APLNR regulates IFN-γ signaling via β-arrestin 1 mediated JAK-STAT1 pathway in melanoma cells

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Abstract

The apelin receptor (APLNR) regulates many biological processes including metabolism, angiogenesis, circulating blood volume and cardiovascular function. Additionally, APLNR is overexpressed in various types of cancer and influences cancer progression. APLNR is reported to regulate tumor recognition during immune surveillance by modulating the IFN-γ response. However, the mechanism of APLNR crosstalk with intratumoral IFN-γ signaling remains unknown. Here, we show that activation of APLNR upregulates IFN-γ signaling in melanoma cells through APLNR mediated β-arrestin 1 but not β-arrestin 2 recruitment. Our data suggests that β-arrestin 1 directly interacts with STAT1 to inhibit STAT1 phosphorylation to attenuate IFN-γ signaling. The APLNR mutant receptor, I109A, which is deficient in β-arrestins recruitment, is unable to enhance intratumoral IFN-γ signaling. While APLNR N112G, a constitutively active mutant receptor, increases intratumoral sensitivity to IFN-γ signaling by enhancing STAT1 phosphorylation upon IFN-γ exposure. We also demonstrate in a co-culture system that APLNR regulates tumor survival rate. Taken together, our findings reveal that APLNR modulates IFN-γ signaling in melanoma cells and suggests that APLNR may be a potential target to enhance the efficacy of immunotherapy.

Keywords: APLNR, mutant receptor, IFN-γ signaling, β-arrestin 1, STAT1 phosphorylation
Introduction

The human apelin receptor (APLNR) is a class A family G protein-coupled receptor (GPCR). Two distinct classes of endogenous peptide agonists, apelin and elabela (apela), have been identified.\(^1,2\) Although they show limited sequence homology, both peptides activate APLNR through G\(\alpha\)i signaling leading to intracellular cAMP inhibition. Activated APLNR subsequently recruits cytoplasmic scaffold proteins, \(\beta\)-arrestins (\(\beta\)-arrs), which mediate receptor desensitization, internalization, and activation of multiple signaling pathways. APLNR is ubiquitously expressed in many tissues and plays important roles in a variety of pathophysiological processes, such as vasoconstriction, muscle contractility, glucose and energy metabolism, angiogenesis and fluid homeostasis.\(^3,4\) Therefore, APLNR has been considered as a promising therapeutic target for the treatment of heart failure, pulmonary arterial hypertension, metabolic and renal diseases.\(^5\)

Recently, several studies revealed an important role for APLNR in tumor development. Increased expression of APLNR and its ligand, apelin, are observed in a number of cancers, such as non-small cell lung carcinomas, gastroesophageal, glioblastoma, colon, hepatocellular, prostate, endometrial, and oral squamous cell carcinoma.\(^6\) APLNR induces endothelial cell migration and proliferation, indicating a role in tumor neo-angiogenesis and tumor vascular maturation.\(^7\) Antagonizing APLNR signaling using APLNR antagonists showed enhanced anti-angiogenic efficacy in mouse tumor model. Zhao et. al demonstrated that targeting APLNR\(^+\) tumor vessels, genetically and pharmacologically, remarkably inhibits tumor angiogenesis and
growth. In addition, APLNR regulates tumor cell apoptosis through PI3K/AKT signaling pathway and promotes mesenchymal stem cell differentiation to cancer stem cells. Apelin-induced tumor vascular maturation enhances the efficacy of immunotherapy and significantly suppresses tumor growth by promoting the infiltration of NK cells and thereby inducing apoptosis of tumor cells. Interestingly, a genome-wide screen of loss-of-function mutations involved in the resistance to T-cell-based immunotherapies using CRISPR-Cas9 technology identified multiple APLNR mutants that reduce effector function of T cells. Functional loss of APLNR impairs CD8⁺ T cell cytotoxicity, which is associated with a lack of IFN-γ signaling in tumor cells, revealing the potential role of APLNR in regulating tumor immunity. However, the signaling pathways modulated by APLNR to influence the intratumoral IFN-γ response remains unknown.

Interferon-γ (IFN-γ) is a cytokine that belongs to interferon family that is produced primarily by T lymphocytes and natural killer cells. IFN-γ signaling, predominantly triggering JAK-STAT signaling cascade, is initiated when IFN-γ binds to its membrane receptor, IFN-γ receptor (IFNG), and induces JAK proteins phosphorylation and activation, following STAT1 phosphorylation and dimerization. The activated STAT1 homodimer translocates into the nucleus where it binds to interferon-stimulated response elements (ISREs), and thereby promotes transcription of IFN-γ-stimulated genes (ISGs). IFN-γ can play both antitumor and protumor roles. IFN-γ plays a major role in activating anti-tumor immunity either by directly exerting anti-proliferative and pro-apoptotic effects on tumor cells and by
promoting the antigen processing and presentation in tumor cells, or by promoting the activity of immune cells, such as Th1 CD4 helper T cells, CD8 cytotoxic T lymphocytes (CTLs), natural killer (NK) cells, dendritic cells (DCs), and macrophages. On the other hand, IFN-γ promotes tumorigenesis through altering IFN-γ signaling sensitivity in tumor cells to avoid immunoregulation, which is the principal intrinsic mechanism for tumor immune escape. Analysis of tumors in patients who did not respond to immune checkpoint blockade therapy (anti-CTLA-4 or anti-PD-1/PD-L1 axis) revealed that loss-of-function mutations occurred in the IFN-γ signaling pathway genes, such as IFNG, JAK1/2, STAT1 and IRF1. In addition, accumulating evidence indicates that chronic IFN-γ exposure ultimately stimulates negative feedback regulation on melanoma cells, thus resulting antitumor immunity suppression. For example, PD-L1, an important immune response negative regulator, is tightly regulated by IFN-γ signaling, which contributes to the acquired immune resistance in melanoma cells. The overall outcome of IFN-γ signaling depends on the intensity of the signal, tumor microenvironment and context of tumor specificity.

