

## Rapamycin Synergizes with Cisplatin in Antiendometrial Cancer Activation by Improving IL-27–Stimulated Cytotoxicity of NK Cells



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### Abstract

Natural killer (NK) cell function is critical for controlling initial tumor growth and determining chemosensitivity of the tumor. A synergistic relationship between rapamycin and cisplatin in uterine endometrial cancer (UEC) *in vitro* has been reported, but the mechanism and the combined therapeutic strategy for endometrial cancer (EC) are still unknown. We found a positive correlation between the level of IL-27 and the differentiated stage of UEC. The increase of IL-27 in uterine endometrial cancer cell (UECC) lines (Ishikawa, RL95-2 and KLE) led to a high cytotoxic activity of NK cells to UECC in the co-culture system. Exposure with rapamycin enhanced the cytotoxicity of NK cells by upregulating the expression of IL-27 in UECC and IL-27 receptors (IL-27Rs: WSX-1 and gp130) on NK cells and further restricted the growth of UEC in Ishikawa-xenografted nude mice. In addition, treatment with rapamycin resulted in an increased autophagy level of UECC, and IL-27 enhanced this ability of rapamycin. Cisplatin-mediated NK cells' cytotoxic activity and anti-UEC activation were independent of IL-27; however, the combination of rapamycin and cisplatin led to a higher cytotoxic activity of NK cells, smaller UEC volume and longer survival rate *in vivo*. These results suggest that rapamycin and cisplatin synergistically activate the cytotoxicity of NK cells and inhibit the progression of UEC in both an IL-27–dependent and –independent manner. This provides a scientific basis for potential rapamycin-cisplatin combined therapeutic strategies targeted to UEC, especially for the patients with low differentiated stage or abnormally low level of IL-27.

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### Introduction

The incidence of uterine endometrial cancer (UEC) as a common gynecological malignancy is increasing worldwide. The majority of women diagnosed with UEC have early-stage disease, which can often be cured with surgery alone. However, chemotherapeutic or hormonal treatment for women with advanced or recurrent disease has met with limited efficiency. There is an urgent need to find an

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additional agent with a low toxicity to increase the anti-UEC efficiency in cooperating with traditional hormonal and cytotoxic agents.

The inhibitors of the mammalian target of rapamycin (mTORs) (such as rapamycin and ridaforolimus) are known for potent antiproliferative and autophagy inducer properties [1] and are currently under evaluation in clinical trials for a broad range of cancers, including UEC [2,3]. Rapamycin is a macrocyclic triene with immunoregulatory properties [4–6], for example, it stimulates the secretion of IL-12p70 and IL-27 by dendritic cells and further promotes allogeneic type 1 polarization modulated by natural killer (NK) cells [6]. Although a synergistic relationship exists between rapamycin and cisplatin in both the inhibition of cell growth and induction of apoptosis in UEC [7], the detailed mechanism for this process has remained elusive.

NK cells are critical for controlling initial tumor growth and determining chemosensitivity through the secretion of interferon- $\gamma$  (IFN- $\gamma$ ) and other proinflammatory cytokines and a cytotoxic response [8–10]. NK cells have the potent ability of antitumor immunity in mice, and their depletion has been linked to metastatic spread [11]. In addition, the presence of tumor-infiltrating NK cells clinically correlates with good patient outcome in various human cancers [12–14]. However, the regulation mechanism of the function of NK cells in UEC is largely unclear.

Interleukin (IL)-27, a member of the IL-12 family of cytokines, is a heterodimeric cytokine that contains Epstein–Barr virus–induced gene 3 and a unique IL-12p35-like protein, IL-27p28, which signals through a receptor composed of IL-27R $\alpha$  (also known as WSX-1 or TCCR) and gp130 (utilized by many cytokines, including IL-6) [15,16]. IL-27 is mainly produced by antigen-presenting cells such as dendritic cells (DCs) and macrophages, and is involved in regulating the balance between protective and pathological immunity and promoting antitumor responses [17,18]. Our group has found that IL-27 secreted by endometrial stromal cells and that macrophages promote the progression of endometriosis by inducing the differentiation of IL-10<sup>+</sup>T help 17 (Th17) cells [19]. However, the expression and role of IL-27 in UEC remain unknown.

Therefore, this study investigated the expression and effect of IL-27 in UEC progression, and the regulation relationship between rapamycin, IL-27, and NK cells in uterine endometrial cancer cells (UECCs) *in vitro* and *in vivo* and further clarifies the mechanism of rapamycin on immune regulation of the UEC microenvironment.

