Autophagy Inhibition by Sustained Overproduction of IL6 Contributes to Arsenic Carcinogenesis

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Abstract
Chronic inflammation has been implicated as an etiologic factor in cancer, whereas autophagy may help preserve cancer cell survival but exert anti-inflammatory effects. How these phenomena interact during carcinogenesis remains unclear. We explored this question in a human bronchial epithelial cell-based model of lung carcinogenesis that is mediated by subchronic exposure to arsenic. We found that sustained overexpression of the pro-inflammatory IL6 promoted arsenic-induced cell transformation by inhibiting autophagy. Conversely, strategies to enhance autophagy counteracted the effect of IL6 in the model. These findings were confirmed and extended in a mouse model of arsenic-induced lung cancer. Mechanistic investigations suggested that mTOR inhibition contributed to the activation of autophagy, whereas IL6 overexpression was sufficient to block autophagy by supporting Beclin-1/Mcl-1 interaction. Overall, our findings argued that chronic inflammatory states driven by IL6 could antagonize autophagic states that may help preserve cancer cell survival and promote malignant progression, suggesting a need to uncouple inflammation and autophagy controls to enable tumor progression. Cancer Res; 74(14): 3740–52. ©2014 AACR.

Introduction
Sterile inflammation refers to a host inflammatory response to physically or chemically induced injuries in the absence of microorganisms. Chronic sterile inflammation caused by continuous exposure to chemical carcinogens has been linked to various steps of tumorigenesis (1). Overproduction of specific cytokines and abnormal activation of some transcription factors have been involved in the oncogenic action of chronic inflammation (2). On the basis of their relation to inflammation, cytokines can be roughly classified as pro- or anti-inflammatory cytokines, although many of them have both pro- and anti-inflammatory functions depending on the situations. The pro-inflammatory cytokines, such as IL1, IL2, IL6, and TNFα, are mostly considered as tumor promoting (3). For example, IL6 is elevated in human colon (4), gastric (5) and oral cancers (6).

Autophagy, a catabolic process, functions in maintaining cellular homeostasis. Dysfunctional proteins and organelles are sequestered in autophagosomes and subsequently degraded by lysosomal machinery. Autophagy has been suggested as a new immunologic paradigm acting basically as a negative mediator of inflammation (7–9). Autophagy is mainly controlled by three systems: (i) LC3 (MAP1LC3A)-PE conjugation system converting LC3-I to LC3-II, which functions in autophagosome membrane elongation, (ii) ULK1 (unc-51 like autophagy activating kinase 1) protein kinase complex, which is regulated by mTOR, and (iii) Beclin-1/Vps34 complex by which Beclin-1 (BECN1) interacts with class III PI3K (Vps34) and other proteins to initiate autophagosome formation.

Arsenic is a carcinogen. Humans are exposed to arsenic via both environmental contamination and occupational exposure. Chronic, low-dose arsenic exposure increases the risks of many cancers, including lung cancer (10). However, the underlying mechanism is not clear. Exposed to external environment, the lung is particularly sensitive to injury and inflammation induced by arsenic. Chronic airway inflammation alters the microenvironment in the bronchial epithelium and lung and may promote pulmonary carcinogenesis (11).

Arsenic-induced cell transformation, a process converting normal cells into a cancer-like state of uncontrolled division, is a critical step for arsenic tumorigenesis. Subchronic exposure of human lung epithelial cells (BEAS-2B) to low-dose arsenic inducing cell transformation has been used as an in vitro model system by us and many others (12, 13) for mechanistic study of arsenic carcinogenesis. Using this model system, we have previously shown subchronic arsenic exposure–induced BEAS-2B cell transformation accompanied with increased reactive oxygen species (ROS) generation and autophagy activation (14). However, the patterns for ROS and autophagy alteration were different. Arsenic...
exposure generated a prolonged and steady increase of ROS levels, whereas the activation of autophagy, after an initial boost by arsenic administration, decreased in response to prolonged arsenic exposure. Our data further indicated that oxidative stress was the force driving cell transformation and autophagy was the cell self-defense mechanism against arsenic-induced ROS generation and cell transformation. With the adequate protection from autophagy, short-term arsenic exposure did not cause cell transformation. However, the activation of autophagy decreased upon sustained arsenic exposure and the decreased protection by autophagy with increased oxidative stress leads to cellular oxidative damage and cell transformation.

