IL6 Mediates Immune and Colorectal Cancer Cell Cross-talk via miR-21 and miR-29b

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Abstract

Tumors are surrounded and infiltrated by a variety of stromal cell types, including fibroblasts, immune cells, and vascular endothelial cells, which interact with malignant cells to generate the tumor microenvironment (TME). This complex environment is thought to be regulated by the tumor in order to promote its survival and progression and thus constitutes a potential target for cancer therapy. However, intercellular communication within the microenvironment is not yet well understood. The current study investigates the mechanism by which cancer and immune cells communicate using an in vitro coculture model. It is demonstrated that IL6, a proinflammatory cytokine, secreted by immune cells promotes colorectal cancer cell invasiveness. In addition, in the presence of IL6, the cancer cells were able to secrete circulating miRNAs miR-21 and miR-29b to further induce immune cell IL6 production. Activated immune cells were also found to release miR-21 into the TME. Taken together, these mechanistic findings provide a better understanding of intercellular communication between immune and cancer cells in the TME and offer insight into some of the key players that mediate this cross-talk.

Implications: This study demonstrates that cocultured cancer and immune cells communicate via IL6 and circulating miRNAs to sustain chronic inflammation and promote prometastatic cancer cell behavior. In addition, critical players are identified that mediate intercellular communication in the TME and suggest possible therapeutic approaches that target the microenvironment.

Materials and Methods

Cell culture

The human colorectal adenocarcinoma adherent cell lines HCT116 and SW480 as well as the human monocytic THP1...
suspension cell line were obtained from the ATCC (LGC Prochem) and used within 6 months of resuscitation. ATCC authenticates cell lines by performing routine cell morphology monitoring, short tandem repeat profiling, and karyotyping. Cells were routinely cultured in RPMI1640 medium (GIBCO; Life Technologies) supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine (GIBCO; Life Technologies).

For coculture experiments, adherent HCT116 cells were seeded at a density of $1 \times 10^5$ cells per well of a 6-well plate, allowed to attach to the bottom of the plate for 24 hours, prior to the addition of $1 \times 10^7$ THP1 suspension cells. All cells were incubated at 37°C in a humidified incubator (5% CO2). Cells between passages 3 and 7 were used for experiments.

Cell treatments
Prior to treatment, cells were maintained in culture medium supplemented with 5% dextran-coated charcoal-stripped FBS for at least 72 hours.

THP1 cells were treated with 1 μg/mL lipopolysaccharide (LPS) for 3 hours to promote proinflammatory cytokine secretion. LPS was purchased as purified from Escherichia coli 0127:B8 by phenol extraction (Sigma-Aldrich) and dissolved in cell culture medium.

HCT116 and SW480 cells do not constitutively express IL6 so human recombinant IL6 (at doses of 0–1,000 pg/mL, chosen within the range released by stromal cells in the colon; ref. 5; HumanKine; Sigma-Aldrich) dissolved in PBS containing 0.1% human serum albumin (Sigma-Aldrich) or conditioned medium (CM) from THP1 cells were added to the cells for 24 hours.

Transwell migration and invasion assays
For the migration assay, $3 \times 10^5$ cells per well were plated in 100 μL of culture medium supplemented with 1% dextran-coated charcoal-stripped FBS in the upper chamber of a 96-well insert system with 8-μm pores (BD Falcon). In the lower chamber, 100 μL of culture medium containing 10% FBS was added as a chemoattractant. For the invasion assay, 20 μL Matrigel was added to the upper chamber and left to set at room temperature prior to addition of the cells. In both cases, treatment was added to the upper chamber, and cells were left to migrate to the lower chamber for 72 hours. The cells were then removed from the upper chamber using a moist cotton swab, and cells in the lower chamber were incubated with 10% AlamarBlue as per the manufacturer’s protocol (Invitrogen; Life technologies) for 2 hours at 37°C. Under these conditions, AlamarBlue is enzymatically converted to resorufin, which can be measured in a spectrophotometer. The enzymatic conversion to resorufin is proportional to cell number. Results are expressed as fold change compared with vehicle control.

RNA extraction
Total RNA was isolated from cells using TRIzol reagent (Invitrogen; Life technologies) according to the manufacturer’s protocol.

