Defective expression of ATG4D abrogates autophagy and promotes growth in human uterine fibroids

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Uterine fibroids (UF) are the most common pelvic tumors in women of reproductive-age and they usually cause heavy menstrual bleeding, pain and infertility. Autophagy is a collection of processes that enables the cells to digest and recycle their cytoplasmic contents, such as toxic protein aggregates, defunct or damaged organelles and invading microorganisms. Dysregulation in autophagy process were described in neoplasms; however, the contribution of autophagy to the pathogenesis of UF remains unknown. In this study, we demonstrate that autophagy is deregulated in human UF as evidenced by significant accumulation of autophagosomes in human UF cells compared to normal myometrium cells. Analysis of the autophagy markers revealed an enhanced initiation of the autophagy in UF tissues compared to their adjacent myometrial tissues (MyoF). However, autophagosome maturation and flux was blocked in UF tissues, as marked by accumulation of LC3-B and P62 protein. This block was associated with defective expression of autophagy-related protein 4 (ATG4) in the UF tissues compared to MyoF in ~90% of patient samples. Silencing of ATG4D in normal human myometrial cells resulted in defective autophagy flux, enhanced cell proliferation and increased extracellular matrix production, which phenocopy UF cell line. This study indicates that impairment of autophagy flux secondary to defective expression of ATG4D expression is a new mechanistic aberration that contributes to UF pathogenesis. Targeting autophagy pathway could provide novel medical therapeutic approach for non-surgical treatment of UF.

RESULTS
Autophagy flux is altered in human uterine fibroid
Autophagy is a process that leads to degradation of subcellular constituents via formation of autophagosomes that fuse with lysosome and generate autolysosomes. We hypothesized that uncontrolled proliferation of myometrial cells and the development of fibroid could be linked to defect in the autophagy pathway. To test this hypothesis, we first evaluated different steps of the autophagy process (i.e., formation of autophagosome and autolysosome) in human UF tissues versus adjacent matched MyoF from the same patient, using transmission electron microscopy (TEM). As shown in Figure 1, human UF tissues exhibited significantly less autolysosomes compared to adjacent

INTRODUCTION
Uterine fibroids (UF) are common benign monoclonal tumors, which arise from the uterine smooth muscle cells. UF causes heavy menstrual bleeding, pain, infertility, and pregnancy complications. Many studies have confirmed the role of estrogen, progesterone and other growth factors including cytokines, chemokine, and miRNA in the etiology of this disease as key regulators of their proliferative growth. The most established risk factors of UF are age, early menarche, low parity and African ancestry. However, obesity and the consistent exposure to estrogen are believed to be etiological factor that increase the incidence of UF. In United States, the annual healthcare and management cost related to UF are calculated to be up to $34 billion. Autophagy is a collection of processes that enables the cells to remove and recycle their cytoplasmic contents as toxic protein aggregates, damaged organelles, and invading microorganisms. Although the process of autophagy is regulated by more than 30 proteins, most of them are involved in the autophagosome biogenesis. These proteins are known as autophagy-related proteins (ATGs). Studies have shown that ATG4 endopeptidase activity is important for late stages of autophagosome maturation in erythroid cells and allows the fusion of autophagosomes with lysosomes. There are four orthologues of the ATG4 mammalian family, also known as autophagin. These are: autophagin-1/ATG4A, autophagin-2/ATG4B, autophagin-3/ATG4C, and autophagin-4/ATG4D. These orthologues contain all the residues required for the catalytic activity of cysteine proteases, including the conserved cysteine residue within the catalytic site. The activities of different ATG4 orthologues can be regulated by microRNAs after transcription. The in vivo model of ATG4 loss of function was done only for ATG4B and ATG4C. Animals deficient in Atg4C show an increased susceptibility to tumorigenesis. Atg4C is not essential for autophagy development under normal conditions but is required for a proper autophagic response under stressful conditions such as prolonged starvation. However, Atg4B knockout mice showed normal survival, with minimal lesions in central nervous system. The connection between human UF and autophagy has not yet been investigated. In this work we demonstrate that autophagy is deregulated in human fibroid due to lack of ATG4D induction. Autophagy induction could be a promising therapeutic strategy to treat UF and remodel abnormal fibroid tissues into normal myometrium.
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myometrium. Furthermore, there was significantly more (20.5 ± 2.12) autophagolysosomes in MyoF controls compared to UF tissues (1 ± 0.04, P = 0.008; Figures 1a and c). This observation was further confirmed in human fibroid cell lines (HuLM) when compared to human normal myometrial cell line (UTSM) (Figure 1b). Consistent with in vivo data, the cell lines showed absence of autolysosomes versus control UTSM cell line (2.5 ± 0.7) and accumulation of autophagosomes in HuLM human fibroid cell line (13 ± 1.41) compared to UTSM (6 ± 1.41, P = 0.038) (Figures 1b and d). These results suggest that human fibroid phenotype is associated with normal autophagy initiation flux, but a block in autophagy flux, most likely due to a defect in the fusion of autophagosomes with lysosome.