Chronic inflammation and autophagy have been suggested for quite a while as an etiologic factor and cellular protective mechanism, respectively, for carcinogenesis (15). However, in our knowledge, their interactions have not been directly investigated. Subchronic exposure of BEAS-2B cells to arsenic causing cell transformation serves as an appropriate model system to study their interactions as well as how the interactions relate to arsenic carcinogenesis. In the present study, we discovered that subchronic exposure of BEAS-2B cells to arsenic steadily increased the production of IL6, which promoted arsenic-induced cell transformation by inhibiting autophagy, enhancing autophagy counteracted the action of IL6. Our data indicate that autophagy was regulated positively by inhibition of mTOR and negatively by overproduction of IL6. Our data further indicated that autophagy was regulated positively for quite a while as an etiologic factor and cellular protective mechanism, respectively, for carcinogenesis (15). However, in our knowledge, their interactions have not been directly investigated. Subchronic exposure of BEAS-2B cells to arsenic causing cell transformation serves as an appropriate model system to study their interactions as well as how the interactions relate to arsenic carcinogenesis. In the present study, we discovered that subchronic exposure of BEAS-2B cells to arsenic steadily increased the production of IL6, which promoted arsenic-induced cell transformation by inhibiting autophagy, enhancing autophagy counteracted the action of IL6. Our data indicate that autophagy was regulated positively by inhibition of mTOR and negatively by overproduction of IL6 in the presence of arsenic. In addition, IL6 regulated autophagy by mediating Beclin-1/Mcl-1 association through STAT3.

Materials and Methods

Materials
Sodium arsenite solution (70039), rapamycin (R0395), puromycin (P8833), and antibodies directed against actin (A5441) and p62/SQSTM1 (P0067) were purchased from Sigma-Aldrich. Anti-LC3 antibody (PM036) was purchased from Medical and Biological Laboratories. Anti-GM-CSF antibody (NB600-632) was purchased from Novus. Anti-IL6 (ab6672), anti-cleaved Bid (ab10640), and anti-Mcl-1 (ab3184) antibodies were purchased from Abcam. Anti-Bcl-2 antibody was purchased from Santa Cruz. The antibodies directed against Bcl-xL (2764), Mcl-1 (5453), Bcl-w (2724), Bad (9292), Beclin-1 (3495 and 4122), p-mTOR (5536), mTOR (2983), p-P70S6K (9324), P70S6K (2708), p-4E-BP1 (2855), 4E-BP1 (9644), and recombinant human IL6 (89045) were obtained from Cell Signaling Technology.

Cell culture and treatments
Human bronchial epithelial cells BEAS-2B and NL-20 from ATCC and human small airway epithelial cells SAEC from Lonza were cultured in growth medium [BEGM (CC-3170), Lonza] for BEAS-2B, SAGM (CC-3118, Lonza) for SAEC, and F-12 with required additives for NL-20. BEAS-2B cells were tested and authenticated in January 2014 by Promega by short tandem repeat (STR) analysis as described in 2012 in ANSI Standard (ASN-0002). SAEC and NL-20 were obtained directly from the cell banks and passaged in our laboratory for fewer than 6 months. Sodium arsenite solution was used for arsenic treatment as previously reported (14). For short-term (24 hours) arsenic exposure, cells were grown to 80% to 90% confluence before being treated with 0.25 µmol/L sodium arsenite. For prolonged (1–16 weeks) arsenic exposure, 1 × 10^6 cells were plated into each 75-cm² flask. On the second day, the cells were rinsed with fresh medium and cultured with new growth media containing 0.25 µmol/L sodium arsenite for 3 days before the media were changed one more time. Sodium arsenite solutions were diluted in the medium before each medium change. After 6 days of growth in the flasks, the cells were detached using trypsin, 1 × 10^6 of these cells were replated into a new flask, and the treatments were repeated for a total of 16 weeks. The rest of the cells/protein samples were collected and reserved for future analysis. Nontreated passage-matched cells served as a control.

Plasmids and transfection
IL6 shRNA plasmid (19959), IL6 scramble control plasmid (19960), and pcDNA3-Beclin-1 plasmid (21150) were purchased from Addgene. GM-CSF shRNA plasmid (TF313703), STAT3 shRNA plasmid (TF301348), and Mcl-1 siRNA (SR302834) were purchased from Origene. For transfection, cells were transfected with plasmids or siRNA using Lipofectamine 2000 (Invitrogen, 11668-019) and selected by G418 (Invitrogen, 11811) or puromycin (Sigma P8833) according to the manufacturer’s protocol.

