




Snail-regulated exosomal microRNA-21 suppresses NLRP3 inflammasome activity to enhance cisplatin resistance

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ABSTRACT

Background Compared with the precise targeting of drug-resistant mutant cancer cells, strategies for eliminating non-genetic adaptation-mediated resistance are limited. The pros and cons of the existence of inflammasomes in cancer have been reported. Nevertheless, the dynamic response of inflammasomes to therapies should be addressed.

Methods Tumor-derived exosomes were purified by differential ultracentrifugation and validated by nanoparticle tracking analysis and transmission electron microscopy. A proximity ligation assay and interleukin-1 β (IL-1 β) level were used for detecting activation of NLRP3 inflammasomes. RNA sequencing was used to analyze the exosomal RNAs. *MIR21* knocked out human monocytic THP cells and *mir21* knocked out murine oral cancer MTCQ1 cells were generated for confirming the exosomal delivery of microRNA (miR)-21. Syngeneic murine models for head and neck cancer (C57BL/6J), breast cancer (BALB/C) and lung cancer (C57BL/6J) were applied for examining the impact of Snail-miR21 axis on inflammasome activation in vivo. Single-cell RNA sequencing was used for analyzing the tumor-infiltrated immune cells. Head and neck patient samples were used for validating the findings in clinical samples.

Results We demonstrated that in cancer cells undergoing Snail-induced epithelial-mesenchymal transition (EMT), tumor cells suppress NLRP3 inflammasome activities of tumor-associated macrophages (TAMs) in response to chemotherapy through the delivery of exosomal miR-21. Mechanistically, miR-21 represses *PTEN* and *BRCC3* to facilitate NLRP3 phosphorylation and lysine-63 ubiquitination, inhibiting NLRP3 inflammasome assembly. Furthermore, the Snail-miR-21 axis shapes the post-chemotherapy tumor microenvironment (TME) by repopulating TAMs and by activating CD8⁺ T cells. In patients with head and neck cancer, the Snail-high cases lacked post-chemotherapy IL-1 β surge and were correlated with a worse response.

Conclusions This finding reveals the mechanism of EMT-mediated resistance beyond cancer stemness through modulation of post-treatment inflammasome activity. It also highlights the dynamic remodeling of the TME throughout metastatic evolution.

INTRODUCTION

The emergence of therapeutic resistance during cancer progression is a major obstacle in combating advanced cancers. The reasons

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Therapeutic resistance emerges during late-stage progression of cancers.

WHAT THIS STUDY ADDS

⇒ The epithelial-mesenchymal transition-undergoing cancer cells deliver microRNA-21-abundant exosomes to suppress inflammasome activities of macrophages for enhancing resistance.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ A dynamic interplay between cancer and microenvironments indicates the importance of real-time adjustment of therapeutic strategies alongside cancer progression.

for the acquired resistance of malignant tumors include the genetic evolution of cancer cells and non-genetic adaptation to therapies.¹ The non-genetic plasticity of cancer cells enables lineage switching to adapt to the environment and facilitates the evasion of anticancer immunosurveillance. Compared with the rapid advances in sequencing techniques and precise targeting of resistant mutants, strategies for eliminating non-genetic adaptation-mediated resistance are relatively limited.

The dynamic switch between epithelial and mesenchymal states is crucial for cancer metastasis and therapeutic resistance.² In addition to the well-established impact of epithelial-mesenchymal transition (EMT) on cancer stemness and therapeutic resistance,³ accumulated evidence, including findings reported by our previous studies, supports the bidirectional interaction between EMT-undergoing cancer cells and host immune cells.^{4–7} EMT is well known for both engendering immunosuppression^{8–10} and for promoting the creation of proinflammatory tumor microenvironments (TMEs).^{5 11 12} Compared with the well-established role of

EMT reported in tumor immunosuppression, the impact of EMT on the modulation of the response of host innate immune cells in response to stimuli/treatments is less clear.

Inflammasomes, the multimolecular complexes that consist of an NOD-like receptor scaffold, caspase activation, adapter proteins such as ASC (also known as Pycard), and caspase-1, are considered major sensors of innate immunity which function in response to danger signals in mammals.¹³ Although chronic inflammation has been noted as a critical event in tumorigenesis and cancer progression, the contradictory roles of inflammasomes in the TME have been demonstrated in different contexts. The pro-cancer and anticancer effects of inflammasomes have both been reported.^{14–17} Diverse stimuli such as ATP, bacterial components, or nucleic acids induce the assembly of inflammasomes, leading to caspase-1 activation and secretion of interleukin-1 β (IL-1 β) and IL-18.¹³ Post-treatment dying cancer cells release danger signals, such as nucleic acids, high-mobility group box 1 (HMGB1) protein, and ATP, which trigger innate immunity and inflammation.^{18–20} However, knowledge about the impact of the post-therapeutic activation of inflammasomes on treatment response is relatively limited.

The present study demonstrated that in cancers undergoing Snail-induced EMT, tumor cells suppressed the activity of inflammasomes of tumor-associated macrophages (TAMs) in response to chemotherapy through the delivery of exosomal microRNA (miR)-21. The miR-21 modulates NLRP3 phosphorylation and lysine 63-ubiquitination to inhibit the assembly of NLRP3 inflammasomes, thereby shaping the post-chemotherapy TME and reducing the chemotherapy response. This finding reveals the mechanism of EMT-mediated therapeutic resistance beyond cancer stemness through modulation of post-treatment inflammasome activities. It also highlights the dynamic remodeling of the TME throughout metastatic evolution.

MATERIALS AND METHODS

Cell lines and plasmids

Human head and neck squamous cell carcinoma (HNSCC) cell line FaDu, human monocytic leukemia cell line THP-1, human embryonic kidney cell line 293T, BALB/c mouse breast carcinoma cell line 4T1, and C57BL/6J mouse lung carcinoma cell line LLC1 were originally sourced from the American Type Culture Collection. The HNSCC cell line OECM-1 and the C57BL/6J murine oral cancer cell line MTCQ1 were kindly provided by Dr Kuo-Wei Chang (National Yang Ming Chiao Tung University of Taiwan). The pCDH-Snail, pCDH-Zeb1 and pCDH-MIR21 plasmids were generated via insertion of full-length cDNA (SNAIL: NM_005985; ZEB1: NM_030751.4, MIR21: NR_029493.1) into the pCDH-CMV-MCS-EF1-puro vector. The pcDNA3-Flag-NLRP3 plasmid was provided by Dr Szu-Ting Chen (National Yang Ming Chiao Tung University of

Taiwan). The 3'-untranslated region (UTR) of *BRCC3* (NM_001018055) was cloned into pMIR-REPORTER to generate pMIR-BRCC3-wt, and site-directed mutagenesis was performed to generate the miR-21 binding-site-mutated pMIR-BRCC3-mut.

Patient with HNSCC samples

Five independent sets of samples were used for the experiments. The informed consent was obtained from all patients in this study. The information of the patient samples is detailed in online supplemental methods and online supplemental tables 1-5.

Single-cell RNA-sequencing

To obtain cells for single-cell RNA-sequencing (scRNA-seq) analysis, 1×10^6 MTCQ1-WT/MTCQ1^{mir21-/-} cells were inoculated into the subcutaneous region of C57BL/6 mice. Cisplatin (5 mg/kg) was administered intraperitoneally on the 14th day, and tumors were harvested on the 17th day. Tumors were dissociated using a tumor dissociation kit (Miltenyi Biotec), and dead cells were removed using a dead cell removal kit (MACS). CD45⁺ tumor-infiltrating immune cells were isolated using microbeads (Miltenyi Biotec). Cell viability detected via trypan blue staining was over 85% for subsequent sequencing. For library construction and sequencing, we used the droplet-based scRNA-seq (10x Genomics Chromium Single Cell 3' Reagent Kit V.3.1, no. 1000121) for single-cell library preparation. After the conduction of reverse transcription, complementary DNA sequencing was performed using the Illumina NovaSeq 6000 (Illumina). The quality control (QC) and filtering steps were performed using a loupe browser. Briefly, data on cells with low total counts and high mitochondrial gene expression were filtered. The scRNA-seq reads were processed using a 10x Genomics Cell Ranger pipeline and analyzed using the Partek Flow software (Partek, St. Louis, Missouri, USA). Clustering of cells in our dataset was performed using a t-distributed stochastic neighbor embedding (t-SNE) algorithm in Partek Flow. To identify the t-SNE subclusters present in different types of immune cells, the average gene expression of each cluster was identified using cluster identity predictor (CIPR).²¹ Gene Ontology (GO) analysis of the differentially expressed genes between clusters was performed using DAVID²² and GSEA.²³ Partek Flow was used for gene-specific analysis, macrophage re-clustering, macrophage trajectory analysis, and volcano plot generation.

Data and code availability

The accession numbers for the data reported in this paper are GEO: GSE99474, GSE172326, GSE178537, and GSE 181300.

Statistical analysis

Statistical analyses were performed using GraphPad Prism V.8 (GraphPad Software). Two-sided independent Student's t-test (normal distribution) Pearson's correlation test was used to analyze the correlation between the

two continuous factors. All statistical data were derived from at least three independent biological replicates, and each experiment contained at least two technical replicates. P value ≤ 0.05 was considered statistically significant (*: ≤ 0.05 , **: ≤ 0.01 , ***: ≤ 0.001).

RNA-seq analysis of patient with HNSCC samples, RNA-seq for exosomal small RNA, preparation of conditional media and macrophages, purification and characterization of tumor-derived exosomes, tissue processing and data generation for Visium spatial gene expression, generation of the *MIR21/mir21* knockout cell lines, quantitative real-time PCR, immunoblotting and immunoprecipitation, immunohistochemistry, multiplex immunofluorescence staining of HNSCC samples, proximity ligation assay, luciferase reporter assay, SYTOX green assay, and animal experiments: The information of the experiments is detailed in online supplemental methods.