## Materials and Methods

### Antibodies

Anti-human IL-27 antibodies (Abs) and anti-human PCNA were purchased from Abcam (USA); Phycoerythrin (PE)-conjugated anti-human IL-27, PE-conjugated anti-human WSX-1, Allophycocyanin (APC)-conjugated anti-human gp130, Brilliant Violet 421 (BV421)-conjugated anti-human CD56, phycoerythrin-cyanine 7 (PE-Cy7)-conjugated anti-human CD16, fluorescein (FITC)-conjugated anti-human NKG2D, phycoerythrin-cyanine 7 (PE-Cy7)-conjugated anti-human NKp44, APC-conjugated anti-human NKp46, PE-conjugated anti-human NKp30, FITC-conjugated anti-human KIR2DL1, PE-conjugated anti-human KIR3DL1, APC-conjugated anti-human IFN- $\gamma$ , PE-conjugated anti-human perforin, Brilliant Violet 421 (BV421)-conjugated anti-human Granzyme B, PE-conjugated anti-human Fas, APC-conjugated anti-human FasL, PE/Cy5.5-conjugated anti-human Ki-67, and

PE-conjugated anti-human Bcl-xL were purchased from BD Biosciences (San Jose, CA).

### Patients and Sample Collection

The protocol for this study was approved by the Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University, and written informed consent was obtained from all participants. All the normal endometrial tissues, highly differentiated UEC, moderately differentiated UEC and poorly differentiated UEC tissues were obtained by laparoscopy from 45 patients (mean age 47.8 years; range 36–54 years) at the Obstetrics and Gynecology Hospital of Fudan University. All of the samples were confirmed histologically. Ten UEC patients had a highly differentiated degree of UEC, 10 UEC patients had a moderately differentiated degree of UEC, and 10 UEC patients had a poorly differentiated degree of the disease. Normal endometrium in the secretory phase of the cycle was obtained through hysterectomy from patients with leiomyoma (15 cases) as normal control samples. No patients took any medications or received hormonal therapy within 6 months prior to surgery.

In addition, the peripheral blood was collected from 72 healthy fertile women (mean age 28.7 years; range 23–37 years).

### Cells Lines

The human endometrial carcinoma cell lines (Ishikawa, RL95-2, and KLE cells) were obtained from the cell bank of Chinese Academy of Science (Shanghai, China). Ishikawa was grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin. RL95-2 and KLE cells were grown in DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin.

### Immunohistochemistry

Paraffin sections (5  $\mu$ M) of normal endometrium and UEC tissues from patients were dehydrated in graded ethanol and then incubated with hydrogen peroxide and 1% bovine serum albumin/TBS to block endogenous peroxidase. The samples were then incubated with mouse anti-human IL-27 (10  $\mu$ g/ml, Abcam, USA) or mouse IgG isotype overnight at 4°C in a humid chamber. After washing three times with TBS, the sections were overlaid with peroxidase-conjugated goat anti-mouse IgG, and the reaction was developed with 3,3-diaminobenzidine and counterstained with hematoxylin.

### Purification of NK Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy fertile women. Human NK cells were isolated from PBMCs using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for *in vitro* co-culture experiments and *in vivo* cell transfer.

### Cell Co-Culture

We obtained the IL-27-overexpressed UECC (IL-27<sup>+</sup>) and control UECC (mock) through transfection with GV230-IL-27 plasmid and GV230-vector plasmid (GenePharma, Shanghai, China), and these UECCs were treated with or without rapamycin (100 nM, Sigma, USA) and/or cisplatin (10  $\mu$ M, Sigma) for 48 hours. Cell supernatants were then discarded, and these cells were washed with phosphate-buffered solution (PBS) and co-cultured with NK cells from peripheral blood for 24 hours. After co-culture, these NK cells were collected for flow cytometry (FCM) analysis or co-cultured with fresh UECC cells for cytotoxicity trials.

### Real-Time PCR (RT-PCR)

The efficiency of IL-27 overexpression in Ishikawa, RL95-2, and KLE cells was verified by RT-PCR according to the standard protocols. In addition, mock and IL-27<sup>+</sup> Ishikawa cells were treated with rapamycin (100 nM, Sigma) for 48 hours, and then the transcriptional levels of *BECN1*, *MAP1LC3B*, *MTOR*, and *SQSTM1* in these cells were analyzed by RT-PCR. The primer sequences were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). The primer sequences of these genes are described in Supplementary Table 1. The fold change in the gene expression of the above genes was calculated using the change in cycle threshold value method ( $\Delta\Delta$  Ct). All values obtained were normalized to the values obtained for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*).