Cytokine production assay
The cytokines productions were evaluated using Multi-Analyte ELISArray kit (Qiagen, MEH-004A). For acute arsenic treatment, cells were plated in 12-well plates at a density of 1 × 10^5 cells/well and treated with 0.25 µmol/L sodium arsenite in BEGM medium for 24 hours and the supernatants were collected and analyzed. For prolonged arsenic treatment, cells were exposed to 0.25 µmol/L sodium arsenite for 1 to 16 weeks as described above. Then, the cells were replated in 12-well plates at a density of 1 × 10^5 cells per well and treated with 0.25 µmol/L sodium arsenite for 24 hours. The supernatants were collected for ELISA. Because the cells kept proliferating in the growth medium with 0.25 µmol/L sodium arsenite, this method measured the cytokine levels that were produced by the same amount of cells.

RNA extraction and quantitative reverse transcription PCR
The mRNA levels were analyzed by quantitative reverse transcription PCR (RT-PCR). Briefly, cells were collected for total RNA extraction by a TRIzol reagent (Invitrogen, 15596–026). The quality of the total RNA was confirmed by the integrity of 28S and 18S rRNA. The first-strand cDNA was synthesized using a Reverse Transcription System (Promega, A3500). PCR was performed on a Lightcycler 480 system (Roche) using a Power SYBR Green PCR Master kit (Invitrogen, 4368706). After finishing the last cycle, a melting curve analysis was performed. Standard —ΔΔCt method was used for determining changes in gene expression. The relative expression level of a given mRNA was assessed by normalizing to β-actin and comparing with control values. The primers used for analysis were as below: IL6 forward:
5′-aacgtgaccttcacaagttg-3′, IL6 reverse: 5′-tgtagctggttctcctcact-3′; GM-CSF forward: 5′-caagtctgtagaggataaga-3′, GM-CSF reverse: 5′-ctggattcagagattt-3′, and β-actin forward: 5′-aagcagagctcctgccttt-3′, β-actin reverse: 5′-agttgagaggtaccttcttt-3′.

**Immunoblotting and immunoprecipitation**

The immunoblotting procedure has been previously described (16). Briefly, aliquots of the protein samples (20–40 μg) were separated by electrophoresis and transferred to nitrocellulose membranes. After blocking, the membranes were probed with primary antibodies and then with a secondary antibody conjugated to horseradish peroxidase. The immune complexes were detected by the enhanced chemiluminescence method (PerkinElmer, NEL105001EA). The blots were stripped and reprobed with an anti-actin antibody.

Immunoprecipitation was performed as previously described (16). Briefly, an aliquot of 200 μg of protein was incubated with the antibody against the target protein overnight at 4°C. About 20 μL of Protein A agarose beads (Cell Signaling Technology, 9863) were added to the lysate, and the mixture was incubated for 3 hours at 4°C. Immunoprecipitates were collected by centrifugation at 10,000 × g for 10 minutes. The pellets were then resuspended in 20 μL of 3× SDS sample buffer and analyzed for the expression of specific proteins by immunoblotting.

**Soft agar assay**

Cell transformation was determined by anchorage-independent growth in soft agar as described previously (14). Briefly, equal volumes of 1.5% agar (in 1× PBS) and 1× BEGM were mixed at 40°C. Two milliliters of the mixture (containing 0.75% agar) was added into 6-well plates as a base agar. Then, 5,000 of control or treated cells that were suspended in 2 mL of second BEGM-agar mixture (containing 0.35% agar) were added in each well as top agar. Top agar was covered with 1 mL of culture medium and the medium was replaced with fresh medium every 3 days. Plates were placed in an incubator at 37°C and 5% CO2; and after 4 weeks, the colonies were stained with 0.005% crystal violet and quantified/photographed.

**Animals and tumorigenicity assay**

Five-week-old male athymic nude mice were purchased from Harlan Laboratories and housed in a specific pathogen-free room in the Animal Facility at the University of Kentucky Medical Center (Lexington, KY). All procedures were performed in accordance with the guidelines set by the NIH and the Animal Care and Use Committee of the University of Kentucky.