RESULTS

Supernatants derived from Snail-expressing cancer cells suppress the NLRP3 inflammasome activity

In the present study, we considered HNSCC as the major model for experiments based on the crucial role of EMT reported in HNSCC progression and on our previous findings reported in HNSCC EMT studies.^{24 25} Since Snail has been identified as the major EMT regulator affecting microenvironments, we performed multiplex immunofluorescence staining to examine the immune cells in HNSCC samples with different expression levels of Snail. A noticeable increase in the infiltration of CD163⁺ TAMs was observed in samples with higher Snail expression (online supplemental figure 1A and online supplemental table 1). Next, we analyzed the correlation between EMT and inflammatory cytokines in the RNA-seq data of HNSCC samples retrieved from The Cancer Genome Atlas (TCGA) and the cohort of Taipei Veterans General Hospital (TVGH) (figure 1A–B and online supplemental table 2). The samples were categorized as EMT^{high} or EMT^{low} according to the calculated EMT score,²⁶ and a higher expression of transforming growth factor β (TGF- β) validated the feasibility of the categorization. Increased expression of cytokines related to cancer progression and immunosuppression, such as IL-6, tumor necrosis factor (TNF- α) (in the TCGA cohort), and IL-10 (in both cohorts), was observed in the EMT^{high} group, which was consistent with previous reports.^{4 27 28} Intriguingly, a trend of reduced levels of IL-1 β (in the TCGA cohort) or IL-18 (in the TVGH cohort) was noted in the EMT^{high} group, indicating the potential influence of EMT on inflammasome activity. We further applied spatial transcriptomic technology in a HNSCC sample to validate the relationship between EMT and inflammasome activation. A trend of a reversed relationship between the gene expression profile of EMT and inflammasome-related genes was shown (online supplemental figure 1C), (online supplemental table 6), indicating the potential of EMT in suppression of inflammasome activity in HNSCC.

We have previously shown that Snail-expressing cancer cells recruit TAMs through the secretion of CCL2, CCL5, and TNF- α to facilitate cancer progression.⁴ Snail-expressing cancer cells secrete exosomes to promote M2 polarization of TAMs.⁶ We examined the influence of Snail on inflammasome activity. Snail was overexpressed in FaDu, a human HNSCC cell line harboring low-endogenous Snail (left panel of figure 1C). Snail knockdown was performed in the high-endogenous Snail human HNSCC cell line OECM1⁴ (left panel of figure 1D). The supernatant derived from FaDu-Snail inhibited the release of active caspase-1 and IL-1 β from activated peripheral blood mononuclear cells (PBMC)-derived macrophages compared with the control cells (figure 1C). Knockdown of Snail in OECM1 potentiated the release of active caspase-1 and IL-1 β (figure 1D). Consistent results were observed in the human monocytic cell line THP1-derived activated macrophages. Supernatants from FaDu-Snail repressed IL-1 β secretion from THP1-derived activated macrophages. Supernatants from OECM1-Snail knockdown cells did not potentiate the secretion of IL-1 β to a significant level (online supplemental figure 2A). In contrast, the supernatant derived from FaDu cells expressing another major EMT regulator, Twist1, did not affect the secretion of active caspase-1 and IL-1 β from THP1-derived activated macrophages (online supplemental figure 2B).

We further confirmed the effect of Snail-expressing cancer cells on NLRP3 inflammasome assembly. When the NLRP3 inflammasome is activated, NLRP3 establishes interactions with ASC to recruit and activate caspase-1. Concurrently, the complexes are assembled into a giant pyroptosome. Proximity ligation assay (PLA) was performed to examine the interaction between NLRP3 and ASC in PBMC-derived and THP1-derived macrophages. The supernatant derived from FaDu-Snail repressed the interaction between NLRP3 and ASC in activated macrophages compared with the control cells (figure 1E and online supplemental figure 2C). The FaDu-Snail supernatant also reduced pyroptosome formation in activated macrophages (figure 1F). As chemotherapy reportedly activates the NLRP3 inflammasome,^{14 20} we examined whether Snail-overexpressing cancer cells suppressed chemotherapy-activated inflammasomes in macrophages. Cisplatin treatment activated inflammasomes in macrophages as expected, and FaDu-Snail supernatant suppressed the activity of inflammasomes, as evidenced by the reduction of active caspase-1 and IL-1 β production (online supplemental figure 2D). The FaDu-Snail supernatant also reduced the NLRP3-ASC interaction in cisplatin-activated macrophages (figure 1G). Altogether, the above-mentioned results indicate that the supernatant derived from FaDu-expressing HNSCC suppresses NLRP3 inflammasome activity.

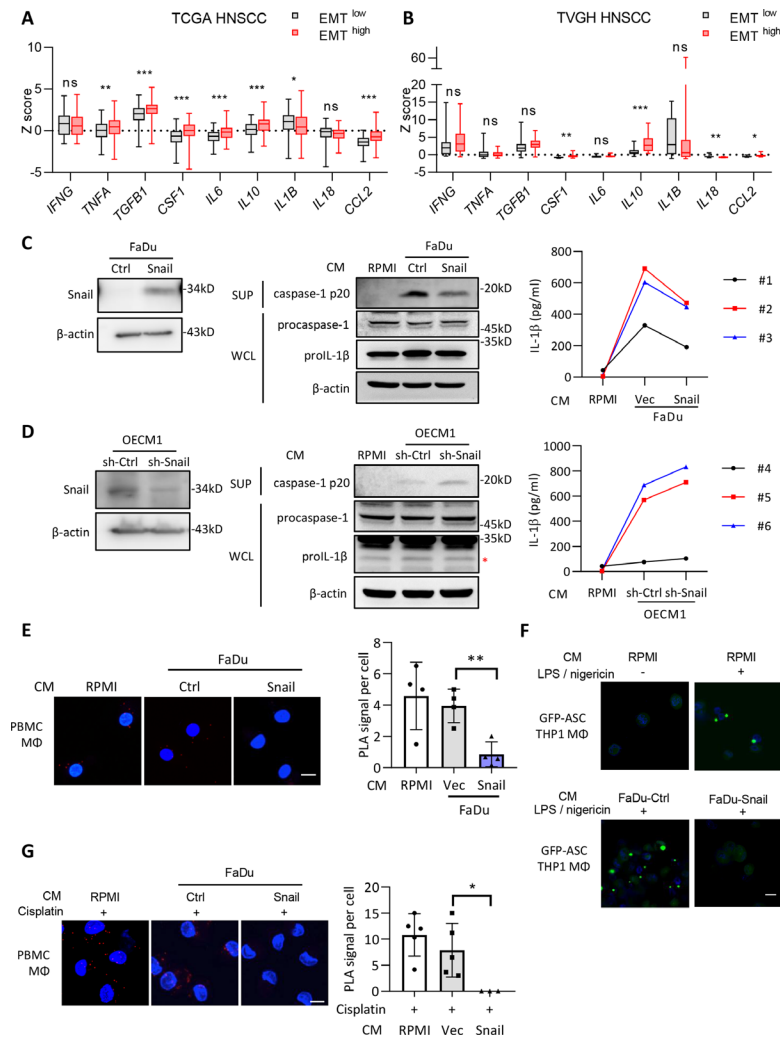


Figure 1 The supernatants of Snail-expressing cancer cells inhibit activation of NLRP3 inflammasomes. (A–B) Analysis of the expression of the inflammation-related genes in the TCGA HNSCC database (A) or Taipei Veterans General Hospital (TVGH) database. The samples were categorized as the EMT^{high} and EMT^{low} group according to the expression of the EMT-related genes (*VIM*, *FN1*, *CDH2*, *ITGB6*, *FOXC2*, *MMP2*, *MMP3*, *MMP9*, *SOX10*, *SNAI1*, *SNAI2*, *TWIST1*, *GSC*, *CDH1*, *DSP*, *TJP1*) (see Chae *et al.*)²⁶ n=521 for TCGA, n=65 for TVGH. The boxplots show the minimum, first quartile, medium, third quartile, and maximum. *p<0.05, **p<0.01, ***p<0.001, ns=no significance by Student's t-test. (C) Left, western blot of Snail in FaDu-Ctrl/Snail. β-actin was a loading control. Middle, immunoblots of cleaved caspase-1 p20 in supernatants (SUP), pro-caspase-1 and pro-IL-1β in whole cell lysates (WCL) of PBMC-derived macrophages cultivated in conditioned media (CM) from FaDu cells stably transfected with Snail or a control vector (Ctrl) for 48 hours. RPMI was a control for CM. β-actin was a loading control for immunoblots of WCL. Right, ELISA for analyzing the level of secreted IL-1β from PBMC-derived macrophages cultivated with the conditional media from FaDu-Snail/FaDu-Vec. Peripheral blood mononuclear cells were isolated from three different healthy donors (case #1, #2, and #3). (D) Left, western blot of Snail in OECM1-sh-Ctrl/sh-Snail. β-actin was a loading control. Middle, immunoblots of cleaved caspase-1 p20 in SUP, pro-caspase-1 and pro-IL-1β in WCL of PBMC-derived macrophages cultivated in CM from OECM1 cells receiving short hairpin RNA against Snail (sh-Snail) or a control sequence (sh-Ctrl) for 48 hours. RPMI was a control for CM. β-actin was a loading control for immunoblots of WCL. Right, ELISA for analyzing the level of secreted IL-1β from macrophages cultivated with the conditional media from FaDu-Snail/FaDu-Vec. Peripheral blood mononuclear cells were isolated from three different healthy donors (case #4, #5, and #6). (E) Left, representative images of proximity ligation assay (PLA) for detecting NLRP3 and ASC interaction in PBMC-derived macrophages cultivated in CM from FaDu-Vec or FaDu-Snail. The red dots indicate the PLA signals. Scale bar, 10 μm. Right, quantification of number of PLA signals per cell. For each group, at least a total of 18 cells from four randomly selected fields were used for PLA quantification. Data represent means±SD. **p<0.01 by Student's t-test. (F) Representative immunofluorescent images of THP1-derived macrophages transfected with GFP-tagged ASC, stimulated with nigericin and cultivated with the CM from FaDu-Ctrl, FaDu-Snail, or RPMI for 24 hours. Scale bar, 10 μm. (G) PLA for detecting NLRP3 and ASC interaction in cisplatin-activated PBMC-derived macrophages incubated with CM from FaDu-Vec/FaDu-Snail. Left, representative images. Red dots indicate PLA signals. Scale bar, 10 μm. Right, quantification of PLA signals per cell. For each group, at least total 16 cells from five random selections (three for FaDu-Snail) were used for PLA quantification. Data represent means±SD. *p<0.05 by Student's t-test. Scale bar, 10 μm. EMT, epithelial-mesenchymal transition; HNSCC, head and neck squamous cell carcinoma; IL, interleukin; PBMC, peripheral blood mononuclear cells; TCGA, The Cancer Genome Atlas.

