Neuropilin-2 is Expressed on Immune Cells Present in the Tumor Microenvironment, and May Contribute to the Suppression of Immune Regulation leading to Progression and Metastasis of Cancer

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Abstract

Neuropilin-2 (NRP2) is a single transmembrane pleiotropic receptor, known to utilize VEGF receptors and plexins for signal transduction. NRP2 has recently been described to play a role in the progression of tumors and their metastasis. Studies have shown the importance of NRP2 in cell migration, antigen presentation, phagocytosis and cell-cell interaction within immune cells, however, the contribution of NRP2 to the progression and metastasis of cancer and immune regulation in the tumor microenvironment is still unknown. Our experiments aimed to identify which immune cells express the NRP2 receptor, and to elucidate the role of NRP2 in the suppression of T cells by myeloid cells that contribute to the progression of tumors. *In vitro*, we show that NRP2 is highly expressed on key immune cells implicated in cancer progression including tumor-associated macrophages (TAMs) generated from triple-negative breast cancer cell lines, and myeloid-derived suppressor cells (MDSCs). We show that both cell types suppress T cell proliferation and activation via flow cytometry by proliferation modelling and by decreased CD25 and CD69 expression in the myeloid cell/T cell co-cultures, respectively. In addition, cytokines in the suppression supernatants were measured utilizing the Meso Scale Discovery platform. A reduction of IFNγ, IL-2, IL-4 and IL-17A is seen in the T cell co-cultures, confirming a reduction in T cell activation. We also show an increase in NRP2 expression on matured dendritic cells (DCs), and that MDA-MB-231 differentiated TAMs prevent their maturation. We demonstrate this with the suppression of the production of anti-tumor IL-12, and by a decrease in CD83, CD86 and HLA-DR expression on DCs in TAM/DC co-cultures utilizing the Meso Scale Discovery platform and flow cytometry respectively. We also demonstrate that inducible T regulatory cells (Tregs) generated *in vitro* express high levels of NRP2. Using a variety of *in vivo* syngeneic models, we confirm that NRP2 is expressed on a variety of immune cells such as TAMS, DCs, MDSCs, and Tregs, demonstrating that our therapeutic target is expressed on important immune suppressive cells in both human and mouse systems. We show for the first time that NRP2 is highly expressed on the immune suppressive cells of the tumor microenvironment. These are key cells implicated in regulating the progression of tumors and their metastasis. These findings indicate the potential of NRP2 as a target for anti-cancer therapeutics, possibly through immune regulation of the tumor microenvironment.

Introduction

- NRP2 is a single transmembrane receptor, known to utilize VEGF receptors and plexins for signal transduction
- NRP2 has been described to play a role in the progression of tumors and their metastasis (Caunt et al, 2008)
- NRP2 has been shown to play a role in cell migration, antigen presentation, phagocytosis and cell-cell interaction within immune cells (Schellenburg et al, 2017)
- NRP2s contribution to the progression and metastasis of cancer and immune regulation in the tumor microenvironment is still unknown
- We aim to determine expression of NRP2 on a variety of immune cells in the tumor microenvironment, and ultimately its role on each of those cells

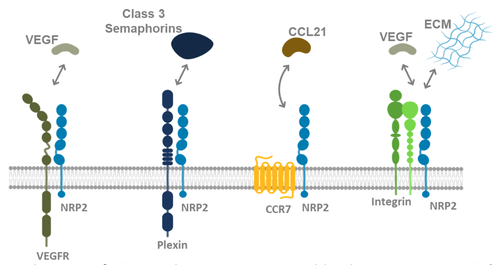


Fig 1. Schematic of NRP2 and its co-receptors and binding partners; VEGFR's with VEGF variants, Plexin with class 3 Semaphorins, CCR7 with CCL21 and Integrin with VEGF variants and the extracellular matrix (ECM)