Nude mouse xenograft assays were performed as described previously (14). Briefly, cells treated with arsenic for 16 weeks and nontreated passage-matched control cells were harvested and adjusted to a cell density of 1 × 107 cell/mL in cell culture medium. About 0.2 mL of the cell suspension was injected subcutaneously into the flanks of the 6-week-old nude mice (n = 6). Tumors were allowed to grow for 3 weeks. The greatest longitudinal diameter (length) and the greatest transverse diameter (width) of a tumor were measured by external caliper 10 days after injection. Tumor volume was calculated by the modified ellipsoidal formula (17): tumor volume (mm3) = 1/2 (x2y); x is the greatest longitudinal diameter and y is greatest transverse diameter.

**Statistical analysis**

Differences among treatment groups were tested by ANOVA. Data are represented as mean ± SEM of three experiments. P < 0.05 was considered statistically significant. In cases in which significant differences were detected, specific post-hoc comparisons between treatment groups were examined by Student–Newman–Keul tests.

**Results**

The secretion and mRNA levels of IL6 and GM-CSF are increased upon subchronic arsenic treatment

Previously, we have shown that exposure of BEAS-2B cells to 0.25 μmol/L sodium arsenite for 16 weeks induced cell transformation evidenced by colony formation in soft agar and tumor formation in nude mice. The justification for the use of sodium arsenite at 0.25 μmol/L has been elucidated in detail (14). Briefly, there are two major reasons: (i) it is an environmentally relevant concentration and (ii) arsenic-induced cell transformation was not dose-dependent among the dosages (0.25, 0.5, and 1 μmol/L) that can induce BEAS-2B cell transformation. Here, we first sought to determine whether inflammatory cytokines were regulated by arsenic during cell transformation. A cytokine production profile was generated after the cells were treated with arsenic for 24 hours, 1 and 16 weeks. After a literature search, 12 cytokines including both pro- and anti-inflammatory ones were selected. Among these cytokines, the production of IL6, IL8, TNFα, and granulocyte-macrophage colony-stimulating factor (GM-CSF) was increased 24 hours after arsenic treatment (Fig. 1A). However, only IL6 and GM-CSF levels stayed upregulated after 1 and 16 weeks. In addition, the increase of IL6 was in a time-dependent manner whereas the increase of GM-CSF was not. The mRNA levels of IL6 and GM-CSF in arsenic-treated cells were also increased (Fig. 1B), indicating that arsenic transcriptionally enhanced the levels of IL6 and GM-CSF.

Overproduction of IL6, not GM-CSF, promotes arsenic-induced cell transformation

To determine whether the increase of IL6 and/or GM-CSF contributed to arsenic-induced cell transformation, IL6 or GM-CSF knocking down (KD) cell lines were established by stably transfected BEAS-2B cells with IL6 or GM-CSF shRNA plasmids, respectively (Fig. 2A). These KD cells were then exposed to arsenic for 16 weeks and cell transformation was tested by soft agar assay. As shown in Fig. 2B, IL6 KD significantly decreased arsenic-induced cell transformation whereas GM-CSF KD did not. These results indicated that, although IL6 and GM-CSF were both upregulated by subchronic arsenic treatment, only IL6 contributed to arsenic-induced transformation. This was further confirmed by a tumorigenicity assay (Fig. 2C) in which BEAS-2B or IL6 KD cells were injected into the nude mice after these cells were treated with arsenic for 16 weeks. As
Figure 1. A, BEAS-2B cells were treated with 0.25 μmol/L sodium arsenite for 24 hours, 1 and 16 weeks. The cytokines in the supernatants of arsenic-treated or passage-matched controlled cells were determined by ELISA. Data are mean ± SEM of three experiments. *, P < 0.05 versus passage-matched controlled cells; #, P < 0.05. B, the mRNA levels of IL6 and GM-CSF in arsenic-treated BEAS-2B cells were determined by qRT-PCR and were normalized to matched actin mRNA levels. The data are shown as relative expression folds compared to passage-controlled cells. Data are mean ± SEM of three experiments. *, P < 0.05.
expected, IL6 KD significantly decreased the tumor sizes compared with the arsenic-treated control group. However, IL6 KD did not change the incidence rate.