For miRNA isolation from cell culture medium, mirVANA PARIS kits were used (Applied Biosystems; Life technologies) with 400 μL of CM according to the manufacturer’s protocol. Synthetic Caenorhabditis elegans (C. elegans) miR-39 (Ambion; Life technologies) was spiked in at an amount of 15 fmol to act as an internal standard.

RNA extracts were quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer; Implen) with purity assessed from 260/280 nm and 260/230 nm ratios. Extracts were stored at −80°C until used.

Reverse transcription and quantitative PCR
Reverse transcription and quantitative PCR were performed as previously described (17). Briefly, for mRNA reverse transcription, a Superscript II reverse transcription kit was used (Invitrogen; Life technologies), and miRNA reverse transcription kit was used for mRNA expression (Applied Biosystems; Life Technologies). QPCR was performed using predesigned expression assays (Taquin; Applied Biosystems; Life Technologies) for IL6 (Hs01054797_g1), GAPDH (Hs99999905_m1), U6 (001973), miR-29b (000413), miR-21 (000397), and C. elegans miR-39 (000200). FAST PCR master mix was used according to the manufacturer’s protocol (Taquin; Applied Biosystems; Life Technologies). GAPDH and U6 expression were used to normalize cellular mRNA and miRNA expression respectively, whereas C. elegans miR-39 was used to normalize extracellular miRNA levels in the culture medium.

ELISA
IL6 was quantified in the conditioned cell culture medium using the Human IL6 Quantikine ELISA Kit according to the manufacturer’s protocol (R&D systems). Absorbance was measured in a Synergy H1 plate reader (Biotek).

Transfection of miRNA mimics
MiRNA mimics were obtained from miRIDIAN mimics (Thermo Fisher Scientific) and transfectioned into the cells using Lipofectamine 2000 (Invitrogen; Life technologies) according to the manufacturer’s protocol. Briefly, cells were seeded at a density of $1 \times 10^5$ cells per well of a 24-well plate, and cell culture medium was replaced by 400 μL/well of Opti-MEM (GIBCO; Life technologies) prior to the addition 150 μL/well of Opti-MEM containing 8 μL of Lipofectamine 2000 reagent and 2.5 μL of 20 μmol/L stock of miRNA mimic or miRIDIAN miRNA negative control. Cells were incubated with the transfection complexes for 24 hours prior to harvest.

Statistical analysis
Data were obtained from measurements made in at least three biologic replicates and presented as a mean ± SEM. Significant differences ($P < 0.05$) were determined either by the Student t test or ANOVA (GraphPad Prism 5; GraphPad Software Inc.).

Results
Immune cells secrete IL6 to promote colorectal cancer cell invasion
In the TME, immune cells interact with cancer cells via a complex mixture of cytokines and chemokines, which includes IL6. To recapitulate this environment, CM from LPS-activated THP1 human monocytic cells containing an array of proinflammatory cytokines, including IL6, IL-1β, and TNFα, was added to the cancer cells. THP1 cells were stimulated for 3 hours with 1 μg/mL LPS to induce their cytokine secretion. Following LPS treatment, THP1 cells and their CM were collected. Stimulated
THP1 cells had increased levels of IL6 mRNA expression (Fig. 1A) and IL6 protein secretion (70 pg/mL, Fig. 1B) compared with unstimulated cells.

HCT116 cells were treated with CM from the stimulated THP1 cells or IL6 alone to investigate cell invasion (Fig. 1C). A significant 4-fold increase in cell invasion was observed in HCT116 cells treated with CM from LPS-stimulated THP1 cells (containing around 70 pg/mL IL6) and with 80 pg/mL of IL6 alone (Fig. 1C), suggesting that this effect on cell invasion by the CM is primarily mediated by the presence of IL6 and not the other proinflammatory cytokines.

Cancer cells sustain surrounding immune cell IL6 production

So as to determine whether cancer cells can promote IL6 production in surrounding immune cells, we cultured HCT116 or SW480 cells with THP1 monocytic cells for 24 hours and measured THP1 cell IL6 mRNA expression and secretion (Fig. 2). Interestingly, coculture alone was not able to significantly induce IL6 production by THP1 cells. However, when the cancer cells were pretreated with IL6 for 24 hours, washed, and then cocultured with THP1 cells, IL6 production in THP1 cells was significantly induced. This suggests that factors released by IL6-treated cancer cells but not untreated cancer cells are responsible for THP1 activation. As miRNAs have previously been shown to activate immune cells (16), we then investigated whether miRNAs play a role in these observed effects.