Snail-expressing cancer cells suppress NLRP3 inflammasome activity through the secretion of miR-21-abundant exosomes

Next, we determined the major player(s) in the suppression of inflammasome activity in the supernatant of Snail-expressing cancer cells. We found that IL-6, IL-8, CCL2, and CCL5 were the major cytokines regulated by Snail.^{4,29} However, neutralization of these cytokines did not affect NLRP3-ASC interaction or pyroptosome formation in activated macrophages subjected to treatment with the supernatant derived from either FaDu-Snail or control cells (online supplemental figure 3A-B). As tumor-secreted exosomes (TEXs) have been critically involved in remodeling microenvironments,^{30,31} we investigated whether Snail-expressing cancer cell-secreted exosomes modulated the inflammasome activity in macrophages. The exosomes were confirmed via direct visualization by performing transmission electron microscopy and nanoparticle tracking analysis, and results showed the presence of 50–150 nm nanovesicles with bilayer membranes (online supplemental figure 3C). The markers of exosomes, including CD9, CD63, CD81, and Alix, were examined to confirm the successful purification of cancer-secreted exosomes (online supplemental figure 3D). We investigated the effect of TEXs on the inflammasome activity of macrophages. A significant reduction in the NLRP3-ASC interaction was noted when activated macrophages were subjected to treatment with FaDu-Snail-secreted exosomes compared with those derived from FaDu-control cells (figure 2A). Consistently, a decrease in IL-1 β secretion was also noted when macrophages were incubated with FaDu Snail-secreted exosomes (figure 2B). These data indicate that Snail-expressing cell-secreted exosomes suppress the activity of the NLRP3 inflammasome in activated macrophages.

MicroRNAs (miRNAs) are important components of regulatory networks in innate immunity.³² We have previously shown that *MIR21* transcription was regulated by Snail, and miR-21-abundant exosomes secreted from Snail-overexpressing HNSCC promote M2-like polarization of TAMs.⁶ We hypothesized that exosomal miRNAs might provide a link between cancer cells and innate immune signaling pathways. To this end, we performed exosomal miRNA sequencing in the human HNSCC cell line FaDu versus immortalized human gingival mucosa cells SG. We investigated the miRNAs that were especially enriched in TEXs, the tumor-specific exosomal miRNAs, by focusing on the top 10 miRNAs in FaDu-derived exosomes. Among them, miR-21 was found to be the most significantly enriched in the TEXs (online supplemental table 7). As EMT is closely linked to miRNA dysregulation,³³ and as Snail dominates the expression of several miRNAs in cancer cell,^{34,35} we explored the major Snail-regulated exosomal miRNA(s) by examining the expression of the top-ranked tumor exosomal miRNAs in FaDu-Snail versus FaDu-control and OECM1 that received a short hairpin RNA (shRNA) against Snail or a control

sequence. Among these, miR-21, miR-10a, and miR-191 showed consistency in upregulated levels of FaDu-Snail-secreted exosomes and downregulated levels of exosomes derived from the Snail-knockdown OECM1 cells (online supplemental figure 4A). We further examined the impact of the three miRNAs on NLRP3 inflammasome activation. Transfection of miR-21, but not miR-10a/miR-191, into THP1-derived macrophages significantly attenuated the interaction between NLRP3 and ASC (online supplemental figure 4B). Consistently, miR-21 reduced the assembly of NLRP3 and ASC (figure 2C) and IL-1 β secretion in PBMC-derived macrophages (figure 2D). Together with our previous finding which highlighted that Snail upregulated exosomal miR-21 levels through direct or indirect (via Zeb1) activation of *MIR21* transcription,⁶ the results indicated the potential involvement of Snail-regulated miR-21 in the suppression of NLRP3 inflammasome activity.

To further elucidate the role of exosomal miR-21 in the regulation of NLRP3 inflammasome activity in macrophages, we generated *MIR21* knockout THP1 cells using the CRISPR-Cas9 technology (online supplemental figure 4C). A significant increase in IL-1 β secretion was noted in the supernatant of THP1^{*MIR21*^{-/-}}-derived macrophages subjected to treatment with LPS and nigericin compared with the wild-type THP1-derived macrophages (figure 2E). Knockout of miR-21 in THP1 increased NLRP3-ASC interaction in THP1-derived macrophages (figure 2F). Treatment of THP1^{*MIR21*^{-/-}}-derived macrophages with exosomes harvested from miR-21-overexpressed FaDu cells reduced NLRP3-ASC interaction (figure 2G). To further confirm the role of Snail-regulated miR-21 in the suppression of inflammasome activity, we knocked down miR-21 in Snail-overexpressing FaDu cells (FaDu-Snail) and examined the impact of the TEXs on IL-1 β secretion of macrophages. The efficacy of the miRZip anti-miR-21 was validated by the reporter assay (online supplemental figure 4D). Downregulation of miR-21 reporter activity was shown in Snail-overexpressing FaDu cells, and the successful knockdown of miR-21 was confirmed by the restoration of reporter activity in FaDu-Snail transfected with anti-miR-21 (online supplemental figure 4E). We next examined the impact of the Snail-miR-21 axis in cancer cells on the inflammasome activity of macrophages through delivering exosomes. The macrophages were treated with TEXs derived from FaDu-control, FaDu-Snail, and FaDu-Snail-anti-miR21. Overexpression of Snail in FaDu cells suppressed IL-1 β secretion of macrophages, and knockdown of miR-21 in FaDu-Snail restored the IL-1 β level (figure 2G). Consistently, overexpression of Snail in FaDu cells reduced NLRP3-ASC interaction (detected by PLA) and knockdown of miR-21 in FaDu-Snail restored the interaction (figure 2H). In summary, these results indicate that exosomal miR-21 from Snail-overexpressing cancer cells attenuates NLRP3 inflammasome activation of macrophages.

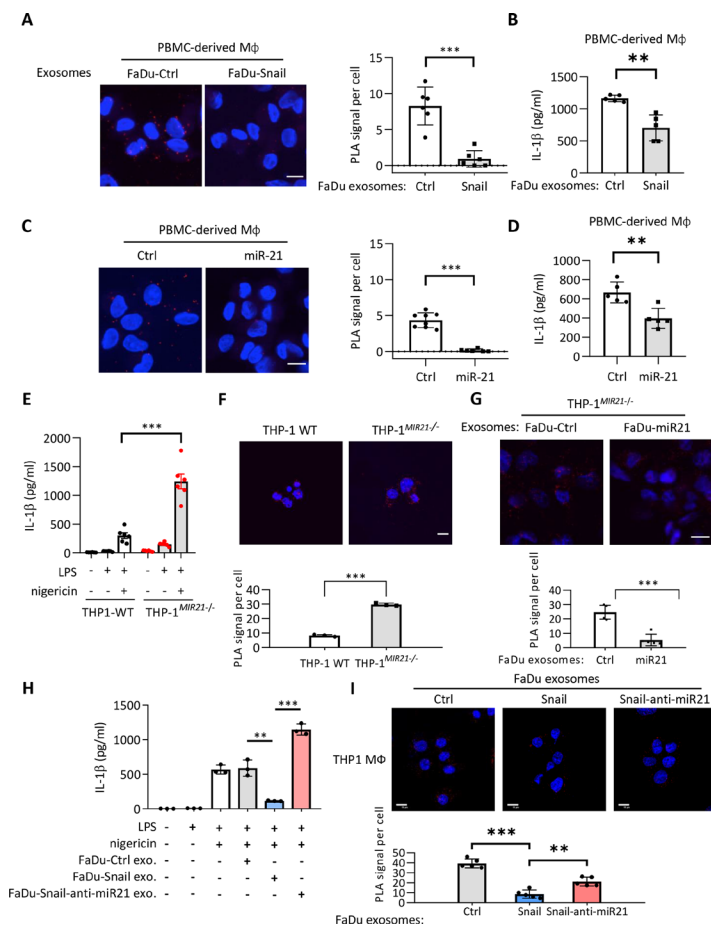


Figure 2 Exosomes from Snail-miR-21 axis activated cancer cells suppress NLRP3 inflammasome activity of macrophages. (A) Representative images of PLA for detecting NLRP3 and ASC interaction in PBMC-derived macrophages incubated with the exosomes from FaDu-Vec/FaDu-Snail. The red dots indicate the PLA signals. Scale bar, 10 μ m. Right, quantification of number of PLA signals per cell. For each group, at least a total of 50 cells from six randomly-selected fields were used for PLA quantification. Data represent means \pm SD. *** p <0.001 by Student's t-test. (B) ELISA for analyzing the level of secreted IL-1 β by PBMC-derived macrophages incubated with exosomes from FaDu-Vec/FaDu-Snail. n =5 independent experiments (each experiment contains two technical replicates). Data represent means \pm SD. ** p <0.01 by Student's t-test. (C) Left, representative images of PLA for detecting NLRP3 and ASC interaction in PBMC-derived macrophages transduced with miR-21 or a control agomir. The red dots indicate the PLA signals. Scale bar, 10 μ m. Right, quantification of number of PLA signals per cell. For each group, at least a total of 60 cells from randomly-selected fields (six for miR-21 group and eight for ctrl group) were used for PLA quantification. Data represent means \pm SD. *** p <0.001 by Student's t-test. (D) IL-1 β ELISA of PBMC-derived macrophage. miR-21 agomir or a control sequence (Ctrl) was transduced to macrophage. n =5 independent experiments (each experiment contains two technical replicates). Data represent means \pm SD. ** p <0.01 by Student's t-test. (E) ELISA for analyzing the level of secreted IL-1 β by macrophages derived from wild-type THP1 (THP1-WT) or *MIR21*-knockout THP1 (THP1^{MIR21-/-}). The macrophages were treated with/without LPS (1 μ g/mL) and nigericin (5 μ M). n =6 (each contains two technical replicates) for each group. Data represent means \pm SD. *** p <0.001 by Student's t-test. (F) Representative images of PLA for detecting NLRP3 and ASC interaction in THP1-WT/THP1^{MIR21-/-}-derived activated macrophages. The red dots indicate the PLA signals. Scale bar, 10 μ m. Right, quantification of number of PLA signals per cell. For each group, at least a total of 400 cells from five randomly selected fields were used for PLA quantification. Data represent means \pm SD. *** p <0.001 by Student's t-test. (G) Representative images of PLA for detecting NLRP3 and ASC interaction in THP1^{MIR21-/-}-derived macrophages incubated with exosomes from FaDu cells transfected with miR-21-expressing vector (FaDu-miR21) or a control vector (FaDu-Ctrl). The red dots indicate the PLA signals. Scale bar, 10 μ m. For each group, at least a total of 400 cells from five randomly selected fields were used for PLA quantification. Data represent means \pm SD. *** p <0.001 by Student's t-test. (H) ELISA for analyzing the level of secreted IL-1 β by THP1-derived macrophages. The macrophages were treated with/without LPS (1 μ g/mL) and nigericin (5 μ M) and the exosomes from FaDu cells transfected with a control vector (FaDu-ctrl), Snail-expressing vector (FaDu-Snail), Snail and anti-miR-21 (FaDu-Snail-anti-miR-21). n =3 (each contains two technical replicates) for each group. Data represent means \pm SD. ** p <0.01, *** p <0.001 by Student's t-test. (I) Representative images of PLA for detecting NLRP3 and ASC interaction in THP1^{MIR21-/-}-derived activated macrophages incubated with exosomes from FaDu cells transfected with a control vector, a miR-21-expressing vector, or Snail together with an antagonist for miR-21 (Snail-anti-miR21). The red dots indicate the PLA signals. Scale bar, 10 μ m. For each group, at least a total of 400 cells from five randomly selected fields were used for PLA quantification. Data represent means \pm SD. ** p <0.01, *** p <0.001 by Student's t-test. IL, interleukin; miR, micro RNA; PBMC, peripheral blood mononuclear cells; PLA, proximity ligation assay.