IL6 negatively regulates autophagy activity

Because autophagy has been shown as a protective mechanism against arsenic-induced cell transformation in this model (14), we next sought to determine whether autophagy activity could be altered by manipulating IL6 levels. We inhibited IL6 using IL6 KD cells and enhanced it using human recombinant IL6 at 10 ng/mL, this concentration of IL6 has been shown to affect cell transformation (18). Arsenic at 0.25 μmol/L increased LC3-II, which has been verified as a result of enhanced autophagy activity in our previous work (14). Thus, we evaluated autophagy activity by measuring LC3-II levels in BEAS-2B cells treated with arsenic, arsenic plus IL6, or in IL6 KD cells treated with arsenic. As shown in Fig. 3A, while co-treatment of IL6 inhibited arsenic-induced LC3-II upregulation, IL6 KD further increased LC3-II, indicating that IL6 may inhibit autophagy activity. To confirm that the alteration of LC3-II reflected the change of autophagic flux, the expression levels of p62 (SQSTM1) were also evaluated. p62 is an autophagosome membrane–associated protein and is degraded in lysosomes after autophagosomes fuse with lysosomes; thus, its level is negatively regulated by autophagic flux. As expected, p62 generally had an opposite alteration pattern compared with LC3-II confirming IL6 inhibited autophagy activity. To determine whether the inhibitive effect of IL6 on arsenic-mediated autophagy activity is cell-type-specific, we performed the same experiment in SAEC cells (a human small airway epithelial cell line) as well as NL-20 cells (a human bronchial epithelial cell line). Both of these cell lines, like BEAS-2B, were isolated from normal human tissue and are nontumorigenic. As shown in Fig. 3B and C, IL6 inhibited arsenic-induced upregulation of LC3-II and downregulation of p62 in both of these cell lines. Therefore, the inhibition of autophagy activity by IL6 is not exclusive in BEAS-2B cells.
Figure 3. BEAS-2B cells (A), SAEC cells (B), and NL-20 cells (C) were treated with arsenic (As) or recombinant IL6 (10 ng/mL) alone or combined; IL6 KD cells were exposed to arsenic (As) for 24 hours. The levels of LC3-II and p62 were measured by immunoblotting. The decreased levels of IL6 by transfection of IL6 shRNA in SAEC or NL-20 cells are shown in the top of B and C, respectively. Data are mean ± SEM of three experiments. *, P < 0.05.
IL6 promotes arsenic-induced cell transformation through inhibition of autophagy

To further investigate whether inhibition of autophagy contributed to IL6-promoted cell transformation, we determined whether upregulation of autophagy can counteract the action of IL6. To upregulate autophagy, we used rapamycin and Beclin-1 overexpression, respectively. Rapamycin is an inhibitor of mTOR and a widely used enhancer of autophagy (14). Beclin-1 (BECN1) is a key regulator of autophagy (19), and overexpression of Beclin-1 increases autophagy activity (20, 21). The enhanced basal as well as arsenic-induced autophagy activities by Beclin-1 overexpression were confirmed by increased LC3-II and decreased p62 levels (Fig. 4A and B). Beclin-1–overexpressed cells as well as regular BEAS-2B cells were exposed to arsenic (As), human recombinant IL6 (10 ng/mL), or rapamycin (Rap, 10 nmol/L) alone or combined for 16 weeks, and then soft agar assays were performed. The number of the colonies in each well was counted and quantified. Data are mean ± SEM of three experiments. *, P < 0.05.

Figure 4. A, immunoblotting shows the increased level of Beclin-1 in BEAS-2B cells stably transfected with Beclin-1 cDNA. B, the levels of LC3-II and p62 in Beclin-1–overexpressed cells (Bec1) and vector control cells (vector) with or without 24 hours arsenic treatment are shown by immunoblotting. C, regular (control) or Beclin-1–overexpressed (Bec1) BEAS-2B cells were exposed to arsenic (As), human recombinant IL6 (10 ng/mL), or rapamycin (Rap, 10 nmol/L) alone or combined for 16 weeks, and then soft agar assays were performed. The number of the colonies in each well was counted and quantified. Data are mean ± SEM of three experiments. *, P < 0.05.
plus IL6 for 16 weeks and soft agar assay was performed thereafter. As shown in Fig. 4C, IL6 enhanced arsenic-induced colony formation, whereas co-treatment with rapamycin or overexpression of Beclin-1 blocked this action of IL6. These results indicated that IL6 promoted cell transformation by inhibiting autophagy.