To further understand the mechanism of APLNR signaling on tumor immunity, with a focus on the crosstalk between APLNR and IFN-γ signaling pathways, we investigated how APLNR regulates IFN-γ signaling in melanoma cells. We demonstrate that APLNR mediated activation of β-arr1 in melanoma cells is required for IFN-γ signaling, which may provide further insight into how APLNR targeting drugs could modulate the tumor resistance to cell-based immunotherapy.
Material and methods

Reagents, siRNAs and plasmids

All commonly used reagents, siRNAs and primers for real-time PCR used in this study are listed in Supplementary Table S1-3 unless otherwise stated. Human APLNR, APLNR I109A, APLNR N112G and STAT1 were cloned into pIRESpuro3 vector with a HA tag at the N terminus. Human ARRB1 and ARRB2 were cloned into pIREShyg3 vector with a GFP tag at the C terminus. Human CD19 was cloned into pIREShyg3 vector. All gene synthesis and cloning were performed by GenScript. pGL4.45 [luc2p/ISRE] vector was purchased from Promega.

Cell culture, transfection and stable cell line generation

Melanoma A-375 (RRID: CVCL_0132) cells were obtained from the Cell Bank of the Chinese Academy of Sciences. HEK293FT (RRID: CVCL_6911) cell line was obtained from the American Type Culture Collection (ATCC). Both A-375 and HEK293FT cell lines were cultured in DMEM medium supplemented with 10% FBS, 1xPenicillin streptomycin (PS), 2 mM Glutamine, 1 mM Sodium pyruvate, 1xMEM non-essential amino acids (NEAA) (Life Technologies). Naïve CD8+ T cells used for co-culture assay were obtained from Allcells and cultured in RPMI 1640 medium supplemented with 10% FBS, 1xHEPES, 1xPS, 2 mM Glutamine, 1 mM Sodium pyruvate, 1xMEM NEAA. All human cell lines have been authenticated using short tandem repeat profiling within the last 3 years at GENEWIZ and all experiments were performed with mycoplasma-free cells. Plasmids were transfected by Lipofectamine LTX and Plus reagent (Invitrogen) and siRNAs were transfected by Lipofectamine
RNAiMAX reagent (Invitrogen) according to manufacturer’s instructions. The A-375 cells were used for all stable cell lines (A-375-ISRE, A-375-CD19, A-375-APLNR, A-375-APLNR I109A/N112G) generation. Briefly, cells were transfected with plasmids using Lipofectamine LTX and Plus reagent. Twenty-four hours after transfection, cells were selected with 250 μg/mL hygromycin or/and 0.5 μg/mL puromycin. And stable cell lines could be generated after 2 to 3 weeks selection.

**IFN-γ-stimulated reporter assay**

The A-375 cells stably expressing interferon-stimulated response elements (ISREs) and a Luc2 reporter gene were seeded onto 96-well plates at a density of 50,000 cells per well and transfected with 0.1 ng/well vector or 5 pmol/well siRNA alone or co-treated with inhibitors (Table S1) for 48 hours. After IFN-γ induction for the indicated time in starvation medium (DMEM with 1% FBS), ONE-Glo luciferase assay reagent (Promega) was applied following manufacturer’s protocol to detect the sensitivity of IFN-γ signaling.

**Whole-cell protein extraction and immunoblot analysis**

Cells were treated with IFN-γ (500 ng/mL) for the indicated time. Whole-cell extracts were obtained in buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM Na-Vanadate, 0.1 mM PMSF, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). SDS-PAGE was used to separate proteins. Anti-α-tubulin (#2144S), anti-PD-L1 (#13684S), anti-β-arrs (#4674S), anti-β-arr1 (#30036S), anti-STAT1 (#9172S), anti-HA (#3724S) antibodies were purchased from Cell Signaling Technology. Anti-phospho-Tyr701 STAT1
(#ab29045) antibody was obtained from Abcam. HRP-conjugated anti-mouse (#61-6520) and anti-rabbit (#65-6120) IgG secondary antibodies were purchased from Invitrogen.

**Flow cytometry**

Freshly harvested cells were plated in 96-well U-bottom plates (up to 8 x 10^5 cells/well) in PBS. Cells were incubated with surface antibodies (anti-human-CD274, Invitrogen Cat#17-5983-42; anti-human CD69, BioLegend Cat#310910) prepared in FACS buffer (1 x PBS with 0.1% BSA) for 30 min at 4 °C. Cells were washed twice with PBS and resuspended in FACS buffer. Flow cytometry was performed using the standard protocol recommended by manufacturer. FACS analysis was performed in IntelliCyt iQue Screener (Sartorius).