### FCM

After co-culture, we collected and analyzed the expression of IL-27Rs (gp130 and WSX-1), CD16, NKG2D, NKp30, NKp44, NKp46, KIR2DL1, KIR3DL1, IFN- $\gamma$ , perforin, and Granzyme B in CD56<sup>+</sup>NK cells by FCM according to the manufacturer's instructions. The samples were analyzed using a Beckman Cyan flow cytometer (Becton Dickinson, USA) and Cellquest software (Becton Dickinson). The statistical analysis was conducted using isotype-matched controls as references.

### Cytotoxicity Trials of NK Cells to UECC [Lactate Dehydrogenase (LDH) Release-Based]

NK cells were first co-cultured with IL-27<sup>+</sup>UECC for 24 hours, and then the NK cells were collected and co-cultured with fresh UECC. The cytotoxicity of NK in response to fresh UECC (Ishikawa, RL95-2, and KLE) was analyzed using a lactate dehydrogenase (LDH) release assay. UECC (2500 to 5000 cells/well) were plated, and the next day, NK cells were added at various ratios (100:1, 10:1, 1:1, 1:3, and 1:10; target cells:effector cells) (all samples in triplicate). After 4 hours of co-culture, an aliquot of 50  $\mu$ l media was used in LDH cytotoxic assay using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, G1780). The value of

corrected experimental LDH release was calculated by subtracting the value of spontaneous LDH release from effector cells at corresponding dilutions. NK cytotoxicity was defined as %cytotoxicity = (experimental value - effector cells spontaneous control - target cells spontaneous control)/(target cell maximum control - target cells spontaneous control)  $\times$  100.

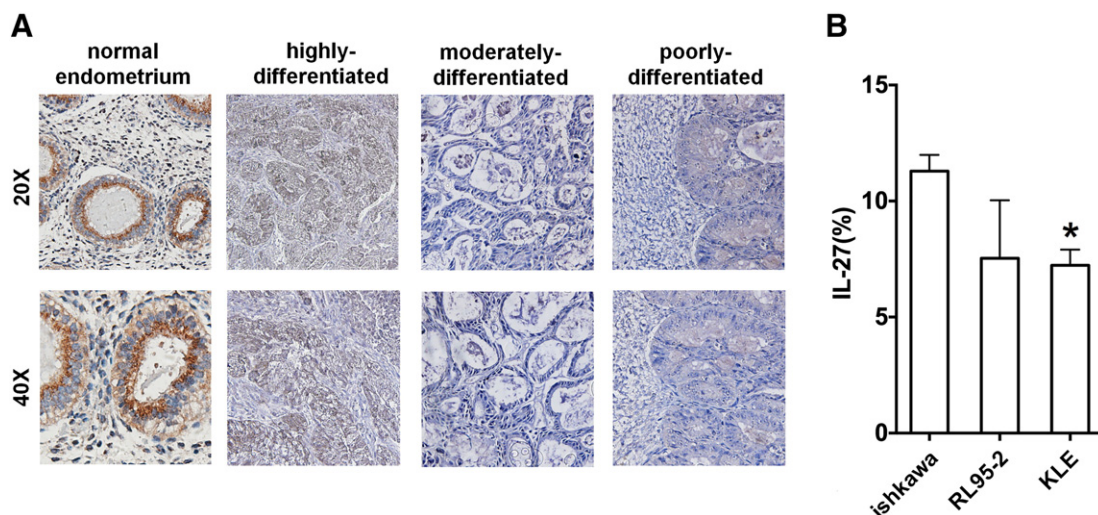
### Enzyme-Linked Immunosorbent Assay (ELISA) for IL-27 Determination

UECC (Ishikawa, RL95-2, and KLE cells) ( $1 \times 10^5$  cells/well) were seeded in 24-well plates and treated with rapamycin (100 nM, Sigma, USA) for 48 hours, and the secretion level of IL-27 in the culture supernatant was analyzed by ELISA (Biolegend, USA).