miR-21 modulates NLRP3 phosphorylation and polyubiquitination to suppress inflammasome activity

We next investigated the mechanism of miR-21-mediated suppression of NLRP3 inflammasome activity. Neither miR-21 agomir nor the miR-21-abundant exosomes (harvested from Snail-expressing cancer cells) affected the messenger RNA (mRNA) levels of *NLRP3/ASC* (online supplemental figure 5A) or the protein stability of NLRP3/ASC (online supplemental figure 5B). Knockout of miR-21 did not downregulate the expression of A20, a negative regulator of nuclear factor kappa B (NF- κ B),³⁶ in LPS/nigericin-stimulated THP1 cells (online supplemental figure 5C). Therefore, we considered that miR-21 repressed inflammasome activity by reducing the interaction between NLRP3 and ASC. Transfection with miR-21 reduced the interaction between NLRP3 and ASC (figure 3A; see also figure 2C). Post-translational modifications of NLRP3, including phosphorylation and K63-ubiquitination, are reportedly crucial for the assembly of NLRP3 inflammasomes for subsequent activation.^{20 37 38} Next, we investigated the mechanisms responsible for the miR-21-regulated NLRP3 inflammasome activation. Regarding phosphorylation-regulated NLRP3 inflammasome activities, PTEN has been shown to dephosphorylate tyrosine 32 of NLRP3 to facilitate NLRP3 inflammasome activation.²⁰ PTEN has been observed to be a direct target suppressed by miR-21.³⁹ Here, we showed that knockout of *MIR21* in THP-1 cells upregulated *PTEN* (figure 3B) and reduced NLRP3 phosphorylation (figure 3C). In addition to phosphorylation, polyubiquitination of NLRP3 is critical in the regulation of inflammasome activity. Furthermore, NLRP3 is the substrate of BRCA1/BRCA2-containing complex subunit 3 (BRCC3), a deubiquitinase that specifically cleaves lysine 63-linked polyubiquitin chains.³⁸ Intriguingly, *BRCC3* contains the seed regions of miR-21 predicted by using TargetScan, and the sequences of the seed regions are preserved across species (online supplemental figure 5D), indicating the potential for miR-21-mediated inflammasome inactivation by targeting *BRCC3*. Ectopic expression of miR-21 in PBMC-derived macrophages downregulated *BRCC3* expression (online supplemental figure 5E). Increased BRCC3 protein and mRNA expression levels were noted in the *MIR21* knockout subline of THP1 cells (figure 3D). Co-incubation of activated macrophages with the supernatants derived from FaDu-Snail versus FaDu-control cells showed that the FaDu-Snail supernatant upregulated miR-21 expression and downregulated the expression levels of *PDCD4* (a known target of miR-21) and *BRCC3* in macrophages (online supplemental figure 5F). A consistent result was observed in OECM1 cells that received shRNA against Snail versus a control sequence. The supernatants derived from Snail-knockdown OECM1 cells reduced miR-21 and increased *BRCC3* and *PDCD4* expression (online supplemental figure 5G). The reporter containing the wild-type or mutated miR-21 binding sites of *BRCC3* 3'-UTR was generated to investigate the regulation of *BRCC3* by miR-21 (online supplemental figure

5H). Ectopic miR-21 suppressed the wild-type *BRCC3* reporter, whereas mutation of the miR-21 binding motifs abrogated this effect (figure 3E).

We examined whether miR-21 inhibited NLRP3 inflammasome activation by suppressing NLRP3 deubiquitination. NLRP3 was immunoprecipitated from HEK293T cells co-transfected with NLRP3 and K63 ubiquitin plus miR-21/control vector. Overexpression of miR-21 increased the level of K63 ubiquitination of NLRP3 (figure 3F). Stable expression of miR-21 inhibited the deubiquitination of NLRP3 in THP-1 cells stimulated with nigericin (figure 3G). Reduced K63 ubiquitination was observed in macrophages derived from THP-1^{MIR21-/-} cells compared with wild-type THP1 cells (figure 3H). Together, these results indicate that miR-21 or miR-21-abundant exosomes from Snail-expressing cells suppress NLRP3 inflammasome activity by targeting *PTEN* to enrich tyrosine phosphorylation of NLRP3, and by targeting *BRCC3* to enhance K63 ubiquitination of NLRP3.

Snail and miR-21 inhibit chemotherapy-induced NLRP3 inflammasome activation and attenuate chemotherapy responses in vivo

NLRP3 inflammasome-produced active caspase-1 and IL-1 β are necessary for chemotherapy-induced anti-tumor immunity.¹⁴ We hypothesized that Snail-regulated miR-21 reduced activation of the NLRP3 inflammasome of macrophages which attenuates chemotherapy-induced antitumor immunity of cancer cells, which contributed to the development of chemotherapy resistance. To this end, we used three syngeneic murine tumor models for the experiments. We first investigated the role of tumorous miR-21 in chemotherapy response in a syngeneic murine HNSCC model⁴⁰ (the oral squamous cell carcinoma cell line MTCQ1 derived from C57B6/J mice) and generated the *mir21* knockout murine HNSCC cell line MTCQ1^{mir21-/-} (figure 4A). We inoculated wild-type and *mir21* knockout MTCQ1 cells into the subcutaneous region of wild-type C57B6/J mice to enable the formation of syngeneic HNSCC tumors, following which treatments were conducted with cisplatin. Tumors were harvested 4 days after treatment to assess caspase-1 activity to indicate inflammasome activity (figure 4B). Cisplatin treatment increased caspase-1 activity of F4/80⁺ TAMs from wild-type MTCQ1 tumors, whereas knockout of *mir21* significantly enhanced caspase-1 activation in TAMs (figure 4C; the gating strategy of caspase 1-positive cells is illustrated in online supplemental figure 6A). To investigate the impact of miR-21-regulated Nlrp3 inflammasomes on chemotherapy responses, we inoculated the wild-type murine MTCQ1 cells or MTCQ1^{mir21-/-} cells to the wild-type or *Nbp3*^{-/-} C57B6/J mice. Cisplatin was intraperitoneally injected to the tumor-bearing mice, and the mice were sacrificed on the 18th day after the commencement of cisplatin treatment (figure 4D). In wild-type mice, knockout of miR-21 in MTCQ1 cells potentiated the tumor suppressive effect of cisplatin, whereas the miR-21 knockout-potentiated chemotherapy response was