**Quantitative reverse transcription-PCR (qRT-PCR)**

Total RNA was isolated from melanoma cells with a RNeasy mini kit (Qiagen). cDNA was synthesized using the high capacity cDNA reverse transcription kit (Applied Biosystems). 5 μl of the RT reaction was used in the quantitative real-time PCR using SYBR Green (Applied Biosystems). The primers used in qRT-PCR were listed in Supplementary Table 3. The relative target gene mRNA level was calculated using the ΔCt method. The threshold cycle (Ct) values of the target gene mRNAs were initially normalized to the Ct values of the internal control GAPDH in the same samples. The fold change was determined using the (2^-ΔΔCt) method. The threshold cycle (Ct) values of the target gene mRNAs were listed in Supplementary Table 4. The relative target gene mRNA level represents an average fold calculated from separate experiments.
**AlphaLISA assay**

A-375 cells were seeded onto a 96-well plate (50,000 cell/well) and transfected with 0.1 ng/well of plasmid. 48 hours after transfection, cells were stimulated with 500 ng/mL IFN-γ for 15, 30 and 60 min, respectively. At the end of treatment, the phosphorylation of STAT1 (Y701) was measured with AlphaLISA assay detection kit (PerkinElmer Cat#ALSU-PST1-A500) according to manufacturer’s protocol. Briefly, cells on 96-well plate were washed 3 times with PBS and lysed with 50 μl lysis buffer. The cell lysates of 10 μl were transferred to a 384-well Optiplate (PerkinElmer Cat#6007290) and incubated with 5 μl acceptor mix at room temperature in the dark. After one-hour incubation, a total of 5 μl of donor mix was added. The plate was incubated for additional one hour before read with Envision plate reader using standard AlphaLISA settings.

**Co-immunoprecipitation**

HEK293FT cells were seeded onto a 6-cm culture dish at 60% confluency and transfected with 2 μg of plasmid. Forty-eight hours after transfection, cells were washed with ice-cold PBS and lysed with ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktail). Cells were collected by scraping and transferred to a pre-cooled microcentrifuge tube. The cells were lysed for 30 min at 4 °C with a slow rotation before centrifuged for 15 min at 13, 200 rpm. Supernatants were aspirated and placed in a fresh tube on ice. The rest of the procedure was performed under the anti-HA magnetic beads according to the manufacturer’s instructions (Thermo Scientific). Briefly, 25 μl of the pre-washed
(TBST, 0.05% Tween-20) magnetic beads were added to the supernatant and incubated at room temperature for 1 hour with rotation. The beads were washed three times with TBST and eluted in IgG elution buffer (Invitrogen). Samples were subjected to Western blot analysis.

**cAMP assay**

Intracellular cAMP was measured by Lance Ultra cAMP kit (PerkinElmer Cat#TRF0263) as described previously. Raw data were normalized on the basis of data from positive and negative control wells. Dose-response curves were fitted using GraphPad Prism with a four-parameter Sigmoidal function.

**Confocal microscopy**

HEK293FT cells co-expressing β-arr1-GFP with HA-tagged APLNR or APLNR N112G were seeded into an 8-well chamber slide and grown for 40 hours before stimulated with agonist. Cells were stained with Alexa Fluor 594 conjugated anti-HA antibody (Invitrogen) and imaged with Leica confocal microscope as described previously.

**Molecular modeling**

The initial model of the wild type human APLNR (amino acids 19-330) was built via homology modeling in Discovery Studio, using the previously published APLNR-AMG3054 structure as a template. The ICL3 loop region was optimized with Prime Refine Loops in Schrödinger. The PPM server was used to calculate rotational and translational positions of APLNR in a membrane. The ligand-free APLNR model was minimized with Prime in Schrödinger considering implicit
membrane environment. Then, it was embedded in POPC membrane with TIP3P explicit water model and counterions under the OPLS3 force field. Sodium and chloride ions were added at a concentration of 150 mM. Molecular dynamics simulation was performed on the system with Desmond in Schrödinger. The system was relaxed before simulation with default protocol. 60 ns simulation was performed in the NPT ensemble with the temperature set at 300 K and the pressure set at 1 bar. N112\textsuperscript{3,35} on APLNR wild type structure model was mutated to Gly to generate the APLNR N112G mutant model, followed by minimization and 60 ns MD simulation as described above.

**T-cell-mediated cytotoxicity assay**

Naïve CD8+ T cells were activated by stimulating with 10 ng/mL IL-2 (Peprotech Cat#200-02), 2 μg/mL anti-CD3 antibody (BD Biosciences Cat#555336) and 2 μg/mL anti-CD28 antibody (BD Biosciences Cat#555725) for 5 days. A-375-CD19 stable cells expressing APLNR or APLNR mutant I109A or N112G were seeded onto 96-well plates at a density of 20,000 cells per well. To each sample well, 10,000 effector T cells (for E: T ratio of 0.5) were added and incubated with different concentrations of blinatumomab (Blinicyto, Amgen) for the indicated time period. At the end of the incubation, cells were washed with PBS 3 times, then lysed, then detected using CellTiter-Glo assay reagents (Promega) and read with Envision plate reader.

**Statistical analysis**

All values are expressed as the Mean ± SD. Statistical analysis was carried out
using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Concentration-response curves for cAMP were fitted to a non-linear regression (four-parameter dose-response curves-stimulation) model to determine pEC50 with Prism software. Values were shown as mean ± SD of at least three independent experiments. Pairwise comparisons were made using Student’s t-test. One-way ANOVA followed by Tukey’s post hoc test was used in the event of multiple group comparisons. Statistical significance is indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s. not significant. P values <0.05 are regarded as statistically significant.