**IL6 inhibits autophagy activity through mediating Mcl-1/Beclin-1 association**

We next investigated the mechanism underlying IL6 inhibited autophagy activity. Because mTOR is a well-known autophagy regulator and inhibition of mTOR by rapamycin ameliorated IL6-enhanced arsenic-induced transformation (Fig. 4C), we first determined whether IL6 inhibited autophagy through mTOR. As shown in Fig. 5A, acute arsenic treatment inhibited mTOR evidenced by decreased phosphorylation of mTOR as well as its substrates eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1 or EIF4EBP1) and P70S6 kinase (P70S6K), and the inhibition was reduced upon prolonged arsenic exposure. Unexpectedly, IL6 KD did not alter the pattern of the change of these marker proteins. Apparently, mTOR was not the target of IL6. Then, as (i) Beclin-1 overexpression reversed the inhibition of autophagy by IL6, (ii) binding of Beclin-1 and Bcl-2 (BCL2) proteins regulates autophagy (22), and (iii) Bcl-2 family is the downstream target of IL6/STAT3 (23), we sought to determine whether Bcl-2 proteins were involved in autophagy inhibition by IL6 upon arsenic treatment. We evaluated the expression levels of anti-apoptotic Bcl-2 proteins, Bcl-2, Bcl-xl (BCL2L1), Bcl-w (BCL2L2), and Mcl-1 (MCL1), as well as pro-apoptotic Bcl-2 proteins, tBid (tBID) and Bad (BAD), after BEAS-2B cells were treated with arsenic for 24 hours, 1 and 16 weeks. These Bcl-2 proteins were chosen because: (i) Beclin-1 binds only to anti-apoptotic Bcl-2 proteins (24), (ii) BH3-only pro-apoptotic Bcl-2 proteins may interfere with the binding between anti-apoptotic Bcl-2 proteins and Beclin-1, and (iii) tBid and Bad represent the activator and enhancer members for the BH3-only Bcl-2 pro-apoptotic proteins (25). Among these Bcl-2 proteins, only the levels of Mcl-1 were changed by arsenic as shown in Fig. 5B. Arsenic treatment at 24 hours inhibited Mcl-1 expression, but this inhibition was reversed and upregulated by prolonged arsenic exposure. The effects of arsenic on Mcl-1 expression in SAEC and NL-20 cells were also examined after arsenic treatment for 24 hours, 1 and 3 weeks. Consistent with our observation in BEAS-2B cells, Mcl-1 expressions were downregulated by arsenic at 24 hours but the downregulations were reversed upon prolonged arsenic exposure in both SAEC and NL-20 cells (Fig. 5C). Because the different effects of acute and prolonged arsenic exposure on Mcl-1 had been observed after 24 hours, 1 and 3 weeks of arsenic exposure, we adopted these time points for arsenic treatment in these cells. Moreover, as Mcl-1 has been shown as a target of IL6 (26), we further determined whether arsenic altered Mcl-1 levels through IL6. As shown in Fig. 5D, IL6 KD did not alter the inhibition of Mcl-1 by acute arsenic treatment but it abolished the upregulation of Mcl-1 by prolonged arsenic exposure, and co-treatment with IL6 further enhanced the upregulation of Mcl-1 upon arsenic treatment. As Germain and colleagues have shown that Mcl-1 inhibited autophagy by binding to Beclin-1 (27), the role of IL6/Mcl-1 in regulating autophagy was further evaluated in Mcl-1 KD cells. As shown in Fig. 5E, Mcl-1 KD blocked IL6-mediated suppression of arsenic-induced LC3-II upregulation. The binding of Mcl-1 and Beclin-1 was further determined by immunoprecipitation. As shown in Fig. 5F, the binding of these 2 proteins was decreased at 24 hours but it was increased after 1 week of arsenic exposure. Because the increased binding can be detected 1 week after arsenic treatment, to further determine the effects of IL6 on Mcl-1 expression as well as Mcl-1/Beclin-1 association in BEAS-2B, SAEC, and NL-20 cells, we treated these cells with IL6 for 1 week. IL6 significantly increased Mcl-1 levels in all 3 cell lines (Fig. 5G). More importantly, along with the increased Mcl-1 expression, the association between Mcl-1 and Beclin-1 was enhanced by IL6. Taken together, these data indicated that autophagy was inhibited by IL6 upon prolonged arsenic exposure through upregulating Mcl-1, which increased the binding of Mcl-1 and Beclin-1.