Autophagy process is initiated in UFs. To further examine the autophagy process in human UF, we evaluated LC3 expression; a marker of autophagosome formation from the phagophore, the SQSTM1/P62 which serves as a link between LC3 and ubiquitinated substrates,17 and Beclin, a marker of early stage of autophagosome initiation process in human UF tissues and HuLM cell lines versus MyoF tissues and UTSM cell line, respectively, by western blots. We found an increase in the autophagosome markers LC3I, LC3II, p62, and Beclin-1 in the fibroid, compared to their adjacent tissue control group from the same patient (Figures 2a and b). The observed increase in both LC3I and LC3II suggest that conversion of LC3I to LC3II by lipidation is hindered in the fibroid and points toward possible deregulation of the autophagy flux. This observation was further supported by FACS analysis of LC3 and p62 level, through intracellular staining using un-conjugated antibody used for the immunoblotting (Figures 2c and d). Furthermore, this data was supported by the evaluation of LC3 and P62 by immunohistochemistry and immunofluorescence, respectively (Figures 2e and f).

Autophagy blockade in UF is linked to defect in ATG4D
The autophagy-related genes (ATGs) have been originally identified in the human genome and are largely associated with formation of the autophagosome membrane. We compared the expression level of several ATGs by real-time PCR between human fibroid versus myometrial cell lines. Our data demonstrated a significantly higher expression in ATG3, ATG5; ATG7, ATG12 and ATG16 in HuLM compared to UTSM cell lines (P < 0.05; Figure 3a) which is consistent with autophagy induction in UF. Importantly, the expression of total ATG4 was not induced showing the same level in both cell lines compared to other selected and analyzed ATGs. (Figure 3a). ATG4 or autophagin is a cysteine protease that has 4 orthologues. Among them, ATG7 and ATG8 are considered as the major regulators of autophagic flux. Therefore, we proceeded to determine their expression levels in UF versus normal myometrial cell lines by real-time PCR. We found a significant decrease in ATG4D mRNA level in fibroid cell line HuLM compared to the control UTSM cell line (P = 0.005; Figure 3e). The expression of the remaining ATG4 orthologues was not significantly different between HuLM and UTSM (Figures 3b and e). To further analyze ATG4D expression at protein level, we examined the ATG4D level by western blot. Our data indicates significant decreased expression of ATG4D normalized to the level of actin in human fibroid versus normal myometrial cell lines (Figures 3f and g). The correlation between decreased expression of ATG4 and the conversion of LC3I to LC3II in HuLM suggest that ATG4D defect may contribute to defective fusion between autophagosome and lysosome in human fibroid lesions.