Results

APLNR regulates intratumoral IFN-γ signaling

To investigate whether APLNR regulates IFN-γ signaling in melanoma cells, we first developed a reporter system by introducing IFN-γ-stimulated response elements (ISREs) that drive luciferase expression into the A-375 cell line to monitor intracellular IFN-γ signaling. In this cell line, IFN-γ stimulation generated a significant response in an IFN-γ concentration-dependent way (Figure S1A). siRNA-mediated silencing of IRF1, the key transcription factor mediating cellular IFN-γ signaling, decreased the IFN-γ response as observed by a significant decrease in luciferase activity (Figure S1B). While, IRF1 knockdown had no effect on IFN-γ response activity without IFN-γ exposure (Figure S1B). The expression of PD-L1, an inhibitor for T cell immunity, was confirmed to be upregulated by IFN-γ in A-375 melanoma cells (Figure S1C). Together, these data suggest that the IFN-γ reporter
system can be used to evaluate the sensitivity of IFN-γ signaling.

Next, we assessed the role of APLNR on IFN-γ signaling by siRNA-mediated APLNR knockdown using this reporter system. IFN-γ-stimulated response in cells transfected with APLNR siRNA was reduced by 2-fold when compared to the control cells transfected with non-targeting siRNA (Figure 1A). Consistent with this result, APLNR knockdown significantly inhibited PD-L1 expression that was induced by IFN-γ in A-375 cells (Figure 1B). siRNA-mediated knockdown of APLNR was confirmed by qRT-PCR (Figure 1C). These results indicate that APLNR directly regulates IFN-γ signaling in melanoma cells. To further test APLNR mediated effects on IFN-γ signaling, we utilized an endogenous APLNR ligand, apelin-17 (Apnl-17), and found that it significantly increased the IFN-γ-stimulated response in A-375 cells overexpressing APLNR (Figure 1D). Together, these results suggest an important role of APLNR in regulating IFN-γ signaling in melanoma cells.

**APLNR mediated recruitment of β-arr1, but not β-arr2, negatively regulates the JAK-STAT1 signaling pathway**

APLNR can signal through G protein and β-arrs pathways. To dissect the contribution of these signaling pathways, we assessed the effect of siRNA-mediated knockdown of β-arrs on IFN-γ signaling using the IFN-γ reporter system. We found that β-arr1/2 dual silencing significantly increased IFN-γ-stimulated response (Figure 2A). This was driven by siRNA-mediated knockdown of β-arr1, as IFN-γ signaling was increased in β-arr1, but not in β-arr2 siRNA treated cells, even though the level of mRNA knockdown for both β-arr1 and β-arr2 were comparable (Figure 2A). PD-L1
expression was increased in β-arrows siRNA-mediated knockdown cells by Western blots (Figure 2B). We also found that knockdown of β-arr1, but not β-arr2, significantly increased PD-L1 levels compared to control group as detected by flow cytometry (Figure 2C). Furthermore, we evaluated the effects of silencing β-arr1 or β-arr2 on IFN-γ-stimulated genes involved in immune regulation, antigen processing and presentation, apoptosis and JAK-STAT signaling. We found that siRNA-mediated knockdown of β-arr1, but not β-arr2, upregulated the expression of many IFN-γ-stimulated genes (Figure 2D). These data indicate that IFN-γ signaling in melanoma cells is negatively regulated by β-arr1. It is well known that β-arrows regulate GPCR downstream signaling pathways through MAPK, MEK/ERK and PI3K/AKT pathways, while JAK-STAT signaling is a dominant pathway in response to IFN-γ. To dissect the β-arr1 downstream signaling pathways that mediate IFN-γ response, we examined the effect of different kinase inhibitors using the IFN-γ reporter assay. After 24-hour IFN-γ induction, among three inhibitors tested, only a JAK inhibitor (CP690550) suppressed the IFN-γ response activity, while the ERK (FR180204) and MEK (U01206) inhibitors had no effect (Figure 3A). Similar results were observed in cells following β-arr1 siRNA treatment, suggesting that β-arr1 regulates IFN-γ response through JAK-STAT signaling pathway (Figure 3A).

STAT1 is a key mediator in IFN-γ signaling and is activated by phosphorylation at residue Y701 (pSTAT1-Y701). We measured the effect of β-arr1 regulation on STAT1 activation by both Western blot and the AlphaLISA assay. Western blots revealed that the level of pSTAT1-Y701 was increased following β-arr1 siRNA
treatment in A-375 cells following a 15-min IFN-γ induction (Figure 3B). Overexpressing β-arr1 in A-375 cells, which validated by Western blot (Figure 3C), significantly reduced STAT1 phosphorylation (Y701) after 15-min IFN-γ treatment in the AlphaLISA assay (Figure 3C). These data suggest that β-arr1 attenuates JAK-STAT signaling by inhibiting STAT1 phosphorylation (Y701) upon IFN-γ exposure. Additionally, co-immunoprecipitation showed that β-arr1, but not β-arr2, interacts with STAT1 in cells overexpressing HA-tagged STAT1 suggesting that β-arr1 may negatively regulate IFN-γ signaling through its physical interaction with STAT1 (Figure 3D and Figure S2).

**APLNR I109A mutant fails to promote intratumoral IFN-γ signaling activity**

To provide additional evidence that APLNR regulates IFN-γ signaling through β-arrs recruitment, we compared the effects of the APLNR agonist on IFN-γ signaling in cells expressing either the wild type or the β-arrs recruitment deficient APLNR mutant, APLNR I109A. APLNR I109A was generated based on the APLNR/peptide ligand crystal structure and has been shown to retain normal G protein activity but lacks β-arrs-mediated signaling. Here, it serves as a pharmacological tool to dissect the contribution of APLNR mediated G protein vs β-arrs signalling induction.