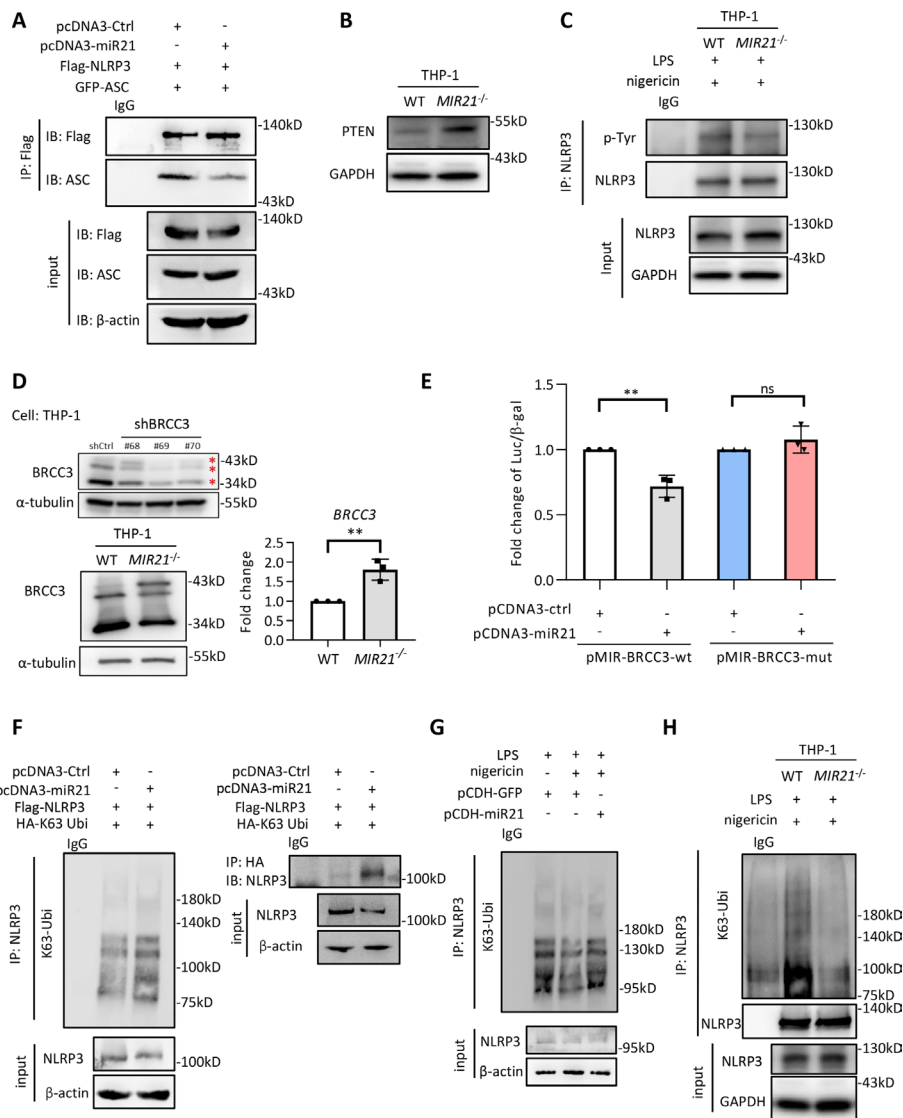


Figure 3 The miR-21-containing exosomes represses *BRCC3* and *PTEN* to reduce NLRP3 inflammasome activity. (A) Immunoprecipitation (IP)-western blots to show the interaction of NLRP3 and ASC in HEK293T cells transfected with Flag-NLRP3, GFP-ASC and miR-21/control vector. IgG is a control for IP. (B) Immunoblots for examining the expression of PTEN in macrophages derived from THP-WT or THP1^{MIR21^{-/-}}. (C) IP-western blots to show the tyrosine phosphorylation of THP-WT/THP1^{MIR21^{-/-}}-derived macrophages primed by LPS (1 μ g/mL) and activated by nigericin (5 μ M). IgG is a control for IP. (D) Left upper, western blot of BRCC3 in THP1 cells receiving short hairpin RNA against BRCC3. α -tubulin was a loading control. Left lower, western blots of BRCC3 in THP-WT/THP1^{MIR21^{-/-}}-derived macrophages. Right, quantitative real-time PCR for examining the relative expression of *BRCC3* in THP-WT/THP1^{MIR21^{-/-}}-derived macrophages. n=3 (each contains two technical replicates). Data shows mean \pm SD. **p<0.01 by Student's t-test. (E) *BRCC3* 3'-UTR reporter assay. The wild-type or miR-21 binding site mutated 3'-UTR reporter constructs of *BRCC3* (pMIR-BRCC3-wt and pMIR-BRCC3-mut), pcDNA3-miR21/control vector and β -galactosidase were co-transfected to HEK293T cells. Data represent means \pm SD. **p<0.01 by Student's t-test. n=3 independent experiments (each contains two technical replicates). (F) IP-western blot to show the K63-ubiquitylated NLRP3 in HEK293T cells transfected with miR-21 or a control vector. NLRP3 (left panel) or HA-tagged K63 ubiquitin (right panel) was immunoprecipitated for immunoblotting. IgG is a control for IP. (G) IP-western blot to show the K63-ubiquitylated NLRP3 in THP1-derived activated macrophages primed by LPS (1 μ g/mL) and transfected with miR-21 or a control vector. Nigericin (5 μ M) was used to activate inflammasome. IgG is a control for IP. (H) IP-western blot to show the K63-ubiquitylated NLRP3 in THP1-WT/THP1^{MIR21^{-/-}}-derived activated macrophages. IgG is a control for IP. miR, micro RNA; UTR, untranslated region.

abrogated in *Nlrp3^{-/-}* mice (figure 4E-F). We examined whether cisplatin influence the viability of TAMs from murine MTCQ-WT and MTCQ^{mir21^{-/-}}-formed tumors by 7-Aminoactinomycin D (7-AAD). The result showed that cisplatin treatment had a slight trend to reduce the viability of F4/80⁺ macrophages in MTCQ-WT-formed

tumors, and the effect was also noted in MTCQ^{mir21^{-/-}}-formed tumors (online supplemental figure 6B). We next investigated the impact of inflammasome activation on pyroptosis of human THP1 cells-derived macrophages. Knockout of miR-21 increased LPS/nigericin-induced cleavage of gasdermin D and IL-1 β . A SYTOX green assay

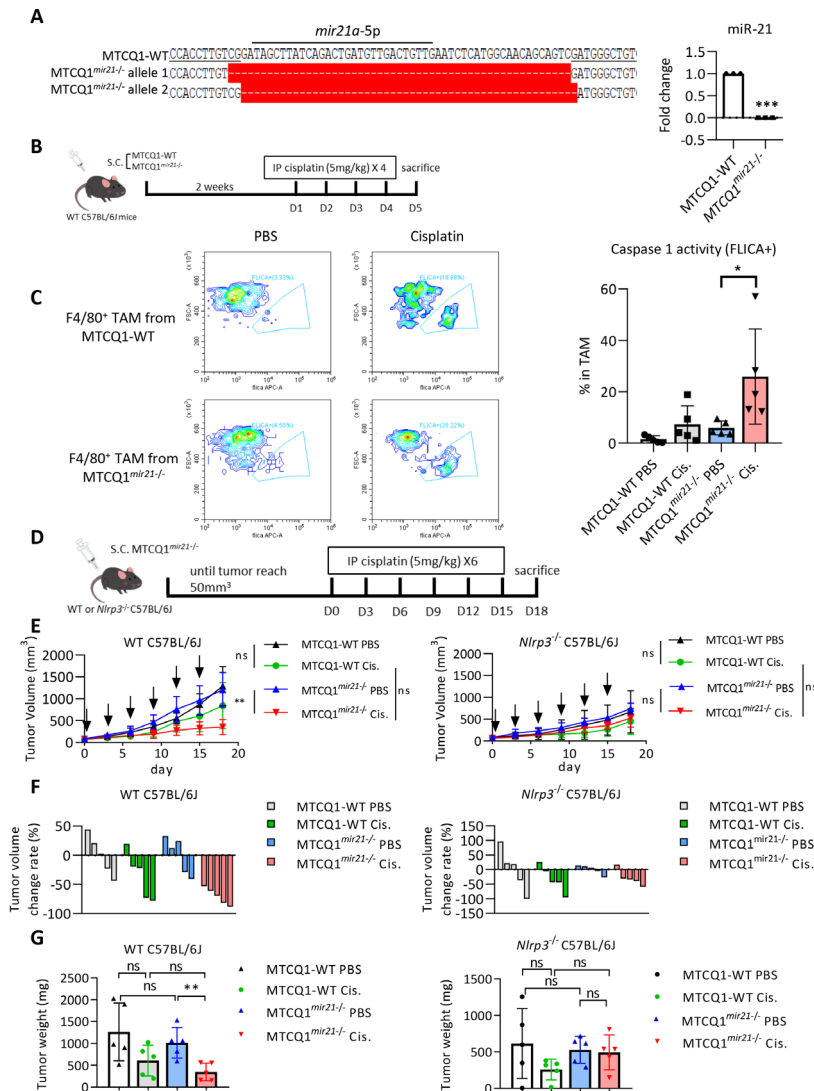


Figure 4 Depletion of mir-21 potentiates response to chemotherapy in oral cancer cells. (A) Left, genomic sequence of mir21a-5p of wild-type murine oral cancer cell line MTCQ1 (MTCQ-WT) and mir-21 knockout subline MTCQ1^{mir21-/-}. Right, quantitative real-time PCR for analyzing the expression of mir-21 of MTCQ-WT and MTCQ1^{mir21-/-}. Data represent means±SD. ***p<0.001 by Student's t-test. n=3 independent experiments (each experiment contains two technical replicates). (B) Schema of the experiment for assaying inflammasome activities of murine tumors. 1×10⁶ of MTCQ1-WT or MTCQ1^{mir21-/-} cells were inoculated to the subcutaneous region of the wild-type C57BL/6J mice for 2 weeks. Cisplatin 5 mg/kg was given intraperitoneally for 4 consecutive days. The mice were sacrificed on the fifth day after the start of cisplatin injection. F4/80⁺ tumor associated macrophages (TAMs) were harvested and caspase 1 activity (FLICA⁺) was analyzed by flow cytometry. (C) Left, representative data of the flow cytometry analysis for the caspase 1 activity of the TAMs (F4/80⁺FLICA⁺) after treated with cisplatin or the control PBS. n=5 for each group. Data shows mean±SD. *p<0.05 by Student's t-test. (D) Schema of the experiment of (E) and (F). 1×10⁶ of wild-type MTCQ1 cells (MTCQ1-WT) or MTCQ1^{mir21-/-} cells were inoculated to the subcutaneous region of the wild-type or Nlrp3^{-/-} C57BL/6J mice. After the tumor size reached 50 mm³ (day 0), intraperitoneal injection of cisplatin (50 mg/kg) or PBS was given every 3 days for a total of six doses, and the tumor size were measured every 3 days. Mice were sacrificed at the 18th day and tumor weight were measured. (E) The volume the MTCQ1-WT/MTCQ1^{mir21-/-}-formed tumors in wild-type or Nlrp3^{-/-} mice treated with cisplatin or PBS. n=5 for each group. Data shows mean±SD. **p<0.01, ns=no significance by Student's t-test. (F) The waterfall plots to indicate the tumor volume change of each mouse. (G) The weight of the MTCQ1-WT/MTCQ1^{mir21-/-}-formed tumors in wild-type or Nlrp3^{-/-} mice treated with cisplatin or PBS. n=5 for each group. Data shows mean±SD. **p<0.01 ns=no significance by Student's t-test. miR, micro RNA; PBS, phosphate buffered saline.

also validated the increased pyroptosis in macrophages derived from THP1^{MIR21-/-} cells. Re-expression of miR-21 in THP1^{MIR21-/-} cells reduced SYTOX green-stained cells (online supplemental figure 6C-D).

We next investigated the effect of Snail on chemotherapy-induced inflammasome activation in vivo.

The murine mammary cancer cell line 4T1 transfected with Snail or a control vector (4T1-Snail/4T1-control; online supplemental figure 7A) was intravenously injected into BALB/c mice to establish pulmonary colonization of tumors. Cisplatin was administered 14 days after the tumor cell injection. Mice were euthanized 3

days later, and the F4/80⁺ macrophages were isolated from the lungs of mice to investigate NLRP3 inflammasome activation by PLA (figure 5A). Overexpression of Snail in murine 4T1 cells significantly reduced the interaction between NLRP3 and ASC in F4/80⁺ macrophages (figure 5B). A reduced level of serum IL-1 β was noted in mice that received orthotopic implantation of 4T1-Snail and were subsequently treated with cisplatin compared with the 4T1-control group (online supplemental figure 7B). We used another syngeneic mouse model to confirm the effect of Snail on the suppression of inflammasome activation. We delivered shRNA against Snail or a control sequence into the murine Lewis lung carcinoma cell line LLC1 (LLC1-shSnail or LLC1-control, respectively; online supplemental figure 7C). LLC1-shSnail/LLC1-control cells were inoculated into the subcutaneous area of wild-type or *Nlrp3*^{-/-} C57BL/6J mice. Cisplatin was injected 10 days after tumor cell inoculation, and the mice were euthanized on the 13th day (figure 5C). In wild-type mice, knockdown of Snail in cancer cells increased the interaction between NLRP3 and ASC in TAMs and the serum level of IL-1 β . NLRP3-ASC interaction in TAMs was not observed in *Nlrp3*^{-/-} mice, as expected (figure 5D). The elevation of serum IL-1 β in the LLC1-shSnail group was also not noted in *Nlrp3*^{-/-} mice (figure 5E).