Defective ATG4D expression in human UFs
The autophagy process is orchestrated by many ATG in order to regulate the cellular homeostasis.29 To corroborate the in vitro data, we quantified the mRNA expression levels of several ATGs in patient UF versus MyoF biopsies. We did not detect significant difference in the expression of ATG3, ATG5, ATG7, ATG10, ATG12, and ATG16 in UF compared to MyoF tissues (data not shown). The ATG4 expression defect was noted in 90% of patients and their average show absence of expression induction in UF (Figure 4a). Our finding was supported by immunohistochemistry (Figure 4b) and immunofluorescence staining of UTSM and HuLM UF cell lines with anti-ATG4D data (Figure 4c). Together, these data suggest that defective ATG4 induction in fibroid tissues and corresponding cell line is more likely due to decreased expression of ATG4D.

Figure 1. Autophagy blockade at the autophagosome stage in Human UF. Autophagosome formation was analyzed in vivo in Fibroid tissues (F) and adjacent normal myometrium (MyoF) collected from different patients as well as in fibroid cell line (HuLM) and normal myometrial cells (UTSM) using transmission electron microscopy (TEM) as described in the Materials and Methods. Representative TEM sections from (a) patient biopsy and (b) UTSM and HuLM cell lines. (c) and (d) semi-quantitative measurement of the number of autophagosomes (consistent with autophagy block) and autolysosomes (consistent with autophagy flux) in patients (n = 5) with fibroid (c) and in cell lines (d). Yellow arrows: Autolysosome (single-layer vacuole with content), Red arrows: autophagosome (double-layer vacuole). Representative images are shown (n = 5). *P < 0.05.
Lack of ATG4s induction and diminishment of lysosome associated membrane LAMP in UFs

LAMP-1 and LAMP-2 deficiency in various metabolic conditions, neurodegenerative diseases and infectious diseases is linked with the accumulation of autophagosomes. We hypothesized that defective fusion of the lysosome and autophagosome could impair the clearance of damaged organelles and aggregated proteins in fibroid cells likely leading to increased cell proliferation.

Figure 2. Initiation of autophagy is enhanced in Human UFs. Fibroid tissues (F) and adjacent normal myometrium (MyoF) collected from different patients, as well as in fibroid cell line (HuLM) and normal myometrial cells (UTSM) were analyzed for the expression of markers of autophagy flux by western blot. (a) Human UFs biopsy (MyoF, adjacent normal myometrium and F, UF). (b) Cell lines UTSM, HuLM. Levels of lysate proteins loaded in the gels were monitored by immunoblotting with an anti-Actin antibody. (c and d) The relative expression of LC3II, P62, ATG4D and Beclin1 was calculated based on the gray scale value of β-actin from patient biopsies and UF human cell lines respectively. (e) Quantification of endogenous LC3 and P62 by flow cytometry. Histograms show a higher expression of LC3 and p62 in HuLM compared to UTSM. Data are from one experiment and representative of three independent experiments. (f, g) Immunofluorescence staining of LC3 A/B in (MyoF) and (f) from UF patients (40 × magnification). (g) Immunochemistry staining for LC3 in human fibroid cells (magnification 63 × ). Representative images are shown. Data are means of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. Altered expression of autophagy-related proteins (ATGs) in Human uterine fibroid. RNA was extracted from frozen myometrium tissue and reverse transcribed as described in the Materials and Methods. (a) Real-time PCR analysis of mRNA levels of several ATGs. Data show significantly higher expression of ATG3, Atg7, ATG12, ATG16, but not ATG4, in HuLM compared to UTSM. (b–e) Quantification of the ATG4D isoforms by real-time PCR showing lower expression of ATG4D isoform. (f, g) Western blot analysis of ATG4D and the expression of ATG4D normalized to Actin. Data represent mean ± SEM of three independent experiments. *P < 0.05.
To test this hypothesis, we first analyzed the markers involved in lysosome fusion process, namely LAMP1 and LAMP2, in HuLM and UTSM cell lines by immunoblotting (Figures 5a and b) and their quantification by real-time PCR (Figures 5c–e). We found no significant change in the expression of LAMP1, however LAMP2 protein level was significantly decreased in (P \leq 0.05) and clear expression induction of Rab7b (Figure 5f) but not in Rab11 significantly lower in HuLM compared to UTSM (P = 0.00018). Furthermore, using immunofluorescence technique, there was significantly less LAMP2 expression in HULM versus UTSM cells (Figure 5g). Our data shows a second defect related to lysosome that may be involved in the observed autophagy blockade in UF.