APLNR activation by two endogenous peptide ligands (apelin and elabela/apela) enhanced the IFN-γ response in cells overexpressing wild type APLNR (Figure 4A). However, these APLNR agonists had no effect on IFN-γ response in cells with endogenous APLNR (Figure S3A) or overexpressing the APLNR I109A mutant suggesting that β-arrs are required for APLNR induction of IFN-γ signaling.
Additionally, Apln-17 stimulation did not enhance IFN-γ-induced expression of PD-L1 or IRF1 in WT APLNR as well as APLNR I109A cells, when compared to the vehicle treated group (Figure 4C-D and Figure S3B-C). Together, these data suggest that APLNR modulates IFN-γ signaling in melanoma cells through β-arrrs signaling.

**APLNR N112G, a constitutively activated receptor, activates IFN-γ signaling**

The residue at position 3.35 on TM3 plays an important role in the activation of class A GPCRs. Mutations at this position such as N1193.35A of CXCR4 or N1113.35G of angiotensin II type-1 (AT1) receptor can induce constitutive activity of the receptor, which is referred to as a constitutively active mutant (CAM). Since APLNR also possesses an Asn at position 3.35, we generated N1123.35G mutant to investigate whether it can similarly lead to constitutive activity. Cells expressing APLNR N112G mutant receptor maintain relatively high activity in a G-protein signaling assay that monitors changes in the level of the second messenger, cAMP, even in the absence of agonist stimulation (Figure 5A). In contrast, wild type APLNR showed agonist dose-dependent activation. Next, we monitored β-arrr recruitment and receptor internalization using fluorescence confocal microscopy. In wild type APLNR expressing cells, Apln-13 treatment caused the APLNR/β-arrrs complex internalization as observed by large cytosolic intracellular vesicles. Interestingly, APLNR N112G exhibited constitutive internalization and β-arrr recruitment in the absence of agonist stimulation (Figure 5B). Results from both the cAMP assay and confocal microscopy indicate that N112G converted APLNR into a constitutively active receptor. Molecular modeling using wild type APLNR and N1123.35G mutants in the absence of
a bound ligand suggest that the Asn to Gly mutation results in decreased size of side chain at position 112 (3.35), leaving more room for adjacent residues (Figure 5C-D). Molecular dynamic (MD) simulations suggest that the adjacent F78\textsuperscript{2.53} side chain in N112\textsuperscript{3.35}G mutant model can form a hydrophobic triad of contacts with M113\textsuperscript{3.36} and W261\textsuperscript{6.48}, which is at the transmission switch CWXP.\textsuperscript{35} This could facilitate APLNR activation and is consistent with the mechanism of activation in CXCR4 N119\textsuperscript{3.35}A CAM.\textsuperscript{36}

Next, we tested the effect of constitutively active APLNR N112G on IFN-\(\gamma\) signaling in the reporter assay. APLNR N112G cells demonstrated enhanced IFN-\(\gamma\) signaling, higher than that in wild type APLNR cells stimulated by Apn-13 (Figure 5E). Consistent with this result, the phosphorylation of STAT1 was increased after 15-min IFN-\(\gamma\) induction in cells expressing APLNR N112G mutant (Figure 5F). Both PD-L1 protein and IRF1 mRNA levels were significantly increased in APLNR N112G mutant after 24-h IFN-\(\gamma\) exposure (Figure 5G-H). Together, these data reveal that the constitutively active APLNR mutant, N112G, positively regulates IFN-\(\gamma\) signaling in melanoma cells. Notably, APLNR N112G failed to enhance IFN-\(\gamma\) signaling in the absence of \(\beta\)-arr1 in A-375 cell (Figure S4), further suggesting that APLNR mediated recruitment of \(\beta\)-arr1 is required for the regulation of IFN-\(\gamma\) signaling in melanoma cells.

Antigen presentation and processing genes are induced by IFN-\(\gamma\).\textsuperscript{37} Since we observed crosstalk between IFN-\(\gamma\) signaling and APLNR signaling in melanoma cells, we wanted to determine if APLNR regulates the genes involved in MHC class I
antigen processing and presentation, such as beta-2-microglobulin (B2M), TAP1, TAP2 and TAPBP, which are important for the recognition and elimination of CD8⁺ T cells. In wild type APLNR expressing cells, agonist stimulation significantly enhanced the expression of all four genes tested but not in APLNR I109A mutant cells that lack β-arrs signaling (Figure S5A-B). Constitutively active mutant APLNR N112G showed increased gene expression without agonist stimulation (Figure S5C). These results suggest that APLNR signaling exerts positive an effect on IFN-γ signaling and is involved in regulating the expression of antigen presentation and processing genes.

**Different activation modes of APLNR exhibit divergent immunoregulation in the co-culture system**

IFN-γ signaling plays an important role in tumor immunity. To further investigate how the immune effects of IFN-γ signaling is regulated by APLNR, we established a functional co-culture system to monitor T-cell-mediated cytolysis (Figure 6A). The co-culture system employs activated effector CD8⁺ T cells, which from naïve CD8⁺ T cells stimulated with IL-2 and anti-CD3/CD28 antibodies (Figure S6A), A-375 tumor cells ectopically expressing antigen CD19, and bi-specific T-cell engager (BiTE) blinatumomab, an anti-CD3 and anti-CD19 bi-specific antibody. Blinatumomab significantly promoted the efficiency of T-cell-mediated tumor killing (Figure 6B and S6B), while itself didn't affect the viability of tumor cells in the absence of effector T cells (Figure S6C), indicating blinatumomab is an effective CD19-directed T-cell engager in our co-culture system. To further optimize the co-culture system, we tested
different blinatumomab concentrations and incubation times at an effector T cell to tumor cell (E: T) ratio of 0.5. Tumor cell survival rates decreased with higher blinatumomab concentrations and longer co-incubation time (Figure S6D). To validate this co-culture system, PD-L1 knockdown by siRNA was performed in tumor cells. As expected, PD-L1 knockdown augmented T-cell-mediated tumor killing in co-culture assay due to its inhibitory role in immune regulation (Figure S6E).