### In Vivo Experiments

Nude mice of 4 to 5 weeks of age were inoculated subcutaneously under the scruff on day 0 with 200  $\mu$ l of  $2 \times 10^6$  Ishikawa cells (IL-27<sup>+</sup> on the right or NC on the left) (NC: Ishikawa cells were transfected with negative control lentivirus; IL-27<sup>over</sup>: Ishikawa was transfected with the lentiviruses expressing human IL-27). Rapamycin (2 mg/kg) and/or cisplatin (2 mg/kg) or PBS was intraperitoneally injected for 3 weeks after xenotransplantation. In addition, human NK cells from peripheral blood ( $5 \times 10^5$  cells/mouse) were adoptively transferred to Ishikawa-xenografted nude mice every 7 days for the first 2 weeks. Tumor growth was monitored by measuring the tumor volume every 2 days. Tumor volume was determined using the formula: volume ( $\text{mm}^3$ ) =  $1/2(\text{length} \times \text{width} \times \text{height})$ . After 30 days, mice were euthanized, and the tumor tissues were collected for analysis of immunohistochemistry (IHC) (proliferating cell nuclear antigen, or PCNA, expression), FCM, and transmission electron microscopy (TEM). For evaluation of survival rate, survive curves of all groups were recorded until 60 days after translation.

For FCM, the tumor tissues of nude mice were perfused thoroughly with cold PBS before cell collection, and then tissues were minced on ice and digested with an enzyme mix of Liberase and Dispase (Invitrogen). We then collected the cells and evaluated the expression of Ki-67, Fas, FasL, and Bcl-xL in CK7<sup>+</sup>UECC and the



**Figure 1.** IL-27 level is positively correlated with the differentiated stage of UEC. (A) The expression of IL-27 in normal endometrium ( $n = 15$ ) and UEC tissues (highly differentiated group = 10, moderately differentiated group = 10, poorly differentiated group = 10) by IHC staining. (B) The level of IL-27 in Ishikawa, RL95-2, and KLE cells was detected by FCM. Data are expressed as mean  $\pm$  SEM. \* $P < .05$ .

expression of IL-27Rs (gp130 and WSX-1), CD16, NKG2D, NKp30, NKp44, NKp46, KIR2DL1, KIR3DL1, IFN- $\gamma$ , perforin, and Granzyme B in CD56<sup>+</sup>NK cells by flow cytometry.

To identify autophagosomes at the ultrastructural level, UEC tissues were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 45 minutes at 4°C, rinsed in cacodylate buffer, postfixed in 1% OsO<sub>4</sub> in cacodylate buffer, dehydrated, and embedded in Eponate. Ultra-thin sections were briefly contrasted with uranyl acetate and photographed with a TEM (Hitachi 7100, Tokyo, Japan). The number of autophagosomes in UECC from UEC tissues was counted in 10 randomly chosen cells per sample. The autophagy grade was based on the average number of autophagic vacuoles and autolysosome/per cell visible by TEM [20].

### Statistics

All values are shown as mean  $\pm$  SEM. Data were analyzed with GraphPad Prism version 5 by *t* test or one-way ANOVA. A log-rank test was performed, and Kaplan-Meier survival curves were plotted. Differences were considered statistically significant at *P* < .05.

