

Interleukin-6 mediates crosstalk between immune and colorectal cancer cells *via* miR21 and miR29b.

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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. 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. In the current study, we investigated the mechanisms by which cancer and immune cells communicate using an *in vitro* co-culture model. We demonstrated that pro-inflammatory cytokine interleukin-6 (IL6) secreted by immune cells promotes colorectal cancer cell invasiveness. Additionally, in the presence of IL6, the cancer cells were able to secrete circulating microRNAs (miRNAs) miR21 and miR29b to further induce immune cell IL6 production. Activated immune cells were also found to release miR21 into the tumor microenvironment. Taken together, these data provide a better understanding of intercellular communication between immune and cancer cells in the tumor microenvironment and offer insight into some of the key players that mediate this crosstalk.

Implication

This study demonstrates that *in vitro* co-cultured cancer and immune cells can communicate *via* IL6 and circulating miRNAs to sustain chronic inflammation and promote pro-metastatic cancer cell behavior. These *in vitro* mechanistic findings identify some of the key players that mediate intercellular communication in the tumor microenvironment and could have significant

implications in designing therapeutic approaches that target the microenvironment.

Introduction

Over 150 years ago, Rudolf Virchow first hypothesized that inflammation plays a role in the development of cancer based on his observations that inflammatory cells infiltrate tumors (1,2). In the last 30 years, extensive research on the topic has validated this idea and rekindled the interest of researchers in the cancer field. Inflammation is now thought to promote various stages of carcinogenesis including cell transformation, proliferation, survival, angiogenesis and metastasis (3).

Malignant tumors are surrounded and infiltrated by inflammatory cells that secrete bioactive compounds into the tumor microenvironment, which include growth factors, chemokines and cytokines. This complex environment is thought to promote cancer progression, metastasis and chemoresistance, and has thus been identified as a potential target for therapy (4). Understanding intercellular communication within the microenvironment is key to this therapeutic approach. Importantly, the tumor is thought to mold its microenvironment for optimal growth by corrupting surrounding immune cells to maintain pro-tumorigenic inflammatory conditions as well as evade the anti-tumor immune response, although it is still unclear how the tumor is able to do this.

Pro-inflammatory cytokine interleukin-6 (IL6) is secreted by cells in the tumor microenvironment (stromal cells have been shown to secrete up to 8000pg/ml (5)) and is thought to play a central role in carcinogenesis (5,6). IL6 induces proliferation and invasion of a variety of cancer cell types (7–9), and is known

to activate the oncogenic STAT3 transcription factor resulting in expression of a number of tumor-promoting genes and microRNAs (miRNAs) (10–12).

Emerging evidence suggest that miRNAs play a crucial role in cancer progression (13). MiRNAs are small RNA molecules (≈ 22 bp in length) able to bind primarily (but not exclusively) to the 3' untranslated region of mRNAs thus regulating gene expression. In recent years, a new role for miRNAs as paracrine signaling molecules has emerged (14). This hormone-like function is particularly apparent in the immune response where miRNAs facilitate the crosstalk between different immune cells types (15) and can also activate human immune cells by binding to Toll-Like receptor 8 (TLR8), resulting in the production of pro-inflammatory cytokines including IL6 (16).

In the current study, we have investigated the potential role of IL6 in mediating intercellular communication between cancer and immune cells using an *in vitro* co-culture model. Our data suggest that IL6 plays a crucial role in the tumor microenvironment: immune cells secreting IL6 promote adjacent cancer cell invasion and miRNA release, which in return sustains immune cell activation inducing further IL6 production, thus maintaining a pro-tumorigenic environment.

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 ATCC (LGC Prochem, Middlesex, UK) 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, Paisley, UK) supplemented with 10% FBS, 100units/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (GIBCO, Life technologies).

For co-culture experiments, adherent HCT116 cells were seeded at a density of 1×10^5 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 1×10^5 THP1 suspension cells. All cells were incubated at 37°C in a humidified incubator (5% CO₂). Cells between passages 3-7 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 1µg/ml lipopolysaccharide (LPS) for 3 hours to promote pro-inflammatory cytokine secretion. LPS was purchased as purified from *Escherichia coli* 0127:B8 by phenol extraction (Sigma-Aldrich, Dorset, UK) and dissolved in cell culture medium.