To evaluate the influence of APLNR mediated signaling on T-cell-mediated cytotoxicity, we compared cell depletion in cells overexpressing APLNR or the APLNR mutants I109A and N112G. Surprisingly, in wild type APLNR cells, Apln-17 stimulation compromised the cytotoxicity of effector T cells (Figure 6C), even though activation of APLNR upregulates intratumoral IFN-γ signaling sensitivity. Consistent with this observation, Apln-17 failed to promote T-cell-mediated cytolysis in the APLNR G-protein biased mutant receptor, I109A, indicating β-ar1 signaling is required for the modulation of intrinsic anti-tumor activity by APLNR (Figure 6C). Constitutively active APLNR, APLNR N112G, slightly enhanced the T-cell-mediated cytolysis (Figure 6C). Overall, these results further highlight the importance of APLNR signaling in regulating melanoma susceptibility to T-cell-mediated cytolysis.

Discussion

IFN-γ modulates the transcription of genes involved in host defense and immune surveillance as well as in the establishment of adaptive immunity through the JAK-STAT signaling cascade.\textsuperscript{16, 38} Recently, APLNR has been reported to be essential for immunotherapy by directly interacting with JAK1 to modulate IFN-γ response in
In this study, we aimed to better understand how APLNR modulates the tumor IFN-γ signaling pathway and tumor immunity. We show that activation of APLNR upregulates IFN-γ signaling in melanoma cells and that this upregulation is mediated through β-arr1 but not β-arr2. Despite the fact that β-arr1 and β-arr2 share high sequence homology and similar functions in GPCR internalization and desensitization, they have different binding affinities, selectivity and distinct subcellular localization. β-arr1 contains a seven-residue nuclear localization sequence (NLS) which is critical to its interaction with several nuclear factors. This contrasts to β-arr2, which has a strong nuclear export signal in its C terminus that excludes it from the sustained presence in the nucleus. As a scaffold protein, β-arcs could interact with many intracellular partners, including JNK, Src and ERK. Here, we demonstrate that β-arr1 directly interacts with transcription factor STAT1 to inhibit STAT1 phosphorylation and negatively regulate IFN-γ signaling. Previous studies reported that endogenous β-arr1 interacts with JAK1 in naïve CD4+ T cells. Our results are consistent with reports showing that β-arr1 directly interacts with STAT1 in the nucleus following IFN-γ treatment, accelerates STAT1 dephosphorylation by recruiting tyrosine phosphatase (TC45) and consequently, negatively regulates the transcription of IFN-γ-induced genes.

GPCRs undergo substantial fluctuations between active and inactive states. Receptor ligands stabilize certain conformations that could specifically recruit cellular binding partners to transduce downstream signals. GPCR mutations are known to stabilize receptors at certain conformations and alter function.
mutation of Ile at TM3 region to Ala in APLNR converted the receptor to a G protein biased receptor. Here, we report a constitutively active mutant (CAM), APLNR N112G. The mutation occurs at position 3.35 on TM3 of APLNR. Similar mutations have been observed in CXCR4 N1193.35 A and angiotensin II type-1 (AT1) N1113.35 G receptors, which induce constitutive activity of the receptors. Naturally occurring constitutively active GPCR mutants are found in several hereditary and acquired human diseases, including cancers. For example, the CAMs of apoprotein opsin, which regulates photoreceptor cell signaling, have been found in retinal degeneration disease. The CAMs of glycoprotein hormone receptors are linked with thyroid, ovary and testis dysfunction and tumorigenesis. The APLNR N112G CAM can contribute to our understanding on the mechanism of receptor activation in the absence of agonist, especially how CAMs recapitulate the receptor active conformation. The mutant could also be an important pharmacological tool to investigate APLNR downstream signaling and physiological functions.

APLNR I109A receptor, which is deficient in β-arrs recruitment, lost the ability to enhance intratumoral IFN-γ signaling sensitivity. While APLNR N112G, a constitutively active mutant receptor that recruits β-arrs even in the absence of agonist stimulation, increases the intratumoral sensitivity of IFN-γ signaling. Based on these results, we proposed a working model for APLNR regulation of IFN-γ signaling (Figure 6D). In this model, activation of APLNR recruits β-arr1 to the membrane-associated receptor, prevents β-arr1 interaction with STAT1, which relieves the inhibitory effect of β-arr1 on STAT1 phosphorylation. Elevated
phosphorylated STAT1 upregulates the activation of IRF1, and eventually the expression of antigen presenting-processing genes, such as B2M and TAP1, and immune checkpoint genes, PD-L1. According to this model, activation of APLNR I109A mutant receptor, which is defective in β-arrs recruitment, has no effect on intratumoral IFN-γ sensitivity (Figure 6D), while in APLNR N112G mutant, β-arr1 is constitutively recruited and tightly associated with internalized receptors, removing the β-arr1 negative regulation on IFN-γ signaling (Figure 6D).