MiRNAs are involved in IL6-mediated cancer immune cell cross-talk

Cancer cells were treated with IL6, and expression of various miRNAs was measured. IL6 treatment of HCT116 and SW480 cells significantly promoted expression of two miRNAs miR-21 and miR-29b and additionally miR-135b was also induced in HCT116 (Fig. 3A). Interestingly, miR-21 and miR-29b are reported circulatory miRNAs able to integrate into surrounding cells acting as paracrine signaling molecules (14, 18–22). In addition, it was recently discovered that miR-21 is able to bind to TLR8 located within endosomes of immune cells to induce an inflammatory response (16).

To investigate whether these miRNAs are secreted by cancer cells into the TME, we measured their expression levels in CM
Figure 3.
MiR-21 and miR-29b are secreted by cancer cells in response to IL6 treatment. A, MiRNA expression in HCT116 and SW480 cells following IL6 treatment (0, 10, 100, and 1,000 pg/mL) for 48 and 24 hours, respectively. Data are presented as fold change compared to unconditioned media only control. B, MiR-21 and miR-29b expression in the CM from HCT116 and SW480 cells. Error bars, SEM for independent cultures (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with control. The lines in A indicate significant trends.
from IL6-treated cancer cells (Fig. 3B). Although miR-21 and miR-29b were detected in the untreated cancer cell CM, their levels in culture medium were significantly increased when the cells were treated with IL6. These observations correlate with our previous finding that only IL6 pretreated cancer cells were able to activate immune cells. Therefore, miR-21 and miR-29b could be the factors released by cancer cells involved in activating immune cells.

To confirm whether these secreted miRNAs are indeed able to induce IL6 expression in their recipient immune cells, miR-21 and miR-29b mimics were transfected into THP1 cells, and IL6 mRNA expression was measured (Fig. 4A). Both miR-21 and miR-29b mimics induced more than a 2-fold induction of IL6 mRNA levels in THP1 cells compared with the control mimic. This suggests that miR-21 and miR-29b secreted by IL6-treated cancer cells are able to further activate surrounding immune cells to produce more IL6, thus generating a feedback loop.

Activated immune cells induce the NF-kB pathway, which is known to promote miR-21 expression (23). As miR-21 is a circulating miRNA and known to be oncogenic (24), we determined whether activated immune cells in the TME could also produce and secrete miR-21 as another means of promoting tumor progression. To investigate this, we measured levels of miR-21 in the CM from stimulated THP1 cells compared with unstimulated cells and found that activated immune cells released higher levels of miR-21 (Fig. 4B). Therefore, miR-21 secretion into the TME by immune cells could be another mechanism in addition to cytokine release involved in their protumorigenic effects.

### Discussion

The TME is a dynamic and complex environment that is closely regulated by the tumor through extracellular signals. The tumor is thought to adapt and mold its environment in order to promote its survival and progression. The tumor and its environment are therefore constantly interacting, and miRNAs have emerged as new mediators of this cross-talk. Here, we suggest that cancer cells secrete miR-21 and miR-29b in a mechanism involving IL6, resulting in sustained IL6 release by immune cell, which promotes cancer cell invasion. Activated immune cells can also secrete oncogenic miR-21 into the TME, which could signal back to the cancer cells and further promote tumor progression (Fig. 5).

Proinflammatory cytokine IL6 was found to promote cell invasion, and CM from LPS-treated THP1 cells containing IL6 had a similar effect. However, previous reports have shown that proinflammatory tumor-infiltrating immune cells, such as M1 macrophages that secrete high levels of IL6, have an antitumor effect and are associated with a better prognosis (25, 26). On the other hand, IL6 is known to promote tumor growth and invasion (7–9), and high levels of IL6 correlate with poor disease outcome (27). The results from the current study suggest that proinflammatory immune cells in the TME are able to promote cancer cell invasiveness through IL6 secretion, likely due to their inability to elicit an anti-tumor response in these in vitro conditions.