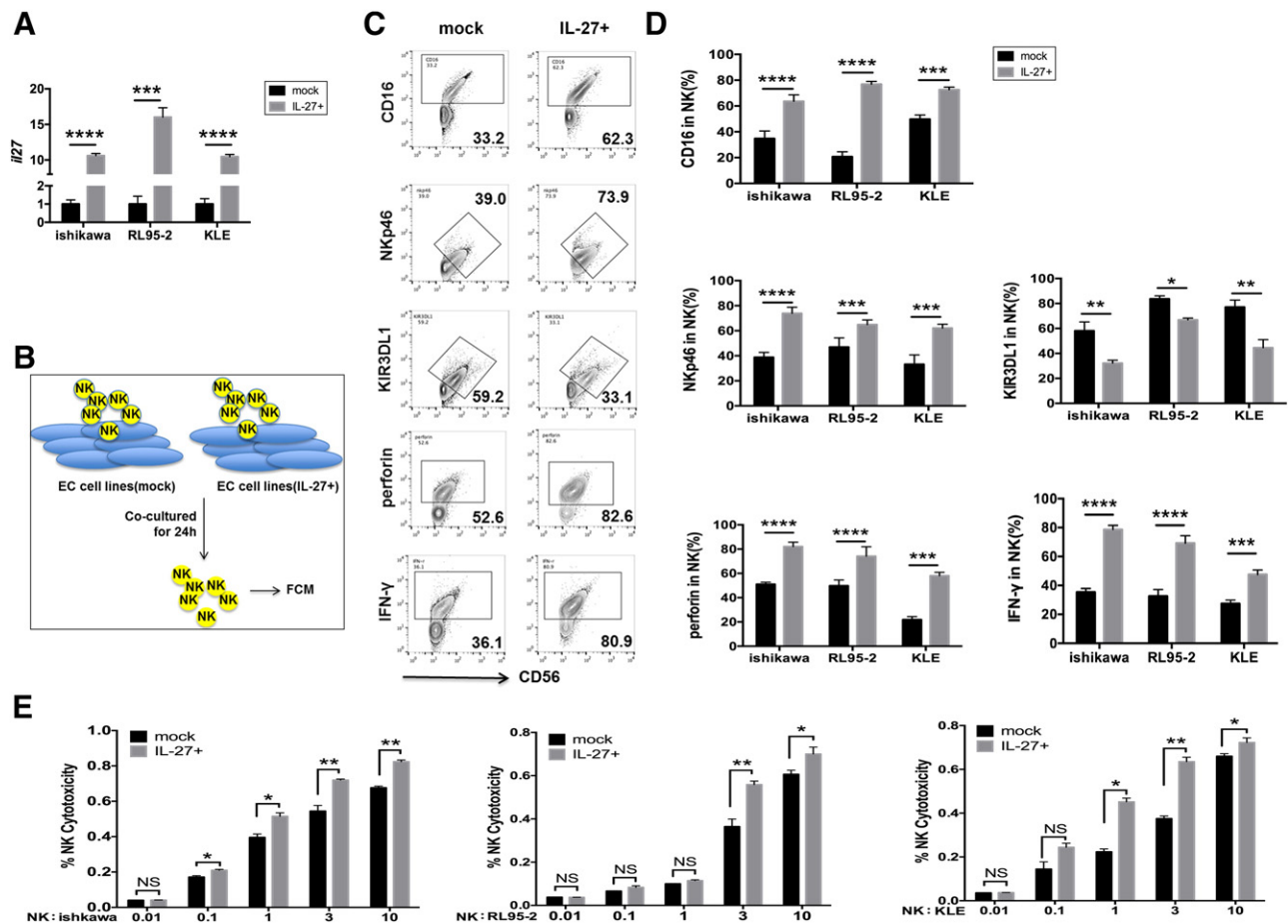
## Results

### IL-27 level Is Positively Correlated with the Differentiated Stage of UEC

To investigate whether IL-27 expression is associated with the pathological stage of UEC, we first analyzed the expression of IL-27 in UEC tissues by IHC staining. As observed in Figure 1A, the staining of IL-27 in normal endometrium was stronger than that in UEC tissues. IL-27 level in UECC was gradually decreased with the decrease of differentiation degree of UEC (Figure 1A). Of note, IL-27 staining in moderately and poorly differentiated UEC was nearly undetected. Compared to Ishikawa and RL95-2 cells, KLE cells had a lower level of IL-27 (Figure 1B). This result indicates that there is a positive correlation between the expression of IL-27 and the degree of UEC differentiation.

### IL-27 Improves the Cytotoxicity of NK Cells to UECC

To investigate the potential effect of IL-27 secreted by UECC on NK cells function, we first constructed IL-27 overexpressed (IL-27<sup>over</sup>) Ishikawa, RL95-2, and KLE cells (Figure 2A) and further co-cultured