It is widely accepted that APLNR plays important roles in cardiovascular function, glucose and metabolism regulation and fluid haemostasis. Recently, the APLNR system has been implicated in cancer development and progression. APLNR regulates endothelial cell migration and proliferation, promotes tumor neo-angiogenesis and tumor vascular maturation. Apelin-induced tumor vascular maturation enhances the efficacy of immunotherapy and significantly suppresses tumor growth. In addition, APLNR augments the IFN-γ responses and improves the efficacy of T-cell-based immunotherapies. Consistent with these observations, overexpression of APLNR N112G mutant increased intracellular IFN-γ response and slightly increase T-cell-mediated cytotoxicity. APLNR I109A, which has no effect on IFN-γ mediated cellular signalling and did not affect T-cell-mediated cytotoxicity. Unexpectedly, Apln-17 mediated APLNR activation in A-375 tumor cells enhanced IFN-γ cellular signaling but impaired T-cell-mediated cytotoxicity. This result may be attributed to HLA class I antigen presentation may have no impact on T cell activation in co-culture system and/or the effect of apelin on tumors. It has been reported that
apelin treatment promotes cell proliferation in several cancer cell lines (i.e. A549, MCF-7, HSC-3 and MzChA-1), and antagonizing APLNR signaling by APLNR antagonist F13A or ML221 inhibits tumor growth in both cancer cell lines and murine tumor models.\textsuperscript{9} Lastly, due to the complex immune response, IFN-\(\gamma\) itself could stimulate either pro- or anti-tumorigenic effect on cancer depending on the tumor microenvironment.\textsuperscript{16}

Previous work indicates that APLNR influences T cell killing through non-canonical G protein-coupled signaling, as stimulating endogenous APLNR by different ligands did not alter T-cell-mediated cell depletion.\textsuperscript{11} While, in our study, we focus on the regulatory role of APLNR signaling by agonist stimulation or APLNR mutant in tumor cells, we found that different activation modes of APLNR indeed exhibit divergent immunoregulation. These inconsistencies may result from differences in APLNR expression or activation models in tumor cells and in co-culture systems. In fact, we did not observe elevated IFN-\(\gamma\) signaling stimulated by APLNR ligands in A-375 cells without APLNR overexpression (Figure S3A). However, in cells overexpressing wild type APLNR, ligand stimulation indeed enhanced tumor cell IFN-\(\gamma\) responses (Figure 4A). Meanwhile, it has been reported that the expression of APLNR is upregulated under hypoxic conditions within tumor microenvironment and elevated APLNR signaling promotes tumor angiogenesis.\textsuperscript{8, 48} Based on these controversial results, the relationship between enhanced IFN-\(\gamma\) mediated signaling and T-cell-mediated cytotoxicity, APLNR activation and tumor development warrant further investigation.
Accumulating evidence suggest that GPCRs are involved in cancer progression and modulating GPCR function could be a new approach for cancer treatment. Here, our findings reveal the mechanism of APLNR modulation of IFN-γ signaling in melanoma cells, which shed light on APLNR as a potential target to enhance the efficacy of immunotherapy. Despite the remarkable clinical responses of immunotherapy, the heterogeneous and evolving tumor cell intrinsic/extrinsic factors restrict their overall utility (Pitt et al., 2016; Sharma et al., 2017). Therefore, understanding the underlying mechanisms of immune regulation of APLNR could enhance the clinical benefits of immunotherapy. However, the dual role of APLNR in tumor angiogenesis and cancer immunity should be considered in future immunotherapy design.
Data Availability

All supporting data in relation to the studies reported here are provided in this manuscript.

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Author contributions

Y.Y.L, X.C.M and H.Y designed, conducted experiments and data analysis, X.L performed MD simulation, X.C.M, Y.L.M, B.A, S.L.L and L.A. H designed and supervised the project. Y.Y.L, S.L.L, B.A and L.A.H wrote the manuscript. All authors read and approved the manuscript.

Declaration of interest

The authors declare no competing interest.

Abbreviations

Apln, apelin; AT1, angiotensin II type 1; β-arrs, β-arrestins; BiTE, Bi-specific T-cell engager; B2M, beta-2-microglobulin; CAM, constitutively active mutant; CTLs, CD8
cytotoxic T lymphocytes; DCs, dendritic cells; GASs, γ-activated sites; GPCR, G protein-coupled receptor; IFN-γ, interferon-γ; ISREs, interferon-stimulated response elements; ISGs, IFN-γ-stimulated genes; MD, molecular dynamics; NLS, nuclear localization sequence; NK, natural killer; TM3, transmembrane helix 3.
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Figure legends

**Figure 1.** APLNR upregulates IFN-γ signaling in melanoma A-375 cells. (A) Effect of APLNR knockdown on IFN-γ signaling. A-375 reporter cells were transfected with siRNAs and subsequently treated with IFN-γ for 24 h before luciferase activity was measured. Data are presented as fold change of relative luciferase activity of mean ± s.d. (n=3). **** represents p<0.0001 (in one-way ANOVA). (B) Effect of APLNR knockdown on PD-L1 expression level. (C) APLNR knockdown efficacy by siRNA was evaluated by RT-PCR. (D) Effect of APLNR activation by apelin-17 (Apln-17) on IFN-γ signaling. Data are presented as fold change of mean ± s.d. (n=3). *** represents p<0.001, **** represents p<0.0001 (in unpaired t-test).

**Figure 2.** β-arrs negatively regulate intratumoral IFN-γ signaling. (A) Effect of β-arr1 and β-arr2 on IFN-γ signaling using the reporter assay (left panel). qRT-PCR analysis of β-arr1 (middle panel) and β-arr2 (right panel) siRNA-mediated knockdown. (B) Western blot analysis of PD-L1 expression in cells treated with β-arrs siRNAs. (C) Quantification of PD-L1 surface expression in A-375 cells by flow cytometry. Data are presented as fold change of mean ± s.d. (n=3). ns, no significance. **** represents p<0.0001 (in one-way ANOVA). (D) Heatmap of selected genes altered by β-arr1 or β-arr2 siRNA treatment in A-375 cells, gene expression was quantified by qRT-PCR.