We next confirmed the role of the Snail-regulated NLRP3 inflammasome in chemotherapy responses. The effect of Snail knockdown exerted on chemotherapy-mediated tumor suppression, the recruitment of interferon (IFN)- γ -expressing CD8⁺ tumor-infiltrating lymphocytes (TILs), and the expression of *Ifng* were examined in the LLC1 syngeneic mouse model (the gating strategy of CD45⁺CD8⁺IFN- γ ⁺ TILs is illustrated in online supplemental figure 7D). When mice were not treated with cisplatin, knockdown of Snail caused a borderline decrease in tumor volume/weight. In contrast, the effect of Snail-knockdown-induced tumor suppression was augmented when mice received cisplatin treatment (figure 5F–G). IL-1 β affects CD8⁺ T cells to enhance their effector function in response to antigen stimulation.^{14 41} The present study found that the knockdown of Snail increased the proportion of IFN- γ -expressing CD8⁺ TILs after cisplatin treatment (figure 5H). Suppression of Snail also enhanced the expression of *Ifng* in the harvested tumors (figure 5I). To further validate the Snail-miR-21 axis in regulating tumor response to cisplatin, we generated the murine LLC1-sh-ctrl, LLC1-sh-Snail, and LLC1-sh-Snail-miR21 sublines for in vivo experiments. The expression of Snail and miR-21 was validated in the corresponding cell lines (online supplemental figure 7E). Snail knockdown potentiated the effect of cisplatin treatment, and the expression of miR-21 in Snail-knockdown LLC1 cells abrogated this effect (online supplemental figure 7F–G). Taken together, these results suggest that Snail attenuates cisplatin-induced NLRP3 inflammasome activation of TAMs, which contributes to the chemoresistance of cancer cells.

Tumorous miR-21 repopulates the infiltrated immune cells in syngeneic tumors

To understand the influence of miR-21 expression in chemotherapy-treated oral cancers on infiltrated immune cells, scRNA-seq was performed using the CD45⁺ cells sorted from the wild-type/miR21 knockout murine oral cancer MTCQ1 cell (MTCQ-WT/MTCQ^{mir21-/-})-derived tumors 72 hours after chemotherapy (online supplemental figure 8A; online supplemental table 8). We used CIPR to annotate the cell clusters of the CD45⁺ cells,²¹ and different types of immune cells were identified accordingly (figure 6A and online supplemental figure 8B). The following pattern of infiltrated immune cells was distinct between the wild-type and miR-21 KO cancer cell-derived tumors: a significant increase in the proportion of macrophages, dendritic cells, monocytes, and natural killer cells and a decrease in the proportion of CD4⁺ T cells and regulatory T cells were noted in murine MTCQ^{mir21-/-}-derived tumors (figure 6B).

Next, we analyzed the TAMs in MTCQ-WT- and MTCQ^{mir21-/-}-derived tumors. The results showed that TAMs from MTCQ^{mir21-/-}-derived tumors harbored an immunoactive gene signature compared with that from MTCQ-WT-derived tumors (figure 6C, online supplemental table 9). GO analysis revealed that the pathways related to immune responses and inflammation were significantly enriched in TAMs of MTCQ1^{mir21-/-}-derived tumors (figure 6D, online supplemental table 10). GSEA demonstrated that compared with the TAMs of MTCQ1-WT-derived tumors, TAMs of MTCQ^{mir21-/-}-derived tumors were associated with a more significant reactive oxygen species-related signature and downregulated expression of the TGF- β -related signature (figure 6E). Since the polarity of macrophages affects the therapeutic efficacy of tumors significantly,⁴² we further analyzed the subclusters of TAMs in these two groups (figure 6F; online supplemental table 11). Re-clustering of macrophages demonstrated a distinct distribution of macrophage subgroups among TAMs from MTCQ^{mir21-/-}- and MTCQ-WT-derived tumors. A significantly higher proportion of cluster 1 was noted in the MTCQ1^{mir21-/-} group, whereas a dominant cluster 2 was observed in the MTCQ1-WT group (figure 6G). Pathway analysis showed that TAMs other than cluster 2 TAMs were associated with an immunoactive signature (online supplemental figure 8C), (online supplemental table 12). However, there is no significant trend of M1/M2 polarity among these seven clusters. Cluster 1 macrophages tended to coexpress both M1 and M2 genes compared with the other clusters (online supplemental figure 8D). Single-cell trajectory analysis of the macrophages/monocytes from tumors revealed that the order of macrophage polarization was cluster 6 to clusters 2 and 3, followed by separation into clusters 1, 4, and 5 (online supplemental figure 9A). In addition to the expression signature in macrophages, the gene expression signature was also distinct between the dendritic cells obtained from the MTCQ1^{mir21-/-} and MTCQ1-WT groups

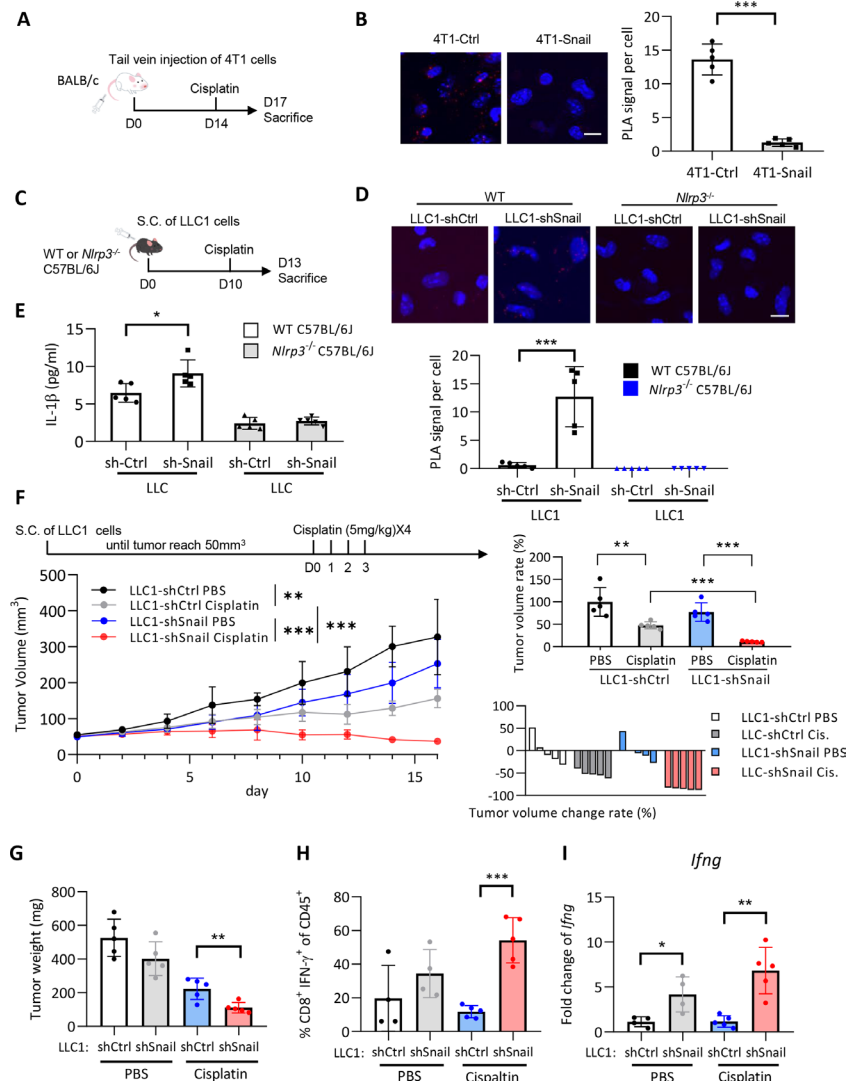


Figure 5 Snail limits chemotherapy-induced NLRP3 inflammasome activation in vivo. (A) Schema of the experiment. 2.5×10^5 4T1 cells expressing Snail or a control vector were intravenously injected into the tail veins of BALB/c mice. 4T1 tumor-bearing mice received a single injection of cisplatin (5 mg/kg) at the 14th day after tumor cell injection. The mice were sacrificed at the 17th day and the F4/80⁺ macrophages isolated from the lungs of the mice were subjected to PLA analysis. (B) PLA signal of NLRP3 and ASC interaction in pulmonary macrophages of 4T1-Snail/4T1-Ctrl. Left, the representative immunofluorescent images for showing the interaction of ASC-NLRP3 in F4/80⁺ macrophages isolated from lungs of mice. The red dots indicate the PLA signals. Right, quantification of PLA signals per cell. For each mouse, four high power fields from random selection were used for PLA quantification. Scale bar, 10 μ m. $n=5$ for each group. *** $p < 0.001$ by Student's t-test. (C) Schema for animal experiment. 5×10^5 LLC1 cells were inoculated into the subcutaneous area of the WT or *Nlrp3*^{-/-} C57BL/6J mice. A single dose of cisplatin (5 mg/kg) was given at the 10th day after tumor cell injection. The mice were sacrificed at the 13th day after tumor injection. The F4/80⁺ TAMs were harvested for PLA for detecting the NLRP3-ASC interaction. (D) PLA signal of NLRP3 and ASC interaction. Upper, the representative immunofluorescent images for showing the NLRP3-ASC interaction in F4/80⁺ TAMs isolated from tumors. For each mouse, at least a total of 30 cells from eight randomly-selected fields were used for PLA quantification. Lower, quantification of PLA signals per cell. Scale bar, 10 μ m. *** $p < 0.001$ by Student's t-test. (E) Serum IL-1 β level of mice 3 days after cisplatin treatment ($n=5$). Data shows mean \pm SD. * $p < 0.05$ by Student's t-test. (F) Left upper, schema of the experiment. LLC1 cells (2.5×10^5) were inoculated to the subcutaneous region of C57BL/6 mice. Cisplatin (5 mg/kg) was given at 0, 1, 2, 3 day after tumor cell injection. The mice were sacrificed at the 16th day after cisplatin/PBS treatment. Left lower, tumor volume curve. Right upper, tumor volume change presented in %. Right lower: a waterfall plot to show the volume change of each tumor. $n=5$ for each group. Tumor volume is shown in mean \pm SD, and tumor volume reduction rate shows mean \pm SD. ** $p < 0.01$, *** $p < 0.001$ by Student's t-test. (G) The tumor weight of LLC1-formed tumors in (F). Data shows mean \pm SD. ** $p < 0.01$ by Student's t-test. (H) Quantification of the percentages of CD8⁺IFN γ ⁺ tumor-infiltrating lymphocytes among CD45⁺ tumor-infiltrating leukocytes in different groups of mice as panel (F). The harvested tumors were dissociated and the tumor-infiltrating leukocytes were analyzed by flow cytometry. For PBS groups, $n=4$; for cisplatin groups, $n=5$. *** $p < 0.001$ by Student's t-test. (I) Quantitative real-time PCR for analyzing the expression level of *Ifng* in different groups of LLC1-formed tumors as panel (F) and (G). For PBS groups, $n=4$; for cisplatin groups, $n=5$. * $p < 0.05$, ** $p < 0.01$ by Student's t-test. IFN, interferon; IL, interleukin; PBS, phosphate buffered saline; PLA, proximity ligation assay; TAM, tumor-associated macrophages.

