRP2-36v2, was selected for further testing.
- Binding of aNRP2-36v2 to human NRP2 was confirmed by ELISA, Western blotting and bio-layer interferometry (BLI). Epitope mapping by ELISA using recombinant NRP2 proteins with truncated domains narrowed down the binding site to residues 642-808.
- aNRP2-36v2 recognizes both NRP2a and NRP2b isoforms but does not recognize NRP2 in which disulfide bonds have been reduced.
- aNRP2-36v2 is specific for human NRP2, does not cross-react with mouse, rat or cynomolgus NRP2 and does not bind NRP1.
- Based on binding to the various recombinant NRP2 proteins, protein sequence differences between human and cynomolgus NRP2, and proximity to cysteine residues that may form disulfide bridges, we identified the likely binding site and generated a synthetic NRP2 peptide of that site.
- We confirmed that the peptide blocks binding of the antibody to NRP2 by Western blotting and BLI.

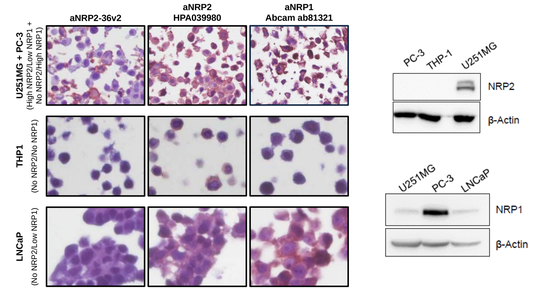
References:
1. Adams et al., Efzofitimod (ATYR1923) Treatment Reduces Pro-inflammatory Serum Biomarkers in Pulmonary Sarcoidosis Patients, ATS 2022, P560
2. Xu et al., ATYR1923 Specifically Binds To Neuropilin-2, A Novel Therapeutic Target For The Treatment Of Immune-mediated Diseases, ATS 2020, P1173
3. Paz et al., Neuropilin-2, The Specific Binding Partner To ATYR1923, is Expressed In Sarcoid Granulomas and Key Immune Cells, ATS 2020, P983

Figure 3. Validation of a novel NRP2 antibody for IHC staining

IHC staining with different aTyr NRP2 antibodies

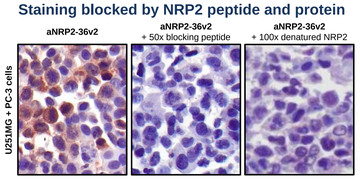


Specificity for NRP2 over NRP1



- Brief IHC protocol: Samples were treated with heat induced epitope retrieval at pH 6 or pH 9, for NRP2 or NRP1, respectively, blocked with BSA, incubated with primary antibody and developed using the VECTASTAIN Universal Elite ABC Kit and ImmPACT NovaRed chromogen.
- Initial IHC validation was done by staining FFPE cell block slices containing a mixture of U251MG and PC3 cells. U251MG express NRP2 while PC3 do not. THP1 cells which do not express NRP2 were used as a negative control.
- aNRP2-36v2 showed strong and specific staining of only the U251MG cells and not PC3 or THP1 cells, whereas two additional NRP2 antibodies (aNRP2-4v2 and aNRP2-37v2) which also performed well in Western blotting showed non-specific staining when used in IHC.
- Lack of binding to NRP1 was confirmed by lack of staining of PC3 and LNCaP cell lines which do express NRP1. aNRP2-36v2 was compared to a commercial antibody (HPA039980) and appears to be more specific when used under the same staining conditions.
- Specificity was demonstrated by addition of excess NRP2 peptide or denatured recombinant NRP2 protein, which blocked staining by aNRP2-36v2.
- Specificity of the antibody for NRP2 in a complex tissue matrix was confirmed by staining sections from Colo357 xenograft samples that had been treated with siRNA to knockdown NRP2.

Staining blocked by NRP2 peptide and protein



Colo357 xenograft samples treated with NRP2 siRNA

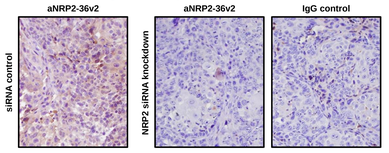
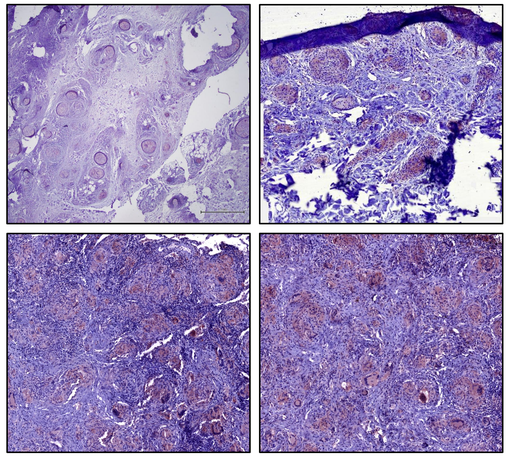
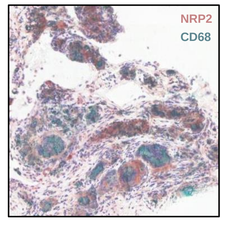


Figure 4. NRP2 is highly expressed in granulomas of sarcoidosis patient tissue as evidenced by IHC staining

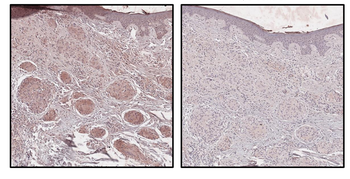
Lung biopsy tissue from sarcoidosis patients stained for NRP2



Lung biopsy tissue from sarcoidosis patient co-stained for NRP2 and CD68



Skin biopsy tissue from sarcoidosis patient



- NRP2 in both skin and lung tissue samples from sarcoidosis patients is highly expressed throughout granulomas but not in the surrounding tissue.
- Lung tissue from a sarcoidosis patient was co-stained for NRP2 and the CD68 macrophage marker. Granulomas in this sample were composed primarily of CD68+ macrophages that express NRP2. Further staining with different markers is planned in order to better understand the various NRP2 positive cell types within granulomas that may be targets for the immunomodulatory effects of efzofitimod.

Conclusions

- We have identified and extensively validated an NRP2 antibody that is highly specific for NRP2 in IHC tissue staining. It demonstrates improved specificity compared to available commercial antibodies.
- IHC staining of sarcoidosis patient tissues from skin and lung biopsies demonstrates high NRP2 expression within granulomas. This antibody is also suitable for co-staining and granulomas that primarily consist of CD68+ macrophages were shown to also express NRP2.
- We are using this antibody to further understand the biology of key immune cells that make up these sarcoid granuloma structures and hope it may lead to identification of biomarkers for sarcoidosis and other indications in which NRP2 plays a role.

aTyr Pharma is open to providing this antibody to clinicians and researchers who might find it useful for research purposes. Kindly contact the authors.

Disclosures:
YEC, DS, EE, LB, KR, SP, CB, AC and LAN are employed by and own stock in aTyr Pharma, Inc. SF, YB and MM received funding from aTyr Pharma for this work.