**Figure 3.** β-arr1, but not β-arr2, modulates JAK-STAT signaling pathway by direct interaction with STAT1. (A) Effect of selected kinase inhibitors on IFN-γ signaling +/-
β-arr1 siRNA treatment. Data represented as the relative fold change in luciferase activity, mean ± s.d. (n=3). * represents p<0.05, *** represents p<0.001, **** represents p<0.0001 (in one-way ANOVA). (B) Effect of β-arr1 knockdown on the phosphorylation of STAT1. pSTAT1 (Y701), total STAT1 and β-arr1 protein were detected by immunoblot. (C) AlphaLISA analysis of STAT1 phosphorylation at Y701 in A-375 cells transfected with β-arr1 (left panel). The expression level of β-arr1 was determined by Western blot (right panel). Data represented as the fold change in AlphaLisa pSTAT1 (Y701) activity, mean ± s.d. (n=3). *** represents p<0.001 (in unpaired t-test). (D) β-arr1 interacts directly with STAT1 demonstrated by immunoprecipitation.

Figure 4. APLNR I109A mutant receptor exhibits IFN-γ signaling insensitivity. (A) WT APLNR stimulated by endogenous agonists enhance IFN-γ signaling. (B) Activation of APLNR I109A mutant had no effect on IFN-γ signaling. (C-D) Activation of APLNR I109A had no effect on IFN-γ-stimulated PD-L1 expression as determined by Western blot (C) or IRF1 mRNA expression (D) in A-375 cells. Data are presented as mean ± s.d. (n=3). ns, no significance. ** represents p<0.01, **** represents p<0.0001.

Figure 5. Constitutively active mutant APLNR N112G enhances IFN-γ signaling in melanoma cells. (A) Dose response of Apln-13 by cAMP assay in WT APLNR and APLNR N112G mutant. Data represented as mean ± s.d. (n=3). (B) Receptor
distribution and β-arrr recruitment in APLNR N112G mutant and WT APLNR cells analyzed by a Leica confocal microscopy. β-arrr1 was visualized with GFP (green channel) and HA-tagged receptors detected by Alexa-594 conjugated anti-HA antibody (red channel). Yellow dots in the overlay images indicate the co-localization of β-arrr1 and receptors. (C-D) MD simulation of wild type and N112G mutant human APLNR in the absence of ligand. Superimposed APLNR wild type (cyan) and N112G (magenta) model before MD simulation (C); Superimposed APLNR wild type (green) and N112G (white) model after 60 ns MD simulation (D). The proteins are shown as cartoons. Residues F782.53, N112/G1123.35, M1133.36 and W2616.48 are shown as sticks. (E) Effect of APLNR N112G mutant on IFN-γ signaling in the reporter assay. (F) Effect of APLNR N112G on STAT1 phosphorylation in A-375 cells after IFN-γ stimulation. pSTAT1 (Y701) and total STAT1 were detected by immunoblot. (G) Effect of APLNR N112G on PD-L1 expression level in A-375 cells in the presence or absence of IFN-γ treatment. (H) Effect of APLNR N112G on IRF1 mRNA level determined by RT-PCR in A-375 cells. Data are presented as mean ± s.d. (n=3). ns, no significance. ** represents p<0.01, *** represents p<0.001, **** represents p<0.0001.

Figure 6. Effect of APLNR activation on T-cell-mediated cytolysis. (A) Schematic of the co-culture assay to evaluate the efficacy of T-cell-mediated cytolysis. (B) Representative images of tumor cells before or after effector T cells and BiTE treatment. White arrows indicate lysed tumor cells. (C) Quantification of
T-cell-mediated cytolysis in WT APLNR (left panel), APLNR I109A (middle panel) or APLNR N112G cells (right panel) with or without Apln-17 stimulation (100 nM) after co-culturing with effector T cells for 72 h. Data are presented as mean ± s.d. (n=4). ns, no significance. * represents p<0.05, ** represents p<0.01 (in unpaired t-test). (D) Models illustrating how APLNR or APLNR mutants modulate the intracellular IFN-γ signaling in melanoma cells. APLNR modulates IFN-γ signaling through β-arr1, which negatively regulates JAK-STAT pathway by directly binding to STAT1. (Left panel) In cells with APLNR I109A, which was deficient in β-arrs recruitment, more β-arr1 was released to inhibit JAK-STAT pathway, leading to intracellular IFN-γ insensitivity and down-regulation of IFN-γ-stimulated genes (i.e. MHC molecules). (Right panel) In cells with constitutively active APLNR mutant (APLNR N112G), which constitutively recruits β-arr1 and forms receptor/β-arr1 complex, APLNR/β-arr1 complex to prevent the interaction of β-arr1 and STAT1, and therefore remove the negative effect on JAK-STAT pathway, and enhance IFN-γ signaling sensitivity and immunotherapy efficacy.
Figure 1.
Figure 4.
Figure 5.

A) cAMP responses

B) APLNR β-arr1 Merge

C) TM1

D) TM2

E) Relative luciferase activity

F) IFN-γ (min) pSTAT1 STAT1 α-tubulin

G) PD-L1 α-tubulin IFN-γ (h) EV APLNR N112G

H) Relative IFN-1 mRNA level