HCT116 and SW480 cells do not constitutively express IL6 so human recombinant IL6 (at doses of 0-1000pg/ml, chosen within the range released by stromal cells in the colon (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, 3×10^4 cells per well were plated in 100 μ l of culture medium supplemented with 1% dextran-coated charcoal-stripped FBS in the upper chamber of a 96-transwell insert system with 8 μ m pores (BD Falcon, Oxford, UK). In the lower chamber, 100 μ l of culture medium containing 10% FBS was added as a chemoattractant. For the invasion assay, 20 μ l 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 spectrofluorimeter. The enzymatic conversion to resorufin is proportional to cell number. Results are expressed as fold change compared to 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 μ l of CM according to the manufacturer's protocol. Synthetic *C.elegans* miR39 (Ambion, Life technologies) was spiked in at a concentration of 0.15 fmol to act as an internal standard.

RNA extracts were quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280nm and 260/230nm 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, Superscript II reverse transcription kit was used (Invitrogen, Life technologies) and miRNA reverse transcription kit was used for miRNA expression (Applied Biosystems, Life technologies). QPCR was performed using pre-designed expression assays (Taqman, Applied Biosystems, Life technologies) for *IL6* (Hs01054797_g1), *GAPDH* (Hs99999905_m1), U6 (001973), miR29b (000413), miR21 (000397) and *C.elegans* miR39 (000200). FAST PCR master mix was used according to the manufacturer's protocol (Taqman, Applied Biosystems, Life technologies). *GAPDH* and U6 expression were used to normalize cellular mRNA and miRNA expression respectively, while *C.elegans* miR39 was used to normalize miRNA levels in the culture medium.

Enzyme-linked immunosorbent assay (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, Abingdon, UK). Absorbance was measured in a Synergy H1 plate reader (Biotek, Potton, UK).

Transfection of miRNA mimics

MiRNA mimics were obtained from miRIDIAN mimics (Thermo Fisher Scientific, Cramlington, UK) and transfected into the cells using Lipofectamine

2000 (Invitrogen, Life technologies) according to the manufacturer's protocol. Briefly, cells were seeded at a density of 1×10^5 cells per well of a 24-well plate and cell culture medium was replaced by 400 μ l/well of Opti-MEM (GIBCO, Life technologies) prior to the addition 150 μ l/well of Opti-MEM containing 8 μ l of Lipofectamine 2000 reagent and 2.5 μ l of 20 μ M 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 biological replicates and presented as a mean $\pm$ standard error (SEM). Significant differences ($p < 0.05$) were determined either by Student's *t*-test or ANOVA (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA).

Results

Immune cells secrete IL6 to promote colorectal cancer cell invasion.

In the tumor microenvironment, 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 pro-inflammatory cytokines such as IL6, IL1 β and TNF α , was added to the cancer cells. THP1 cells were stimulated for 3 hours with 1 μ g/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 (Figure 1 A) and IL6 protein secretion (70 pg/ml, Figure 1 B) compared to unstimulated cells.

HCT116 cells were treated with CM from the stimulated THP1 cells or IL6 alone to investigate cell invasion (Figure 1 C). A significant 4-fold increase in cell invasion was observed in HCT116 cells treated with CM from LPS stimulated THP1 cells (containing around 70pg/ml IL6) and with 80pg/ml of IL6 alone (Figure 1 C), suggesting that this effect on cell invasion by the CM is primarily mediated by the presence of IL6 and not the other pro-inflammatory 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 (Figure 2). Interestingly, co-culture alone was not able to significantly induce IL6 production by THP1 cells. However, when the cancer cells were pre-treated with IL6 for 24 hours, washed and then co-cultured 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 crosstalk.

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, miR21 and miR29b and additionally miR135b was also induced in HCT116 (Figure 3 A). Interestingly, MiR21 and miR29b 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 miR21 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 tumor microenvironment, we measured their expression levels in CM from IL6-treated cancer cells (Figure 3 B). While miR21 and miR29b 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 pre-treated cancer cells were able to activate immune cells. Therefore, miR21 and miR29b 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, miR21 and miR29b mimics were transfected into THP1 cells and *IL6* mRNA expression was measured (Figure 4 A). Both miR21 and miR29b mimics induced more than a 2-fold induction of *IL6* mRNA levels in THP1 cells compared to the control mimic. This suggests that miR21 and miR29b 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 κ B pathway, which is known to promote miR21 expression (23). As miR21 is a circulating miRNA and known to be oncogenic (24), we determined whether activated immune cells in the tumor

microenvironment could also produce and secrete miR21 as another means of promoting tumor progression. To investigate this, we measured levels of miR21 in the CM from stimulated THP1 cells compared to unstimulated cells and found that activated immune cells released higher levels of miR21 (Figure 4 B). Therefore, miR21 secretion into the tumor microenvironment by immune cells could be another mechanism in addition to cytokine release involved in their pro-tumorigenic effects.