ATG4D silencing in normal myometrial cells generates a fibroid-like cell profile

To directly investigate the contribution of ATG4D in the control of cell proliferation, and extracellular matrix production, the two hallmarks of uterine fibroids, we knockdown the expression of ATG4D in normal UTSM myometrial cell line. The ATG4D was silenced in UTSM cell line by ATG4D shRNA using scramble shRNA as a negative control. The transfected cells were selected by

Figure 4. ATG4s are not induced in Human UFs patient biopsies and fibroid cell line. (a) Real-time PCR quantification of total ATG4 mRNA expression and ATG4 isoforms. (b) Immunohistochemistry staining with human anti-ATG4 and anti-ATG4D. (c) Immunofluorescence staining for ATG4D in UTSM and HuLM cell lines, Green ATG4D and Blue is DAPI. (n = 15, *P < 0.05).

Figure 5. Defective expression of lysosomal markers in UFs. (a, b) Western blot analysis and semi-quantitative ratio to actin of LAMP1 and LAMP2. (c-f) Real-time PCR of LAMP1, LAMP2 and Rab11 respectively. (g) Immunofluorescence staining for LAMP2 expression with fluorescein (green) and DAPI (blue) used to counterstain nucleus shown in single stain and corresponding merged form. (\*P < 0.05).
puromycin and the positive transfected cells were positive for GFP, a cis-cistronic marker, as well (Figure 6a). The ATG4D silencing expression was confirmed by real-time PCR and western blot as compared to scramble control (Figures 6b and c). Remarkably, loss of ATG4D expression in UTSM cells resulted in significant increase in cell proliferation compared to the scramble control cell as evidenced by significant increase in the mean fluorescence intensity (MFI) of Ki67, proliferative marker (10346 ± 258.82 versus 1860 ± 281.42, P = 0.001; Figure 6d). In addition, MTT assay was performed to investigate proliferation and viability of UTSM ATG4D shRNA cells versus scramble control at different time point 24, 48, 72, and 96 h. Our data revealed a significant increase in the proliferation and viability/survival of UTSM with loss of function in ATG4D (P < 0.05; Figure 6c). This data suggests that expression of ATG4D in myometrial cells is essential for myometrial cell proliferation and viability, and that disruption of ATG4D expression and function generate a fibroid-like cell phenotype likely via abrogation of the autophagy process.

Loss of function of ATG4D by shRNA in normal myometrium cell line abrogates autophagy and enhances inflammation. ATG4D is a pivotal protein for dilapidation of LC3B from LC3A that leads to fusion of autophagosome with lysosome. We hypothesized that silencing ATG4D will block autophagy in human UTSM normal myometrial cells. To test this hypothesis, we examined accumulation of LC3A and P62 proteins by immunoblotting and flow cytometry in ATG4D-KD UTSM versus scramble control. Our data show a significant increase in the MFI of LC3I in ATG4D knockdown UTSM cells versus scramble control (P < 0.005; Figures 7a and b). Furthermore, western blot analysis also revealed significant increase in P62 expression in ATG4D-KD UTSM versus scramble control (Figure 7b). Thus, loss of function of ATG4D in normal myometrium cell line resulted in alteration of autophagy process that phenocopied UF tissues and HuLM cell lines.