Experimental Procedures

Fig 2. Generation of Primary Human MDA-MB-231 TAMs

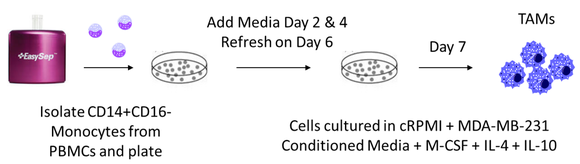


Fig 3. Generation of Primary MDSCs

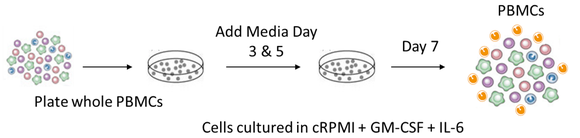


Fig 4. Generation of Primary Human Dendritic Cells

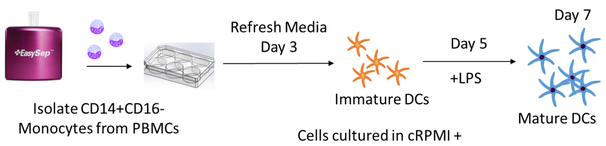
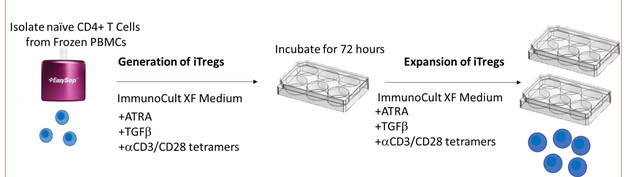


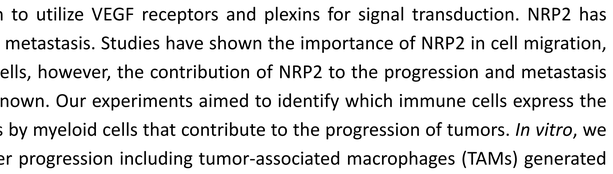
Fig 5. Generation of Primary Human inducible Tregs



Figs 2-5. Schematic representation showing the experimental procedures to generate: human TAMs from MDA-MB-231 conditioned media (Figure 2); human MDSCs (Figure 3); human dendritic cells (Figure 4) and human inducible Tregs (Figure 5). NRP2 levels were measured using flow cytometry and were analyzed using FlowJo and statistical analysis performed using Prism.

Fig 6. T cell Suppression Assay

Fig 7. DC Suppression Assay



Figs 6-7. Schematic representation showing the experimental procedures determining: suppression of T cells by TAMs or MDSCs (Figure 6) and suppression of DCs by TAMs conditioned media (Figure 7). Proliferation, activation and suppression were measured using flow cytometry. Cytokines measured by Mesoscale Discovery technology and statistical analysis performed using Prism.

In vivo Procedures

Table 1. Mouse Syngeneic Tumor Models

Tumor Model	Cell Line Name	Implantation Site	Organs Collected for Immunophenotyping
Breast Cancer	4T1	Flank	Tumor, TDLN
Colorectal Carcinoma	CT26.WT	Flank	Tumor, Spleen
Renal Adenocarcinoma	RENCA	IV	Lungs, Spleen

Table 2. Mouse Syngeneic Tumor Models

Cell Types	Markers Utilized to Identify Cell Types
TAMs	CD45+CD4-CD8-CD19-CD11b+GR1-F4/80+
MDSCs	CD45+CD45+CD4-CD8-CD19-CD11b+GR1+
DCs	CD45+CD4-CD8-CD19-CD11b^{low}CD11c+
Tregs	CD45+CD11b-CD11c-CD3+CD4+CD25+ (GITR+ or Foxp3+)

Table 1-2. *In vivo* syngeneic models (Table 1) utilized to determine NRP2 expression on isolated immune cells (Table 2) and the markers used to identify specific immune cell subsets. NRP2 levels were measured using flow cytometry and were analyzed using FlowJo and statistical analysis performed using Prism.