Discussion

The tumor microenvironment 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 crosstalk. Here, we suggest that cancer cells secrete miR21 and miR29b 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 miR21 into the tumor microenvironment, which could signal back to the cancer cells and further promote tumor progression (Figure 5).

Pro-inflammatory 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 pro-inflammatory tumor-infiltrating immune cells such as M1 macrophages that secrete high levels of IL6 have an anti-tumor 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 pro-inflammatory immune cells in the tumor microenvironment are able to promote cancer cell invasiveness through IL6 secretion.

We observed miRNA expression changes in response to IL6, in particular increased expression of miR21 and miR29b. To the best of our knowledge, this is the first report of IL6 regulating miR29b expression. Moreover, miR21 is an important miRNA in oncogenesis and is upregulated in a variety of cancer types (24). It mediates repression of pro-apoptotic 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 miR21 concurs with previous observations.

Previous reports have shown that functional miRNAs can be secreted by cells and exert their effect in receiving cells. Valadi *et al.* 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 to 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 *et al.* reported that miR21 and miR29a can bind TLR8 to activate the NFkB pathway in recipient immune cells resulting in expression of a variety of pro-inflammatory 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. MiR29b is closely related to miR29a and contains a GU motif in region 19-23 (GUGUU), thus it has the potential to interact with TLR8 in the tumor-surrounding immune cells. These previous reports thus support our findings that miR21 and miR29b 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 miR21 in the CM from activated immune cells. Indeed, immune cell activation results in induction of the NFκB pathway, which is known to promote cytokine production but also miRNAs such as miR21 (23). MiR21 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 non-malignant cells and promote their transformation. Further understanding the mechanisms involved in intercellular crosstalk in the tumor microenvironment is crucial so as to counter cancer cell corruption of its environment and instead be able to manipulate it 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 crosstalk.

Acknowledgements

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Figure legends

Fig. 1 Immune cells secrete IL6 to promote cancer cell invasion via STAT3 signaling. THP1 cells were stimulated with 1µg/ml LPS for 3hours prior to harvesting the treated cells and collecting their CM. (A) *IL6* mRNA expression in THP1 cells. (B) IL6 secretion from THP1 cells. (C) HCT116 cell invasion following treatment with CM from THP1 or IL6 alone (80pg/ml). Error bars represent the SEM for independent cultures (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

Fig. 2 Cancer cells pre-treated with IL6 can promote IL6 production in adjacent immune cells. (A) *IL6* mRNA expression in THP1 cells co-cultured with HCT116 and SW480 pre-treated with or without IL6 (n=6). (B) THP1 cell IL6 secretion following co-culture with HCT116 and SW480 pre-treated with or without IL6 (n=3). Error bars represent the SEM for independent cultures. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

Fig. 3 MiR21 and miR29b 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 1000pg/ml) for 48 and 24 hours respectively. (B) MiR21 and miR29b expression in the CM from HCT116 and SW480 cells. Error bars represent the SEM for independent cultures (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control. The lines in A indicate significant trends.

Fig. 4 MiR21 and miR29b can induce immune cells activation. (A) *IL6* gene expression in THP1 cells following miR21 and miR29b mimic transfection. (B) MiR21 expression in the CM from activated THP1 cells

(treated with 1µg/ml LPS for 3 hours). Error bars represent the SEM for independent cultures (n=3). * $p<0.05$, ** $p<0.01$ compared to control.

Fig. 5 Proposed model for IL6-mediated crosstalk 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 miR21 and miR29b, which are then secreted into the tumor microenvironment and are taken up by the tumor-associated immune cells. MiR21 and miR29b then bind to TLR8 contained within endosomes in the immune cells, thus activating NFκB signaling and inducing further secretion of IL6 and miR21 into the microenvironment to promote cancer cell progression.