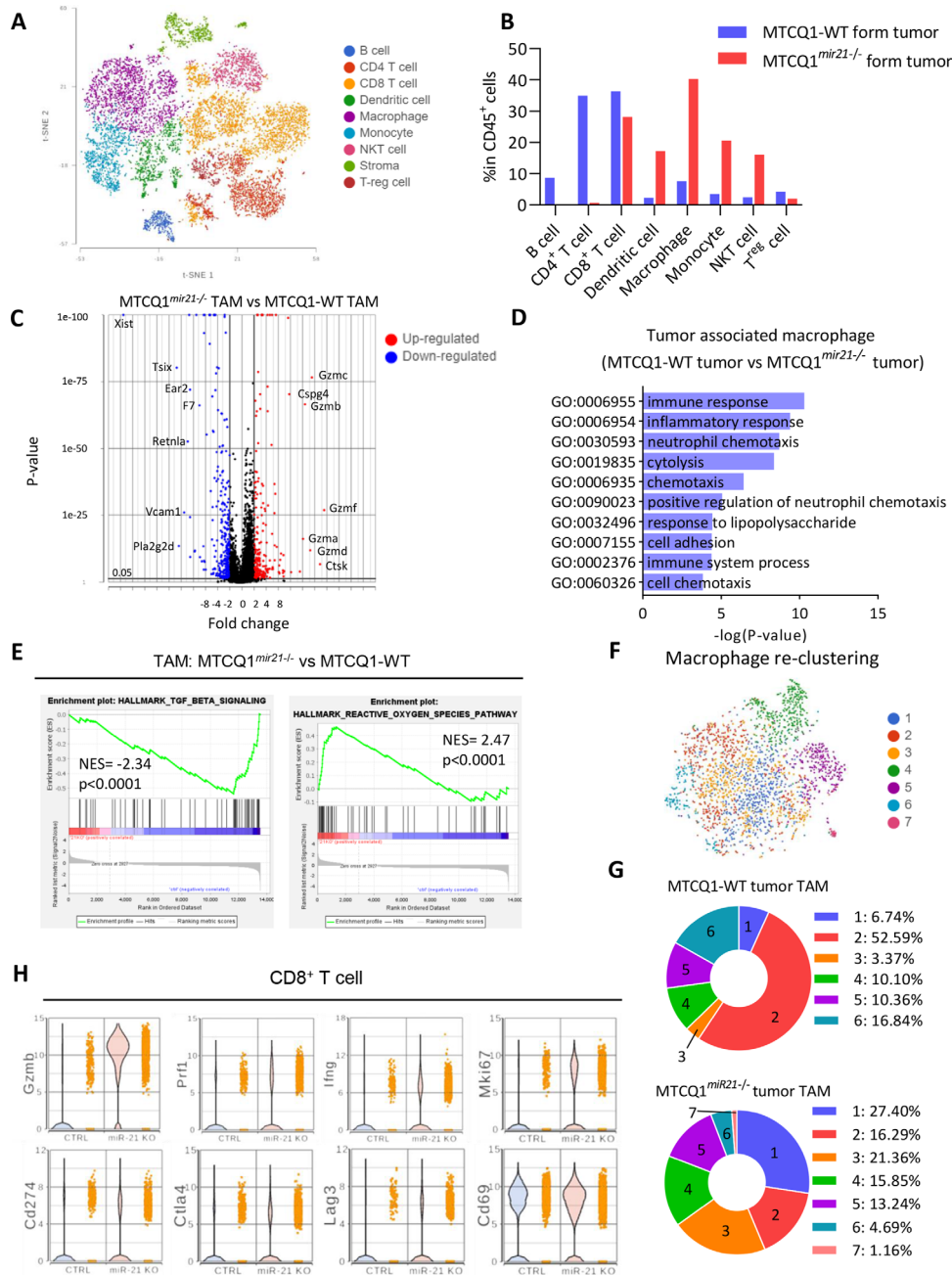


Figure 6 Tumorous mir-21 shapes the infiltrated immune cells of syngeneic murine oral cancers. (A) The t-distributed stochastic neighbor embedding and immune cell type clustering (see Liu *et al*²⁸ of CD45⁺ cells in MTCQ1-WT and MTCQ1^{mir21-/-}-formed tumors 3 days after cisplatin (5 mg/kg) injection. (B) Different immune cell type distribution between MTCQ1-WT and MTCQ1^{mir21-/-}-formed tumor. (C) Volcano plots of the differential expressed genes of the TAMs from MTCQ1^{mir21-/-} versus MTCQ1-WT-formed tumors. Red, upregulated genes; blue, downregulated genes. (D) GO enrichment analysis of the biological pathways of the TAMs from MTCQ1^{mir21-/-} versus MTCQ1-WT-formed tumors. (E) GSEA of the TGF- β signaling gene set (M5896) and reactive oxygen species pathway gene set (M5938) in MTCQ1^{mir21-/-} TAMs versus MTCQ1-WT TAMs. (F) Re-clustering of the TAMs from MTCQ1-WT and MTCQ1^{mir21-/-}-formed tumors. (G) Distribution of the TAM clusters of MTCQ1-WT or MTCQ1^{mir21-/-} TAM. (H) Violin plots for showing the expression of the T cell activation genes (*Gzmb*, *Pfr1*, *Ifng*, *Mki67*) and immune checkpoint genes (*Cd274*, *Ctla4*, *Lag3*, *Cd69*) expression in CD8⁺ T cells of the MTCQ1-WT and MTCQ1^{mir21-/-} tumors. GSEA, Gene Set Enrichment Analysis; GO, Gene Ontology; miR, micro RNA; TAM, tumor-associated macrophages; TGF- β : transforming growth factor β .

(online supplemental figure 9B), (online supplemental table 13).

Next, we investigated the influence of tumorous mir-21 expression on the infiltrated T cells. The violin plots showed that knockout of mir-21 in tumor cells increased

the expression of the cytotoxic genes (*Grmb*, *Prf1*), activated T-cell genes (*Ifng*, *Mki67*) in T cells, suggesting that the activity and cytotoxicity of T cell was enhanced in mir-21 knockout tumors. A mild increase of the immune checkpoints (*Cd274*, *Ctla4*, *Lag3*, and *Cd69*) was noted in

TILs of mir-21 knockout tumors (figure 6H). Regarding the expression of the genes related to T cell exhaustion, a reduced *Tcf1* and an increased *Tbet* was noted. The level of *Tim3* and *Pd1* was not changed significantly (online supplemental figure 9C). In summary, these data suggest that an increased antitumor activity of both macrophages and CD8⁺ T cells was observed in the infiltrated immune cells of mir21^{-/-} tumors after chemotherapy.

The Snail-miR-21 axis correlates with the suppression of NLRP3 inflammasome activity and a worse response to chemotherapy in patients with head and neck cancer

Finally, we validated the clinical significance of the Snail-miR-21 axis in the regulation of inflammasome activity and chemosensitivity in patients with HNSCC. We first examine the prognostic impact of Snail, miR-21, and BRCC3 in the HNSCC TCGA database. The result showed that in the total population of TCGA-HNSCC, a higher expression of *SNAIL* was correlated with a worse overall survival ($p=0.03858$). The expression of *BRCC3* and *MIR21* did not have significant impact on the overall survival of HNSCC. We further analyzed the subgroup of patients with HNSCC receiving pharmaceutical treatment ($n=53$). *SNAIL* or *MIR21* upregulation correlated with worse an overall survival, whereas *BRCC3* expression did not have a significant prognostic impact (figure 7A). This result indicates that the Snail-miR-21 axis has a negative impact on patient with HNSCC survival especially in patients receiving pharmaceutical therapies, which is consistent with our hypothesis that the Snail-miR-21 axis influences the treatment responses of patients with HNSCC.

We next confirmed the findings in three independent sets of samples which were used in this study. In the first group, we retrospectively analyzed the expression of *SNAIL* and IFN- γ gene expression signatures in 50 HNSCC samples. Increased expression of *SNAIL* correlated with higher miR-21 and downregulated expression of IFN- γ signature genes *IFN- γ* , *CXCL9*, and *CXCL10* (figure 7B). In the second group, we prospectively collected serum samples from 19 patients with HNSCC who received chemotherapy. The samples were obtained 1 day before and 1 day after chemotherapy to examine the level of IL-1 β to indicate the activity of inflammasomes. The expression of Snail in the corresponding tumors was also examined. In patients with low Snail expression, upregulated serum IL-1 β levels were observed after chemotherapy. When tumors expressed a higher level of Snail (Snail + ~ ++), chemotherapy could not induce a post-treatment surge in IL-1 β levels (figure 7C). The treatment response was concordant with the expression of Snail, that is, a low percentage of tumor response to chemotherapy was noted in patients with Snail +++ (figure 7D). In the third group, we examined the correlation between Snail and activated NLRP3 inflammasome in TAMs in five tumor samples. PLA could detect activation of the NLRP3 inflammasome in tumor samples probed with anti-NLRP3 and anti-ASC antibodies. Immunofluorescence staining

of CD68 was performed to indicate the activation of the NLRP3 inflammasome in TAMs. Reduced abundance of CD68⁺PLA⁺ TAMs was observed in Snail-overexpressing HNSCC cells (figure 7E). In summary, the clinical sample data support that the Snail-miR21 axis regulates the activity of the NLRP3 inflammasome to attenuate the response to chemotherapy in HNSCC.

We summarize our finding in online supplemental figure 10. In cancer cells with high expression of Snail, the tumor-derived exosomes are enriched with miR-21. The miR-21 suppresses the expression of *PTEN* and *BRCC3* in macrophages, which results in the phosphorylation and K63 polyubiquitination of NLRP3, leading to the disassembly and inactivation of NLRP3 inflammasomes. NLRP3 inflammasome inactivation lowers IL-1 β secretion, thereby the chemotherapy-induced inflammation and immunogenic cell death is reduced.