**IL6 and mTOR regulate Mcl-1 through STAT3 in response to arsenic exposure**

Because rapamycin reversed IL6-enhanced cell transformation (Fig. 4C), we further investigated the roles of mTOR and IL6 in regulating Mcl-1 expression. As shown in Fig. 6A, rapamycin, an inhibitor of mTOR, did not alter the inhibition of Mcl-1 by arsenic at 24 hours but it abolished the upregulation of Mcl-1 after 1 week of arsenic exposure (compared with Mcl-1 in Fig. 5B). Considering that (i) acute arsenic treatment inhibited mTOR (Fig. 5A), (ii) the inhibition was decreased and Mcl-1 expression was increased upon prolonged arsenic exposure (Fig. 5B), (iii) arsenic increased IL6 production in a time-dependent manner (Fig. 1A and B), and (iv) co-treatment of IL6 further enhanced Mcl-1 expression with prolonged arsenic exposure (Fig. 5D), IL6 and mTOR may regulate Mcl-1 expression in response to different durations of arsenic treatment. Acute arsenic treatment inhibited mTOR and downregulated Mcl-1, whereas prolonged arsenic exposure upregulated Il6, which enhanced Mcl-1 expression. We speculated that a downstream target of IL6/mTOR might exist and through which IL6 and mTOR regulated Mcl-1 expression. A possible candidate for such a downstream regulator would be STAT3, a transcriptional factor that controls Mcl-1 expression (28) and its own activity can be regulated by both IL6 and mTOR (29–31). To test this hypothesis, we knocked down STAT3 by STAT3 shRNA (Fig. 6B). Because 1 week is the turning point for the alteration of Mcl-1 in response to acute or prolonged arsenic treatment (as shown in Figs. 5B–D and F and 6A), we tested the effects of STAT3 KD on arsenic-induced Mcl-1 alteration 24 hours, 1 and 3 weeks after arsenic exposure. As shown in Fig. 6C, STAT3 KD abolished both acute and prolonged arsenic-induced alterations of Mcl-1 (top, compared with Fig. 5B). Furthermore, STAT3 KD inhibited the alterations of Mcl-1 by co-treatment of IL6 (middle, compared with right of Fig. 5D) or rapamycin (bottom, compared...
Figure 5. A, the levels of p-mTOR, p-P70S6K, and p-4E-BP1 were determined by immunoblotting after regular BEAS-2B cells (BEAS-2B) or IL6 KD BEAS-2B cells (IL6 KD) were exposed to arsenic for 24 hours, 1 and 16 weeks. B, the levels of Bcl-2, Bcl-xL, Mcl-1, and Bcl-w as well as Bad and tBid were determined by immunoblotting after BEAS-2B cells were treated with arsenic for 24 hours, 1 and 16 weeks. C, the expression levels of Mcl-1 were determined after SAEC (top) and NL-20 cells (bottom) were treated with arsenic for 24 hours, 1 and 3 weeks. (Continued on the following page.)
with Fig. 6A). Together, these data indicate that mTOR and IL6 regulate Mcl-1 expression upon arsenic exposure through STAT3.

Discussion

Cytokines are small signaling molecules produced by virtually all nucleated cells, but especially endothelial/epithelial cells and resident macrophages, in response to microbes or tumor antigens. They mediate intracellular signaling and regulate homeostasis of the immune system. Dysregulated cytokine secretion from sustained inflammation can drive oncogenesis (32). The role of pro-inflammatory cytokines in arsenic carcinogenesis has become more evident recently. Increased secretion of IL8 and IL6 has been shown to positively correlate with arsenic-induced malignant transformation of urothelial (33) as well as bronchial epithelial cells (18). This study aimed to investigate a possible interaction between autophagy and chronic inflammation and how the interaction relates to arsenic carcinogenesis. Using a model system of arsenic-induced cell transformation, we identified pro-inflammatory cytokine IL6 playing a promoting role in arsenic carcinogenesis by inhibiting autophagy. Although arsenic acutely increased the secretion levels of IL6, IL8, TNFα, and GM-CSF, only IL6 and GM-CSF were overproduced upon subchronic arsenic exposure, and it was IL6, among these cytokines, that contributed to arsenic-induced transformation. It is worth noting that IL6 KD did not decrease the incidence rate of tumor formation (Fig. 2C), indicating a promoting role rather than a causal role of IL6 in arsenic-induced cell transformation.