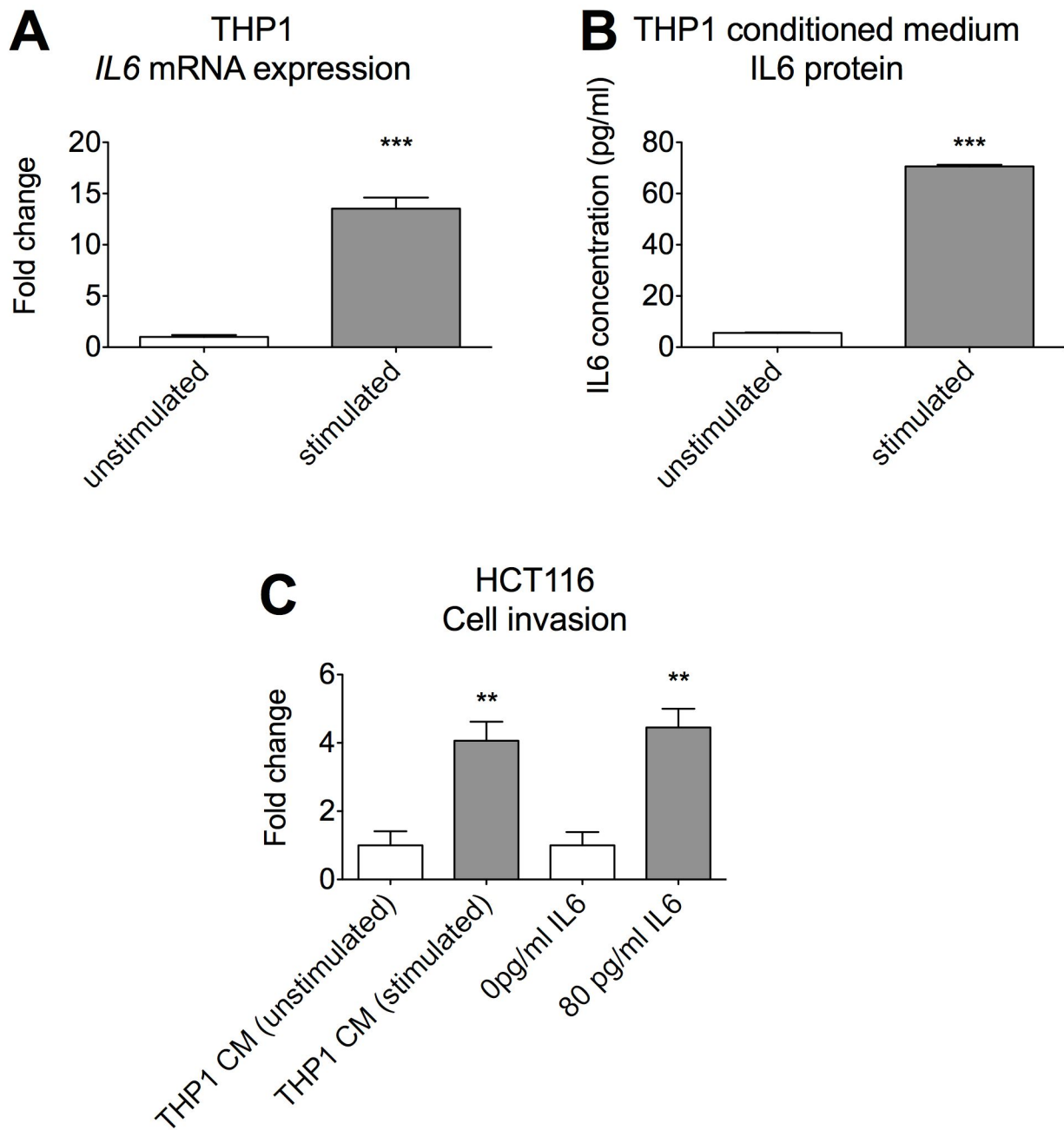
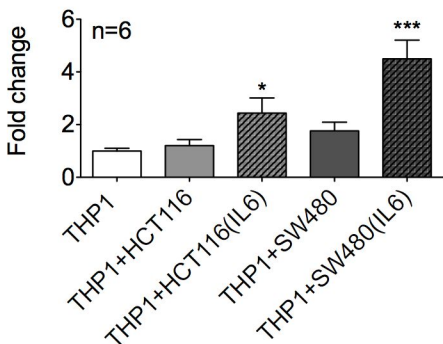