Results

Fig 8. *In vitro* NRP-2 Expression on Primary Human Immune Cells

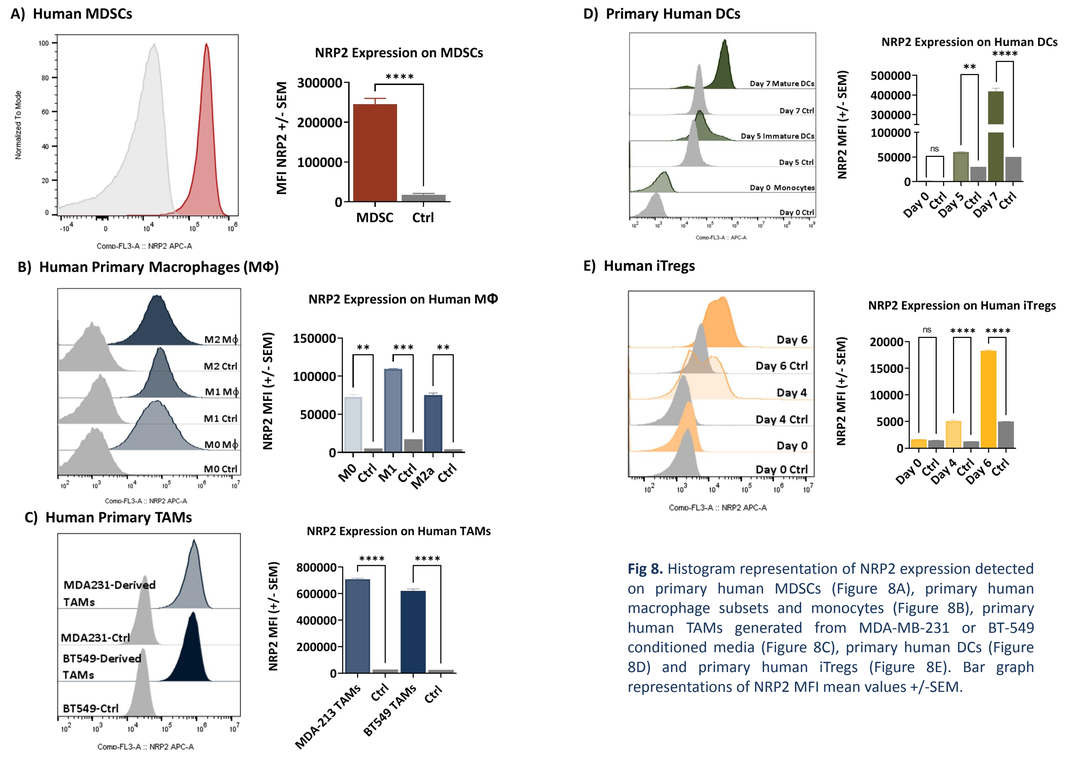


Fig 8. Histogram representation of NRP2 expression detected on primary human MDSCs (Figure 8A), primary human macrophage subsets and monocytes (Figure 8B), primary human TAMs generated from MDA-MB-231 or BT-549 conditioned media (Figure 8C), primary human DCs (Figure 8D) and primary human iTregs (Figure 8E). Bar graph representations of NRP2 MFI mean values +/-SEM.