Previous studies indicated that UF pathogenesis is marked by an increase of extracellular matrix (ECM) due to excessive secretion of interstitial collagen, inflammatory immune cells infiltration and fibrosis. To examine the impact of ATG4D knockdown on ECM, we have used two markers: Fibronectin as glycoprotein of ECM and plasminogen activator inhibitor-1 (PAI-1). The analysis by western blot of extracted protein from UTSM stable cell lines treated with shRNA ATG4D showed significant increase in Fibronectin and PAI-1, compared to shRNA scramble controls (Figure 7c). This suggested that defective expression of ATG4D enhances extracellular matrix and fibrosis development.

Another emerging characteristic of UF is the promotion of inflammation as reported by our group and others.\(^{24,25}\) Furthermore, recent reports connected aberration of autophagy with altered inflammatory state.\(^{26}\) In order to explore the impact of ATG4D silencing on the inflammatory response of normal human UTSM myometrial cells, we examined the expression level of proinflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\) and TGF-\(\beta\) in ATG4D-KD-UTSM versus scramble control cells. As shown in Figure 7d, silencing of ATG4D expression by shRNA was able to activate proinflammatory cytokine release and enhanced inflammatory status. The intracellular staining for TGF-\(\beta\), TNF-\(\alpha\) and IL-1\(\beta\) was significantly increased in ATG4D-KD-UTSM cells compared to scramble control infected cells (P < 0.05; Figure 7d).

**DISCUSSION**

The autolysosome pathway plays an important role in degrading and recycling different cellular components.\(^{27,28}\) Abrogated autophagy has been reported in several different diseases but not in uterine fibroid. We show here, for the first time, that autophagy is initiated in UF but blocked at the autophagosome stage. TEM analysis of UF tissues collected at different stage of menstrual cycle from patients of different ethnicity exhibited accumulation of autophagosome bilayer vacuoles, which failed to fuse with lysosomes. In contrast, autophagy flux was complete in normal myometrium tissue as evidenced by the presence of the different characteristic structures corresponding to the different steps of autophagy process beginning with the formation of

Figure 6. Knock-down of ATG4D by shRNA in UTSM fibroid cell line increase proliferation. The expression of ATG4D in UTSMs was knocked-down by ATG4D-specific shRNA using lentivirus particles. Control cells were infected with lentivirus containing non-specific shRNA vector. (a) Silencing the expression of ATG4D in UTSM by shRNA and scramble control both tagged with GFP construct. (b) Real-time PCR of ATG4D mRNA expression. (c) Western blot of ATG4D protein. (d) Proliferation assay analysis by FACS intracellular staining for Ki67. (e) The cells (3000/well) from either ATG4D knockdown or scrambled control were seeded onto 96-well tissue culture plates. The MTT assay was performed at different time points 24, 48, 72, and 96 h. The Averaged cell numbers from triplicate wells were used in preparing the showed data graph. Each data point is the mean (±S.D.) from an individual experiment performed in triplicate (n = 3). *P < 0.05, **P < 0.01.
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Figure 7. Knock-down of ATG4D by shRNA in UTSM cell line mimic UF phenotype. (a) Autophagy markers analysis by FACS with intracellular staining and mean fluorescence intensity MFI for LC3, and (b) by western blot for LC3 and P62. (c) Extracellular matrix markers analysis by Western blot of fibronectin, PAI-1, and Actin. (d) Endogenous quantification of pro-inflammatory cytokines expression was done by intracellular staining for TNF-α, IL-1β, TGF-β and IL-10 using flow cytometry analysis. The expression level was represented as mean fluorescence intensity (MFI). Data from the histogram are shown as mean ± S.D. and are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