DISCUSSION

Despite the arguments put forth regarding inflammasome activation in cancer progression and therapeutic responses, recent studies support the role of inflammasome activation in augmentation of the chemotherapy response via enhancement of antitumor immunity. Chemotherapy-induced damage-associated molecular patterns include extracellular ATP, a potent activator of the NLRP3 inflammasome,⁴³ which activates the inflammasome of dendritic cells to produce IL-1 β and IL-18 to expand immune signals and to recruit CD8⁺ T-cells for antitumor immune responses.¹⁴ A recent study has shown that phosphorylation of NLRP3 in myeloid cells reduces NLRP3-ASC interaction and inhibits inflammasome activity, resulting in the development of chemoresistance.²⁰ Intriguingly, our clinical data showed that in patients with advanced HNSCC harboring a higher Snail expression, the basal level of serum IL-1 β was relatively high compared with the Snail-low cases. However, the post-treatment surge in IL-1 β levels was not observed in the Snail-high HNSCC and was associated with a worse chemotherapy response (figure 7C–D). The high basal level and lack of response of IL-1 β in Snail-high advanced HNSCC cases implicate pro-tumorous inflammatory microenvironments of aggressive tumors with blunted chemotherapy-triggered inflammasome activation. Together with these findings, our study clarifies the context-dependent role of the inflammasome of innate immune cells in cancer, in which inflammasome-induced proinflammatory signals favor tumor growth. In contrast, therapy-induced inflammasome activation facilitates chemotherapy-induced antitumor immunity and treatment responses.

Our data showed that miR-21 was the most abundant exosomal miRNA in HNSCC, consistent with reports regarding the significant oncogenic role and abundance of miR-21 in different types of cancers.⁴⁴ The miR-21 exerts its oncogenic effects by targeting different tumor suppressors, such as *PTEN*,³⁹ *PDCD4*,⁴⁵ ⁴⁶ and

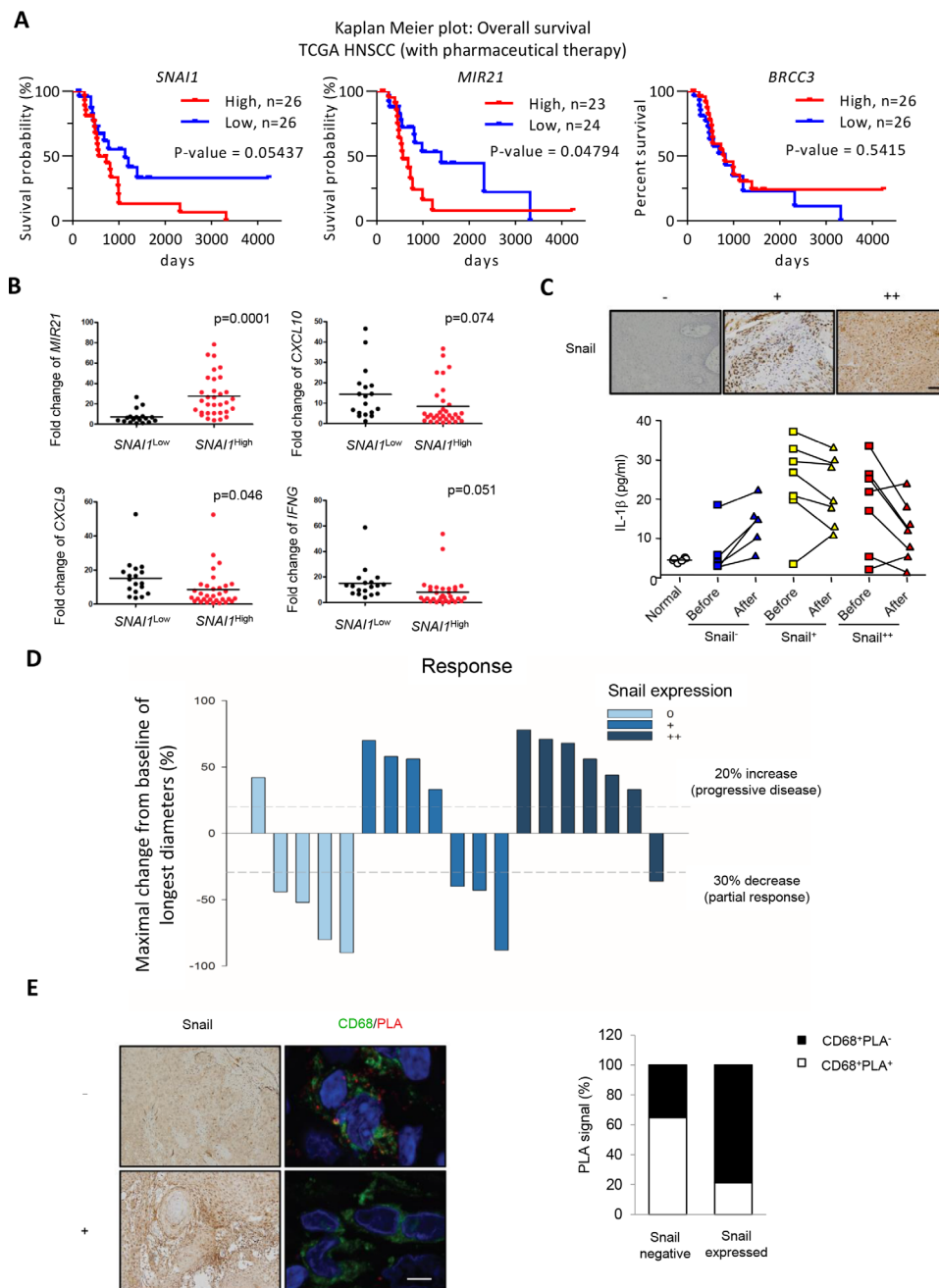


Figure 7 Snail limits chemotherapy-induced NLRP3 inflammasome activation in patients with HNSCC. (A) Kaplan-Meier plots for analyzing the influence of differential expression of *SNAI1*, *MIR21*, and *BRCC3* on overall survival of patients with HNSCC with pharmaceutical therapy from TCGA database. The log-rank p value is shown in each panel. (B) Quantitative real-time PCR for analyzing the expression level of *MIR21*, *CXCL10*, *IFNG* and *SNAI1* in HNSCC samples. $n=50$. The *SNAI1*^{High} ($n=32$) is defined as the level higher than mean value, and the *SNAI1*^{Low} ($n=18$) is defined as the level lower than mean value. (C) Upper, IHC staining of Snail with differential expressions in representative HNSCC samples. Scale bar, 50 μ m. Lower, serum IL-1 β level in patient with HNSCC before and 1 day after chemotherapy. (D) A waterfall plot for illustrating the response to chemotherapy of patients with HNSCC in (C). (E) Left, IHC staining of Snail (left) and immunofluorescent staining (right) of CD68 (green)/NLRP3-ASC PLA (red) in representative HNSCC samples. Right, quantification of the results. Three Snail-negative and two Snail-positive HNSCC samples were used in the experiment. For each sample, at least five CD68⁺ TAMs were quantified for PLA signals. The result is shown as the percentage of PLA-positive among CD68⁺ TAMs. Scale bar, 5 μ m. HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; PLA, proximity ligation assay; TAM, tumor-associated macrophages; TCGA, The Cancer Genome Atlas.

*IGFBP3*⁴⁷ to promote tumor growth. However, the role of miR-21 in immune cells is more complicated than its prominent pro-tumorous effects in cancer cells. It is

well established that miR-21 is predominantly expressed in M2 macrophages.^{6 48 49} In this study, we showed that miR-21 suppresses NLRP3 inflammasome activation in

TAMs. Nevertheless, miR-21 has been reported to activate NLRP3 inflammasome in septic shock patients by repressing A20 to activate NF- κ B for increasing the transcription of inflammasome components.³⁶ The possible explanation for the differential role of miR-21 in regulating NLRP3 inflammasome in different disease context is as follows. NLRP3 inflammasome activation can be regulated at both the transcription level of the inflammasome components for regulating the amount of inflammasome subunits and post-translational modification of NLRP3 for regulating inflammasome assembly. In patients with cancer, the primary TMEs contain abundant inflammatory cytokines,⁵⁰ and macrophages in primary tumors expressed both the M1 and M2 phenotype.⁵ We consider that the basal level of the components of NLRP3 inflammasome is relatively high in patients with cancer, the assembly of inflammasome is therefore crucial for regulating its activities. In patients with septic shock, activation of inflammasomes is the triggering event for the subsequent immune response, therefore induction of components of the expression of NLRP3 inflammasome is important.

Compared with the prominent oncogenic role in cancer cells, contradictory findings were noted regarding the antitumor immunity of miR-21. Knockout of mir-21 in mice slowed the proliferation of both CD4⁺ and CD8⁺ cells and accelerated the growth of engrafted tumors.⁵¹ However, tumorous miR-21 is inversely associated with the densities of CD3⁺ and CD45RO⁺ cells in colorectal cancers.⁵² Furthermore, miR-21 promotes M2 polarization,⁴⁹ and our previous work has shown that miR-21-containing TEXs are potent regulators of TAMs through the promotion of M2-like activation.⁶ Here, we further investigated the role of Snail-miR-21 axis in antitumor immunity. Snail-driven miR-21-abundant TEXs inhibited NLRP3 inflammasome activity of macrophages. In HNSCC, an increased infiltration of TAMs was observed in samples with a higher expression of Snail (online supplemental figure 1A). Knockout of mir-21 in the murine oral cancer cell line MTCQ (MTCQ^{mir21-/-}) upregulated caspase-1 activity of TAMs when treated with cisplatin (figure 4C). In the scRNA-seq analysis, knockout of mir-21 reduced the proportion of CD4⁺ TILs but activated CD8⁺ TILs. Regarding the innate immune cells, depletion of mir-21 prominently enriched and shaped the gene expression signature of innate immune cells such as macrophages and dendritic cells to an antitumor profile (figure 6). Taken together, we consider that the malignant behaviors of miR-21-abundant cancers are attributed to both the oncogenic effect of intracancerous miR-21 and the TME-modulating effect of exosomal miR-21. In tumor-infiltrated immune cells, miR-21 may majorly affect innate immune cells in response to stimuli, highlighting the importance of exosomal transmission of miR-21.

In conclusion, we demonstrate the development of an immune adaptation-related chemoresistance driven by Snail during metastatic evolution. The plasticity of cancer

cells dynamically shapes the tumor-infiltrated immune cells in response to therapeutic stimuli, indicating the necessity of real-time adjustment of treatment strategies alongside tumor progression.

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Contributors M-HY is the guarantor of the study and supervised the whole study. M-HY, H-YC, and C-HH conceived and designed the experiments. H-YC and C-HH performed most of the experiments with the help of P-HL, Y-TC, and DS-SH; and they analyzed the data. M-HY, S-KT and P-YC provided clinical samples and patient care. M-HY collected all demographic data, interpreted the immunohistochemistry results, analyzed the relevant clinical parameters, and performed the statistical comparisons. H-YC, C-HH and M-HY wrote the paper.

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