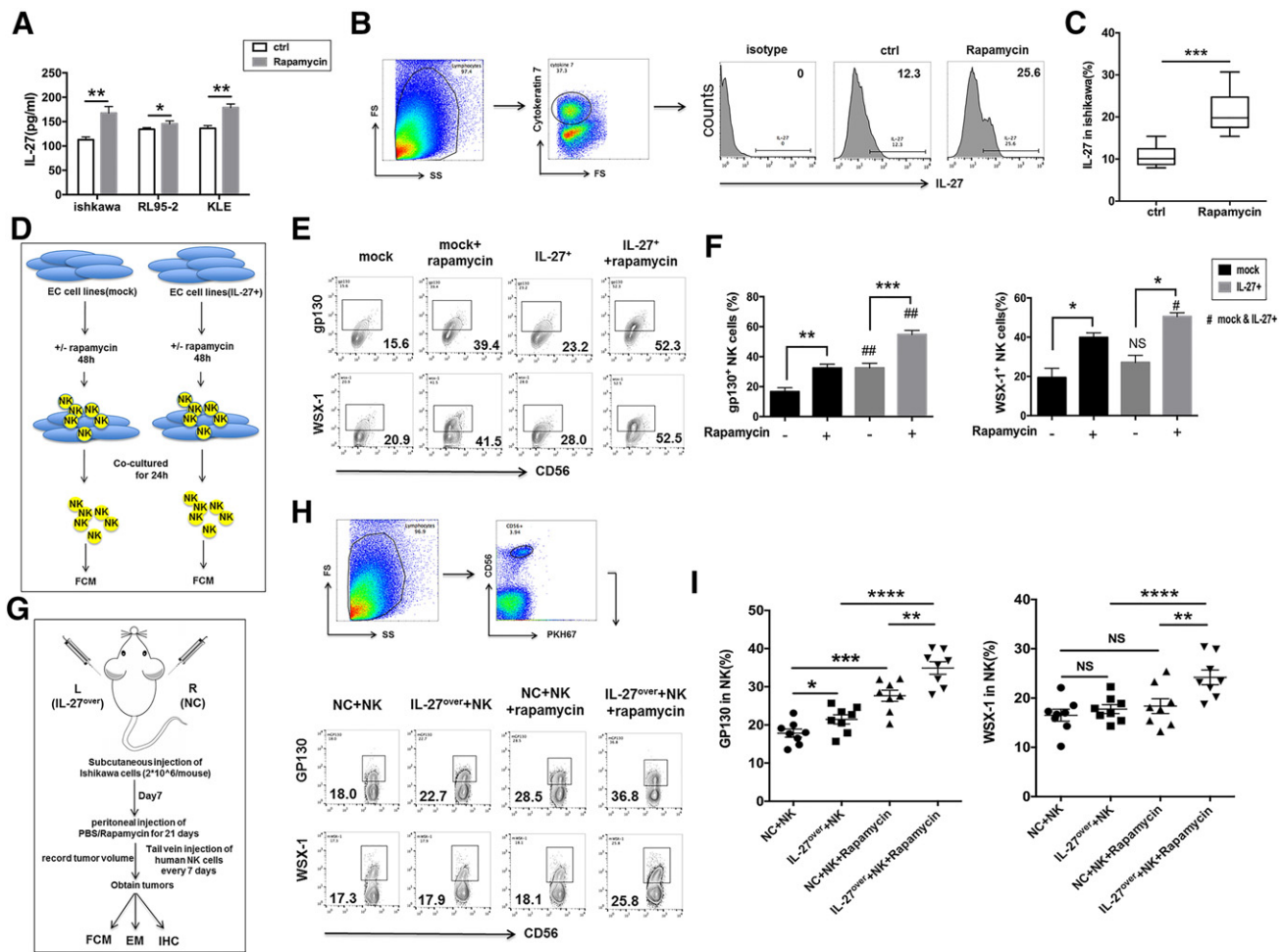


**Figure 2.** IL-27 improves the cytotoxicity of NK cells to UECC. (A) The transcription level of IL-27 in mock and IL-27-overexpressed (IL-27<sup>+</sup>) UECC (Ishikawa, RL95-2, and KLE cells) was measured by RT-PCT. (B-D) NK cells (*n* = 6) from peripheral blood were respectively co-cultured with mock or IL-27<sup>+</sup> UECC (Ishikawa, RL95-2, and KLE cells) for 24 hours, and then the expression of KIR3DL1, NKp46, CD16, IFN- $\gamma$ , and perforin in NK cells was analyzed by FCM. (E) After first being co-cultured with mock or IL-27<sup>+</sup> UECC (Ishikawa, RL95-2, and KLE cells) for 48 hours, NK cells (*n* = 6) were then co-incubated with fresh UECC for the cytotoxicity assay at different E/T ratios (1:100, 1:10, 1:1, 3:1, or 10:1) for 3 hours. Mock: control UECC transfected with GV230-vector plasmid; IL-27<sup>+</sup>: UECC transfected with GV230-IL-27 plasmid. Data are expressed as the mean  $\pm$  SEM. \**P* < .05, \*\**P* < .01, \*\*\**P* < .001, and \*\*\*\**P* < .0001. NS: no statistical difference.