autophagosome and ending with fusion of autophagosome with the lysosome to form autolysosome. The analysis of classical markers of autophagy by immunoblotting showed an accumulation of LC3I and LC3II, Beclin and P62 in patient samples as well as in cell lines; in vitro model of UF, supporting the TEM results. As the molecular regulation of autophagy is orchestrated by ATG proteins, we evaluated the autophagic process by examining expression of ATGs. The molecular screening of several ATGs mRNA expression in UF human biopsies show high variability in their expression. However, the ATG4 expression was not induced in 90% of our patient samples. The ATG4 exist in four orthologues: ATG4A, ATG4B, ATG4C and ATG4D. Intriguingly, our data reveal that ATG4D expression was significantly decreased and almost absent at the protein level as revealed by immunohistochemistry and immunofluorescence from both patient samples as well as cell lines USTM and HuLM. ATG proteins encoded by AuTophaGy-related (ATG) genes, which have been extensively investigated in yeast, are important for autophagy process.9 Notably, ATG4D appears to be the most frequent ATG4 protease involved in other gynecologic cancers such as breast cancer, followed by 4B and 4C. Deregulation of ATG4 is frequent in tumors of the female reproductive system such as ovarian, uterine and breast cancers reflecting its importance in stem cells homeostasis.14,15 Thus, it is possible that ATG4D is the master regulator of autophagy flux in several gynecologic benign and malignant tumors. How ATG4 is regulated in these tumors is not clearly understood. However, studies have shown that ATG4 can be regulated by miR-34a that specifically targets ATG4B,11 miR-376b that target intracellular levels of ATG4C,12 and the tumor suppressor miR-101 that inhibits autophagy by targeting ATG4D.13 At the functional level, the biological role of ATG4D in gynecologic tumor biogenesis and progression is not well understood. However, studies using zebrafish indicated that ATG4D is critical for autophagy-mediated neuronal homeostasis in the central nervous system, where knockdown of ATG4D in neuronal cells resulted in abnormal autolysome formation and degradation and cytoplasmic vacuolization. Further studies suggested that ATG4 is responsible of LC3 dilipidation at the autophagosome stage. ATG4s is expressed at low level in a wide variety of human tissues at low levels. The cleavage of ATG4D is related to its unique roles in both autophagy and apoptosis.23 The majority of ATG4s alterations have been detected in female reproductive tissue tumors, including ovarian and uterine cancers.30

UF is proliferative disease that is characterized by uncontrolled cellular proliferation, inflammatory tumor microenvironment and immature extracellular matrix. To understand and directly evaluate the role of ATG4D in the pathogenesis of UF, we knocked-down ATG4D by shRNA in normal myometrium cell line USTM. We found that ATG4D loss of function in UTSM promotes proliferation of myometrial cells as evidenced by increased intracellular staining for Ki67 (Figure 6d) and supported by the MTT proliferation assay at different points in culture (24, 48, 72, 96 h; Figure 6e). Notably, unlike the expected decrease in cell viability upon genetic manipulation by shRNA, our data indicate that deficiency of ATG4D enhanced the survival of shRNA-treated UTSM compared to the controls (Figure 6e). These data are consistent with other studies showing that ATG4D silencing in HeLa cells sensitize them to the controls (Figure 6e). These data are consistent with other studies showing that ATG4D silencing in HeLa cells sensitize them to starvation-induced cell death, suggesting that ATG4D mediated autophagy contributes to the survival response in starved cells.31 Consistent with the in vivo and in vitro phenotypes of UF tissues and HuLM cells, respectively, knockdown of ATG4D in UTSM also resulted in increased autophagy initiation, but block of autophagy flux as marked by increased expression of LC3 and p62 in UTSM treated with shRNA ATG4D. Further analysis of the fibronectin and PAI-1; markers of extracellular matrix, by western blot demonstrated a high expression of these ECM in UTSM in the absence of ATG4D. The induction of ECM markers correlate with the release of inflammatory cytokines such as TGF-β, that lead to fibrosis
through structural remodeling of the myometrium. Furthermore, the mRNA expression of multiple ECM genes in UF is decreased when the TGF-β pathway is downregulated.32 Thus, based on these observations regarding autophagy in UF, our data unravel a novel mechanistic pathway in UF pathogenesis where ATG4D loss of function in normal myometrial cells was able to induce UF. Our data suggest that autophagy contributes to the accumulation of autophagic vacuoles containing abnormal proliferative cells by apoptosis. In conclusion, the present study suggests that the impairment of autophagy is a causative factor of UF formation primarily due to the mechanism of lysosomal fusion stage. Further studies will examine the intriguing observation regarding autophagy in UF, our data unravel a novel mechanism that de...
Silencing ATG4D expression
Lentiviral shRNA (Origent Technologies Inc., Rockville, MD, USA) targeted to autophagy-related protein-4D (ATG4D) RNA was used to knockdown ATG4D protein expression. For infection, lentivirus particles were added to each well of a six-well plate containing 1 x 10⁵ cells. Control cells were infected with lentivirus containing non-specific shRNA vector. Cells were incubated with lentivirus for 12 h and then transferred to a 75 mm flask. Infected cells were selected following treatment with puromycin (1 μg/ml) and based on positivity for GFP expression. The ATG4D knockdown cell lines showed reduced expression level compared to scramble control. Cells treated with scramble shRNA vector are hereafter referred to as autophagy-competent. ATG4D protein knockdown cells are hereafter referred to as autophagy blocked.