Figure 1

A

IL6 mRNA expression in THP1 monocytes

**B**

IL6 ELISA in THP1 conditioned media

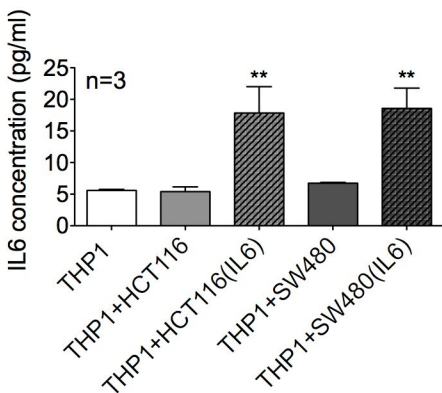
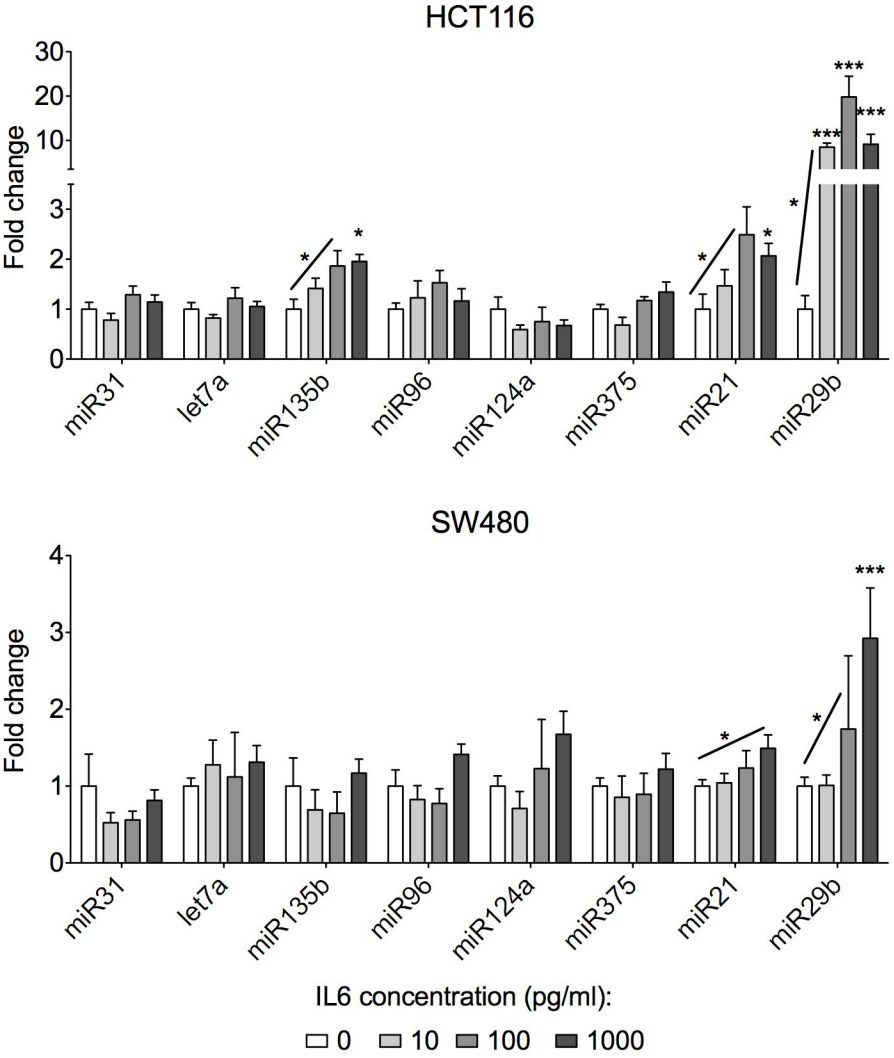
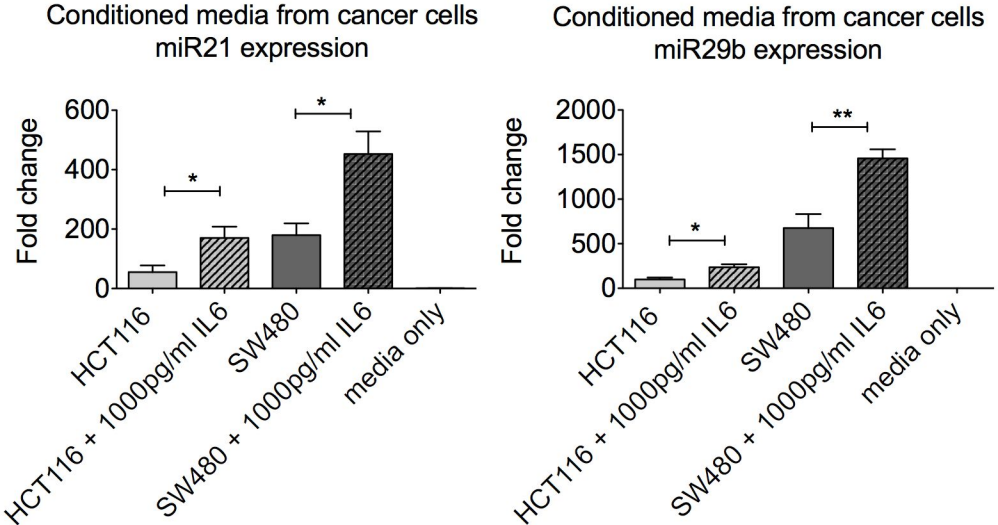
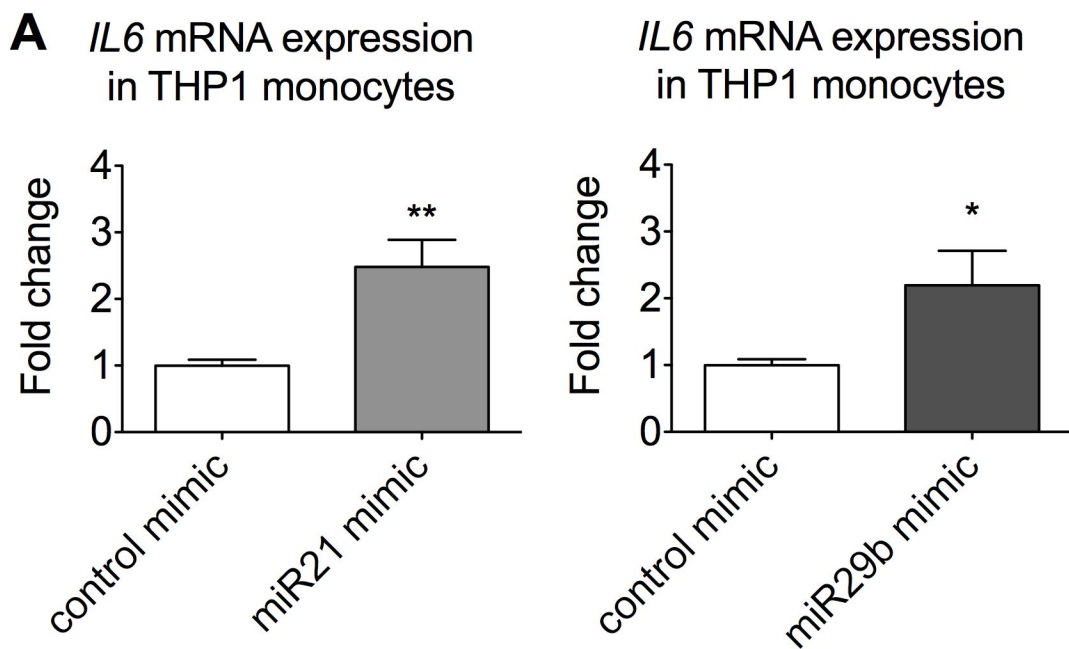