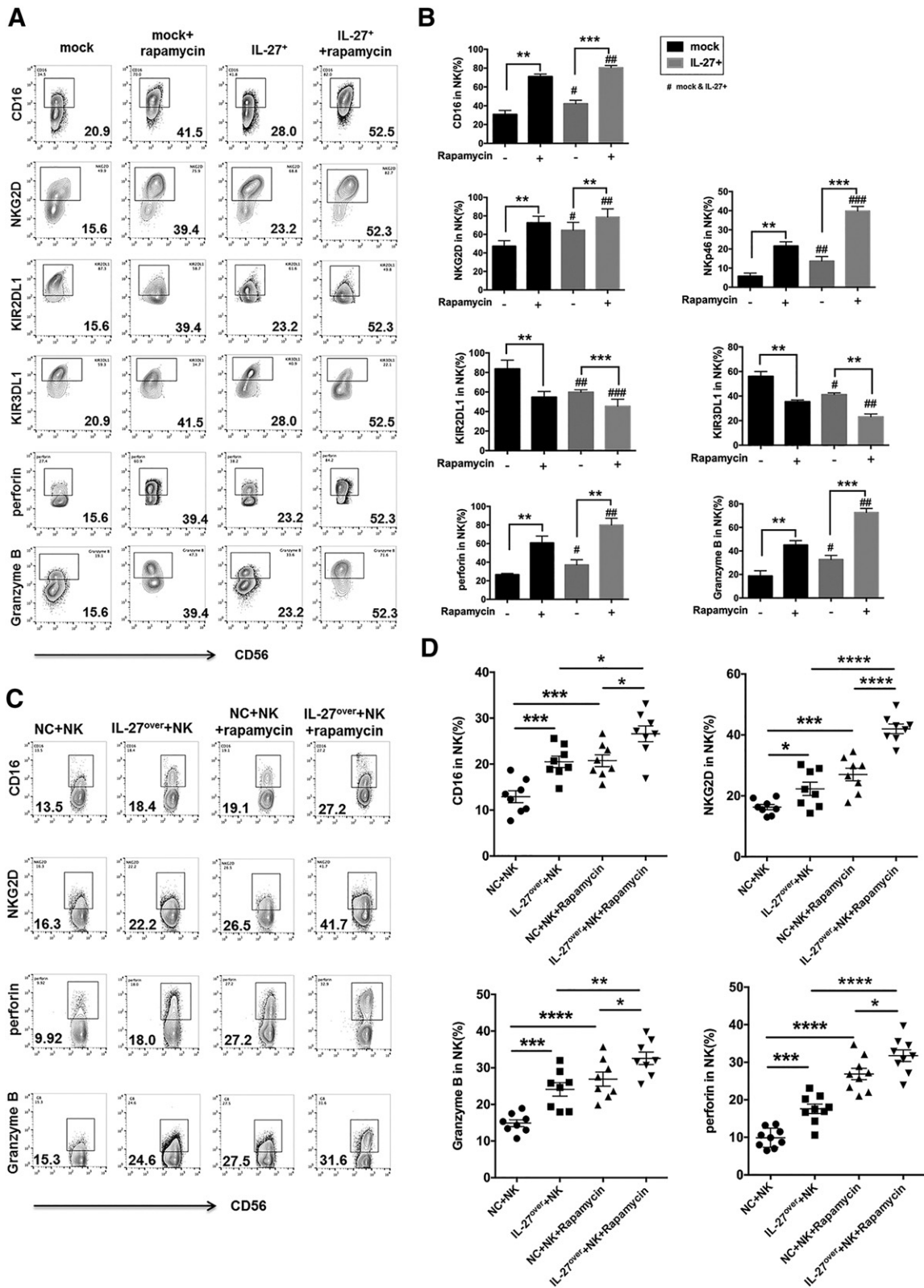
them with NK cells from peripheral blood, respectively (Figure 2B). As shown, compared to control cells, IL-27<sup>over</sup> Ishikawa, RL95-2, and KLE cells led to the increase of CD16, NKp46, NKp30, NKG2D, perforin, IFN- $\gamma$ , and Granzyme B and the decrease of killer cell immunoglobulin-like receptor (KIR) 3DL1, KIR2DL1, IL-10, and transforming growth factor (TGF)- $\beta$  in co-cultured NK cells (Figure 3, C and D, Supplementary Figure 1). Subsequently, we collected these co-cultured NK cells and further co-cultured with fresh Ishikawa, RL95-2, or KLE cells in a different cell ratio of NK and UECC (1:100, 1:10, 1:1, 3:1, or 10:1). As presented in Figure 2E, NK co-cultured with IL-27<sup>over</sup>UECC had more powerful cytotoxic activity than the control UECC, especially with a cell ratio of NK and UECC of 3:1 or 10:1. Taken together, these data suggest that IL-27 secreted by UECC enhances the cytotoxic activity of NK cells to UECC.