Fig 9. TAMs and MDSCs Suppression of T cells & DCs

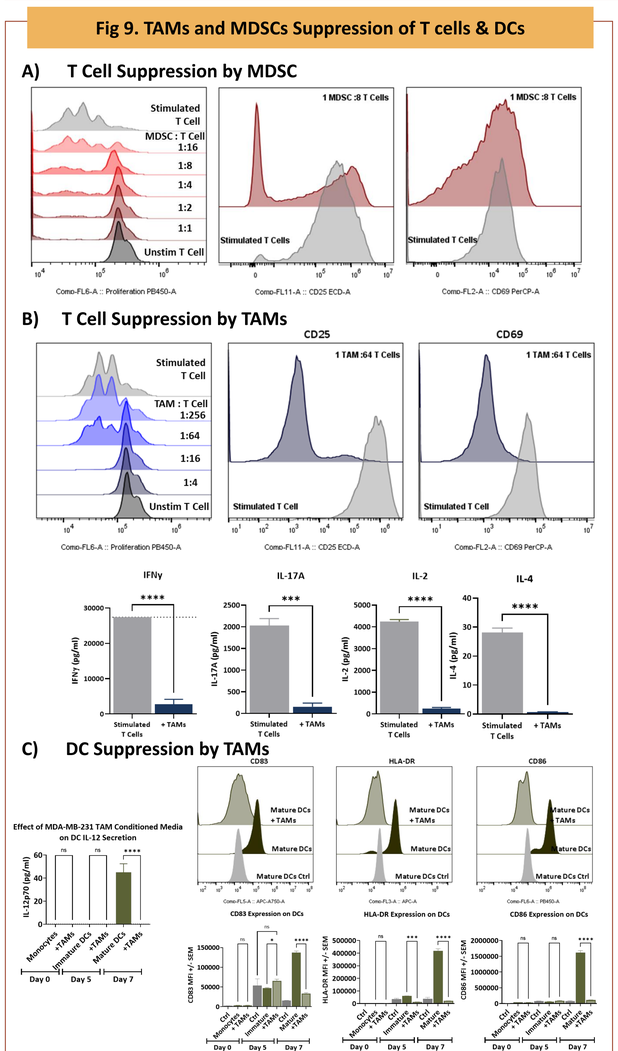


Fig 9. A) Suppression of T cells by MDSCs. Histogram representation of T cell proliferation (left panel) and activation markers CD25 and CD69 (right panels). **B)** Suppression of T cells by TAMs. Histogram representation of T cell proliferation (left panel) and activation markers CD25 and CD69 (right panels). Cytokine analysis of IFNγ, IL-17A, IL2 and IL-4 is represented in the bar graphs. **C)** Suppression of DCs by TAMs (MDA-231) conditioned media. Cytokine (IL-12) production (left panel), histogram representation of DC markers CD83, HLA-DR and CD86 (Top right panel) and corresponding bar graphs (Bottom right panel)

Fig 10. *In vivo* NRP-2 Expression on Murine Immune Cells

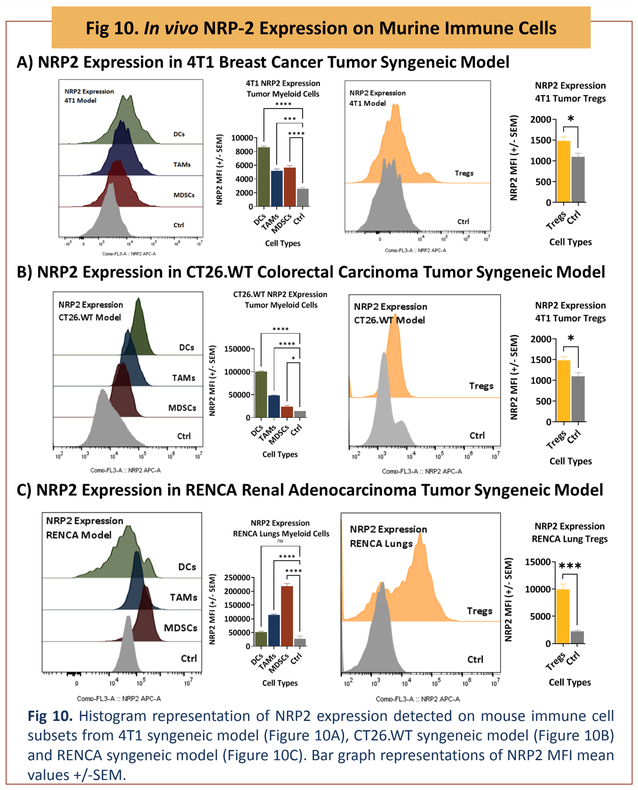


Fig 10. Histogram representation of NRP2 expression detected on mouse immune cell subsets from 4T1 syngeneic model (Figure 10A), CT26.WT syngeneic model (Figure 10B) and RENCA syngeneic model (Figure 10C). Bar graph representations of NRP2 MFI mean values +/-SEM.

Conclusions

- NRP2 was shown to be highly expressed on MDSCs, TAMs generated from triple negative breast cancer cell lines (MDA-231 & BT-549), mature DCs, and inducible Tregs, all of which are cells that contribute to suppression of the immune system and cancer progression
- MDSCs and TAMs generated were shown to be functionally active by their ability to suppress T cell proliferation, T cell activation markers (CD69 & CD25) and T cell cytokine production (IFNγ, IL-17A, IL-2 and IL-4).
- TAM conditioned media was sufficient to impair DC maturation as observed by a lack of IL-12 cytokine production and a reduction in CD83, HLA-DR and CD86 activation markers.
- NRP2 expression on TAMS, DCs, MDSCs and Tregs was confirmed in mouse cells using various *in vivo* syngeneic tumor models (4T1, CT26.WT and RENCA).
- We clearly show that NRP2 expression can be detected on key suppressive immune cells known to play an important role in regulating cancer progression. These findings highlight the potential of NRP2 as a target for anti-cancer therapeutics

References

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