Figure 2

A**B****Figure 3**



B THP1 conditioned medium
miR21 expression

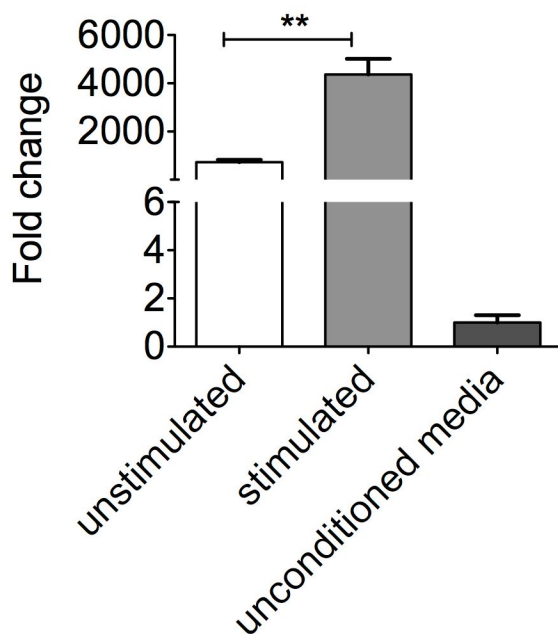


Figure 4

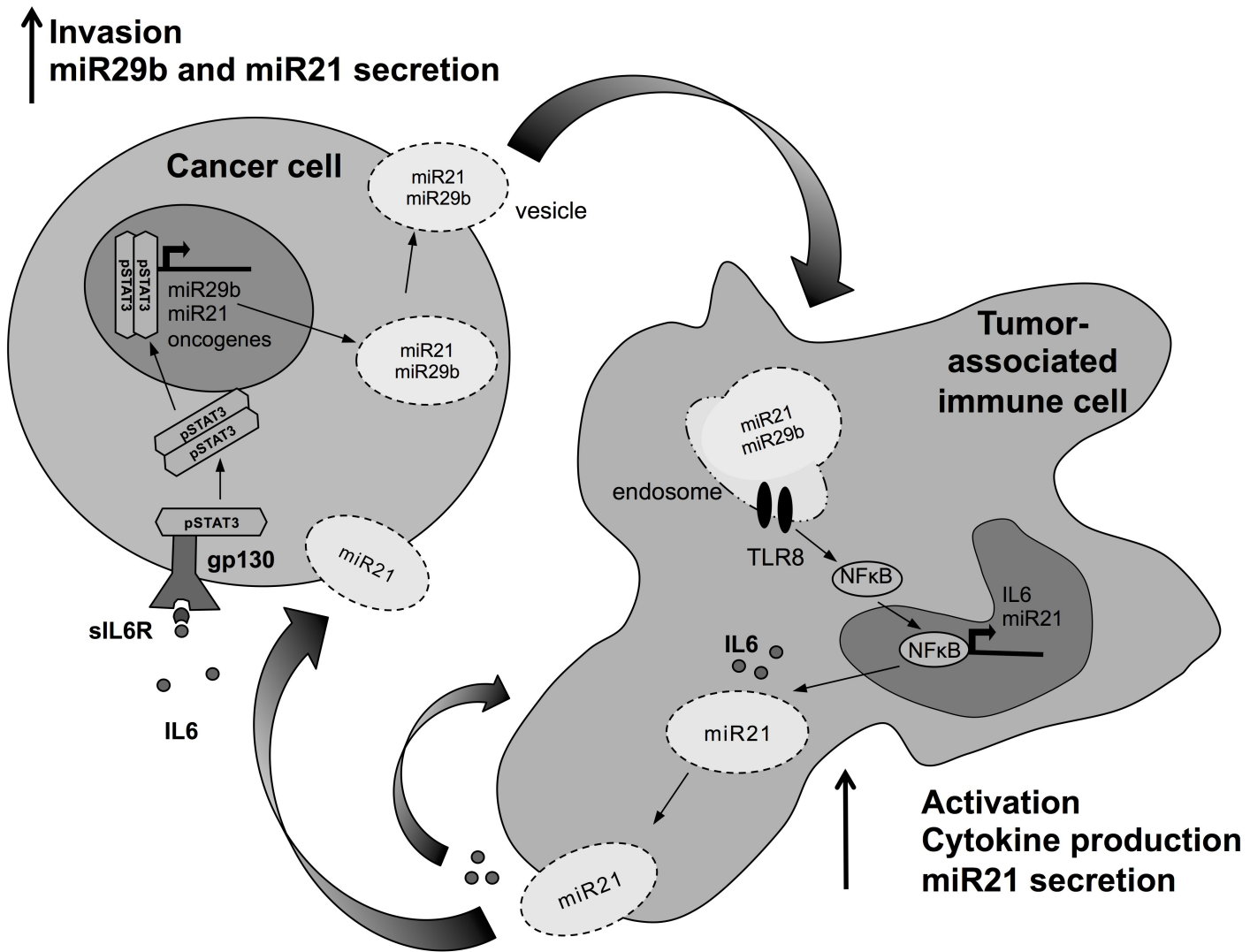


Figure 5