Histology and immunohistochemistry
Patient MyoF and UF tissues were collected, fixed in 4% paraformaldehyde, and embedded in paraffin. Tissue sections were stained with haematoxylin-eosin (H&E) staining as well as with human anti-ATG4 (ab111110) from Abbeex Ltd (Cambridge, UK), anti-ATG4 (ab137621), anti-LC3 antibodies (D3U4C) from Cell Signaling as mentioned above, followed by detection with a biotin anti-rabbit antibody and staining with the ABC kit purchased from Vector Laboratories. The samples processing and staining was done using standard techniques. The slides analyses were performed using Olympus Las 4.1 software (Center Valley, PA, USA).

Immunofluorescence
Patient samples were embedded in paraffin and processed in the histology core facility of Augusta University using standard techniques. The biopsy samples were fixed with 4% paraformaldehyde. After permeabilization by 0.3% Triton X-100 and incubated with goat serum, cells were stained with appropriate antibody for overnight at 4 °C. Then, cells were incubated with a secondary antibody at 37 °C for 1 h and DAPI (0100-20) purchased from Southern Biotech (Birmingham, AL, USA) for 10 min. Finally, samples were examined with a Nikon confocal microscope (Nikon C1-Si, Tokyo, Japan).

Transmission electron microscopy analysis
The cell samples was fixed and prepared as described previously. Serial ultrathin sections were cut on an LKB-III ultratome from LEICA, and were stained with uranyl acetate and lead citrate both of them purchased from TED PELLA. The sample sections were examined using a Hitachi H7600 electron microscope at an accelerating voltage of 100 kV, at the histology core facilities of Augusta University using standard techniques. The counting methods of autophagosome and autolysosome vacuoles was published maps and institutional affiliations.

Flow cytometry and cytokines staining
The intracellular staining for autophagy and inflammation markers was done after fixation and permeabilisation of cells (554714) using BD Cytotox/Cytoperm purchased from BD Bioscience according to the manufacturer’s protocol. The anti-human marker for autophagy LC3 (D3U4C) that was conjugated with anti-rabbit IgG Fab2 (44145), purchased from Cell signaling and PE62 (ab56616) that was conjugated with Goat Dyelight 488 IgG (ab90873) purchased from abcam. Proliferation marker Ki67 (350514) and anti-human proinflammation cytokine markers such us TNF-α (502943), TGF-β (349607) and IL-10 (506804) were purchased from Biolegend except IL-1β (IC8406A) that was purchased from R&D.

Statistical analysis
Statistical comparison of differences between the two test groups was evaluated by one-way ANOVA test or Student’s t-test analysis as appropriate. A P-value of less than 0.05 was considered statistically significant. All values are expressed as mean ±SEM. and analyzed using Prism Graph-Pad Software Inc (La Jolla, CA, USA).

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Competing Interests
The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Cell Death Discovery website (http://www.nature.com/cddiscovery)