We observed miRNA expression changes in response to IL6, in particular increased expression of miR-21 and miR-29b. To the best of our knowledge, this is the first report of IL6 regulating miR-29b expression. Moreover, miR-21 is an important miRNA in oncogenesis and is upregulated in a variety of cancer types (24). It mediates repression of proapoptotic PTEN expression (24) and is also known to be regulated by STAT3 (28), which is activated by IL6. Therefore, our current finding that IL6-treated cancer cells express and secrete miR-21 concurs with previous observations.

Previous reports have shown that functional miRNAs can be secreted by cells and exert their effect in receiving cells. Valadil and colleagues were the first to demonstrate that exosomes from mouse and human cells contained not only protein but also mRNA and miRNA molecules, some of which were expressed at higher levels in the vesicles compared with their cell of origin, suggesting certain miRNAs are loaded specifically into vesicles while others are kept inside the cell (14). These miRNA-containing vesicles are able to integrate into surrounding cells and release functional miRNAs into these cells (29, 30).

More interestingly, Fabbri and colleagues reported that miR-21 and miR-29a can bind TL8 to activate the NF-kB pathway in recipient immune cells resulting in expression of a variety of proinflammatory cytokines, including IL6, and that this binding event was dependent on the presence of a GU motif in the nucleotide region 18–21 of the miRNAs. MiR-29b is closely related to miR-29a and contains a GU motif in region 19–23

![Figure 4](mcr.aacrjournals.org)
Thus, it has the potential to interact with TLR8 in the tumor-surrounding immune cells. These previous reports thus support our findings that miR-21 and miR-29b are able to promote receiving immune cell activation and IL6 expression. Furthermore, we demonstrate that immune cells are also able to secrete miRNAs as we found miR-21 in the CM from activated immune cells. Indeed, immune cell activation results in induction of the NF-κB pathway, which is known to promote cytokine production but also miRNAs such as miR-21 (23). MiR-21 is a known oncogenic miRNA, and thus its secretion by surrounding immune cells could be another mechanism (in addition to IL6 release) by which these cells are able to promote tumor progression. Secreted miRNAs (by immune or tumor cells) could also integrate into nearby nonmalignant cells and promote their transformation. Further understanding of the mechanisms involved in intercellular cross-talk in the TME is crucial so as to counter cancer cell corruption of its environment and instead be able to manipulate the TME to be tumor-suppressive, thus potentially leading to the development of novel therapeutic strategies.

It is important to note that the current study was performed using in vitro cell culture models and offers mechanistic support of intercellular communication. However, whether similar mechanisms operate in tumors in vivo needs to be confirmed.

In conclusion, we demonstrate that cancer and immune cells cultured in vitro interact via IL6 and miRNAs to sustain IL6 production, generating a microenvironment that promotes tumor invasiveness. These data provide further insight into the potential mechanisms by which cancer cells can communicate with their environment and demonstrate that IL6 and miRNAs are involved as mediators of this cross-talk.

Disclosure of Potential Conflicts of Interest
N.J. Gooderham is a consultant/advisory board member for Flavour and Extract manufacturers Association Expert Panel. No potential conflicts of interest were disclosed by the other author.

Authors’ Contributions
Conception and design: S.A.A. Patel, N.J. Gooderham
Development of methodology: S.A.A. Patel, N.J. Gooderham
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.A.A. Patel, N.J. Gooderham
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.A.A. Patel, N.J. Gooderham
Writing, review, and/or revision of the manuscript: S.A.A. Patel, N.J. Gooderham

Figure 5.
Proposed model for IL6-mediated cross-talk between immune and cancer cells. IL6 is secreted by tumor-associated immune cells; upon binding to the IL6 receptor (IL6R) on the cancer cell surface, pSTAT3 is translocated to the nucleus to induce expression of a number of oncogenes and miRNAs, including miR-21 and miR-29b, which are then secreted into the TME and are taken up by the tumor-associated immune cells. MiR-21 and miR-29b then bind to TLR8 contained within endosomes in the immune cells, thus activating NF-κB signaling and inducing further secretion of IL6 and miR-21 into the microenvironment to promote cancer cell progression.
References

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