Autophagy functions in cellular defense against excessive inflammation (34). Defects in autophagy under the condition of chronic inflammation contribute to oncogenesis (15). It is generally accepted that defects in autophagy contribute to inflammation as autophagy functions in cellular homeostasis. Failure to remove cellular garbage in autophagy-defective cells/tissues results in cell death, which certainly is an inflammatory stimulus and creates a cancer-prone environment. However, not many studies have investigated how inflammation regulates autophagy activity. Only a few have shown conflicting results regarding how acute inflammation regulates autophagy (35–37), and little research has been conducted to investigate how chronic inflammation may regulate autophagy activity as well as its consequence related to carcinogenesis. The current study is the first one in our knowledge revealing that sustained inflammation inhibits autophagy activity and the compromised protective capability of autophagy enhances inflammation-promoted carcinogenesis.

Beclin-1 is an essential autophagy protein involved in diverse biologic functions. Beclin-1 binds to class III PI3K (Vps34) and other proteins, forming a complex that is required for autophagosome initiation. Binding to proteins of the Bcl-2 family through its BH3 domain sequesters Beclin-1 from forming complexes with Vps34 and thus inhibits autophagy activity (38). Regulation of Bcl-2/Beclin-1 interaction represents a central mechanism by which autophagy is turned on or off (39). In this study, overexpression of Beclin-1 ameliorated the inhibition of autophagy by IL6, indicating that IL6 inhibited autophagy through sequestering Beclin-1. Our results identified a new action of IL6: regulating autophagy activity through mediating Mcl-1/Beclin-1 interaction. Notably, the inhibitive effect of IL6 on autophagy was shown here in three different human lung epithelial cell lines derived from normal tissue and none of which are tumor cells. We noted that Kang and colleagues reported that in Panc02 mouse pancreatic tumor cells, IL6 enhanced autophagy activity, which promoted cancer cell growth (40, 41). Thus, it is possible that the effects of IL6 on autophagy are different between tumor and nontumor or human and rodent cells. Indeed, it has been reported that autophagy may function as a tumor suppressor in normal cells and tumor promoter in cancer cells (42).

In our previous work, we have shown that arsenic treatment inhibited mTOR, which activated autophagy. The inhibition of mTOR as well as autophagy activation were decreased upon prolonged arsenic treatment (14). In the present study, arsenic enhanced IL6 secretion in a time-dependent manner (Fig. 1A) and IL6 inhibited autophagy upon prolonged arsenic treatment. These observations are coincident with that whereas Mcl-1 was downregulated by acute arsenic treatment, it was upregulated in response to prolonged arsenic exposure, and the upregulation of Mcl-1 can be reversed by rapamycin. A reasonable interpretation for these data is that acute arsenic treatment inhibits mTOR, which downregulates Mcl-1, decreases the binding of Mcl-1/Beclin-1, increases the amount of free Beclin-1, and thus facilitates autophagy activity. In contrast, prolonged arsenic exposure, with increased IL6 production and decreased mTOR inhibition, upregulates Mcl-1, which enhances the binding of Mcl-1/Beclin-1, decreases the amount of free Beclin-1, and inhibits autophagy activity. Therefore, mTOR and IL6 regulate Mcl-1 expression in response to different durations of arsenic treatment. Our...
data further indicated that mTOR and IL6 regulate Mcl-1 through STAT3, as knocking down STAT3 abolished arsenic-induced Mcl-1 alteration. Thus, STAT3 could be an important regulator of autophagy by mediating the association between Bcl-2 proteins and Beclin-1. Indeed, a recent publication has suggested STAT3 as a new autophagy regulator (43). Finally, we also noticed that in different cells, Beclin-1 may bind with Bcl-2 or Bcl-xl rather than Mcl-1 to regulate autophagy activity (44). Hence, which Bcl-2 proteins binds to Beclin-1 and mediate autophagy may be cell-type- or tissue-specific.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors' Contributions**
Conception and design: Y. Qi, M. Zhang, G. Chen
Development of methodology: Y. Qi, M. Zhang, G. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Qi, M. Zhang, H. Li
Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): Y. Qi, M. Zhang, C. Wang, G. Chen
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Dai, H. Liu, J.A. Frank
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References
33. Escudero-Lourdes C, Wu T, Camarillo JM, Gandolfo JG. Interleukin-8 (IL-8) over-production and autocrine cell activation are key factors in monomethylarsonous acid (MMAlIII)-induced malignant transformation of urothelial cells. Toxicol Appl Pharmacol 2012;258:10–8.

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