### Rapamycin Upregulates IL-27 Production of UECC and IL-27R Expression on NK cells

To investigate the role of rapamycin in NK cell function, we first analyzed the effect of rapamycin on the IL-27 and IL-27R expression. Treatment with rapamycin directly increased the secretion of IL-27 in Ishikawa, RL95-2, and KLE cells *in vivo* (Figure 3A) and the expression of IL-27 in cytokeratin (CK)7<sup>+</sup>UECC of cancer lesions from Ishikawa-xenografted nude mice (Figure 3, B and C). Additionally, control and IL-27<sup>over</sup> Ishikawa, RL95-2, and KLE cells were stimulated with rapamycin and then co-cultured with NK cells, respectively (Figure 3D). As shown, IL-27<sup>over</sup>UECC also increased gp130 but not WSX-1 expression on NK cells compared with control UECC (Figure 3, E and F). Rapamycin-pretreated UECC could upregulate the expression of gp130 and WSX-1 on NK



**Figure 3.** Rapamycin upregulates IL-27 production of UECC and IL-27R expression on NK cells. (A) After treatment with rapamycin (100 nM) for 48 hours, the secretion levels of IL-27 from UEC cells UECC (Ishikawa, RL95-2, and KLE cells) were evaluated by ELISA. (B, C) The expression of IL-27 in cytokeratin (CK)7<sup>+</sup>UECC of cancer lesions from Ishikawa-xenografted nude mice ( $n = 8$  mice/group) after intraperitoneal injection of rapamycin (2 mg/kg) or PBS for 3 weeks was analyzed by FCM. (D-F) The mock or IL-27<sup>+</sup> UECC (Ishikawa, RL95-2, and KLE cells) were prestimulated with or without rapamycin (100 nM) for 48 hours and co-cultured with NK cells for 24 hours, and then the IL-27 receptors (gp130 and WSX-1) in these NK cells were evaluated by FCM. (G-I) Nude mice of 4 to 5 weeks of age were inoculated subcutaneously under the scruff on day 0 with 200  $\mu$ l of Ishikawa cells ( $2 \times 10^6$ ) (IL-27<sup>over</sup> Ishikawa cells on the right or NC Ishikawa cells on the left). After 7 days, Ishikawa-xenografted nude mice ( $n = 8$  mice/group) were intraperitoneally injected rapamycin (2 mg/kg) or PBS for 3 weeks. Moreover, human NK cells from peripheral blood labeled with PKH67 were adoptively transferred to Ishikawa-xenografted nude mice every 7 days for the first 2 weeks. After 3 weeks, the tumors were collected for FCM, TEM and IHC. (H, I) FCM was used to analyze the IL-27 receptors on PKH67-labeled NK cells in cancer lesions of Ishikawa-xenografted nude mice. NC: Ishikawa cells were transfected with negative control lentivirus; IL-27<sup>over</sup>: Ishikawa was transfected with the lentiviruses expressing human IL-27. Data are expressed as mean  $\pm$  SEM. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , and \*\*\*\* $P < .0001$ .



**Figure 4.** The increased cytotoxicity of NK cells to UEC cells induced by rapamycin is partly dependent on IL-27. (A, B) After co-culture with or without rapamycin (100 nM)-pretreated mock or IL-27<sup>+</sup> Ishikawa cells, the expression of KIR2DL1, KIR3DL1, NKG2D, CD16, NKp46, Granzyme B, and perforin in NK cells ( $n = 6$ ) was analyzed by FCM. (C, D) The expression of KIR2DL1, KIR3DL1, NKG2D, CD16, NKp46, Granzyme B, and perforin in PKH-67 labeled NK cells was treated as described in Figure 3G. Data are expressed as mean  $\pm$  SEM. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , and \*\*\*\* $P < .0001$ .

cells and amplify the stimulatory effect of IL-27 on WSX-1 on NK cells in the co-culture system (Figure 3, E and F). Subsequently, Ishikawa-xenografted nude mice were studied, and the results of *in vivo* trials further echoed these phenomena (Figure 3, G-I).

**The Increased Cytotoxicity of NK Cells to UECC Induced by Rapamycin Is Partly Dependent on IL-27**

Next, we analyzed the effect of rapamycin on NK cell function and found that rapamycin-pretreated Ishikawa cells significantly elevated the expression of CD16, NKG2D, NKp46, perforin, and Granzyme B and downregulated the expression of KIR3DL1 and KIR2DL1 in co-cultured NK cells (Figure 4, A and B). In addition, these effects in the group of rapamycin pretreated IL-27<sup>over</sup> Ishikawa cells were further augmented (Figure 4, A and B). As shown, both rapamycin and IL-27 could enhance the cytotoxic activity of NK cells *in vivo* (Figure 4, C and D).

**Rapamycin/IL-27 Restricts the Growth of UECC by Increasing Cytotoxicity of NK Cells**

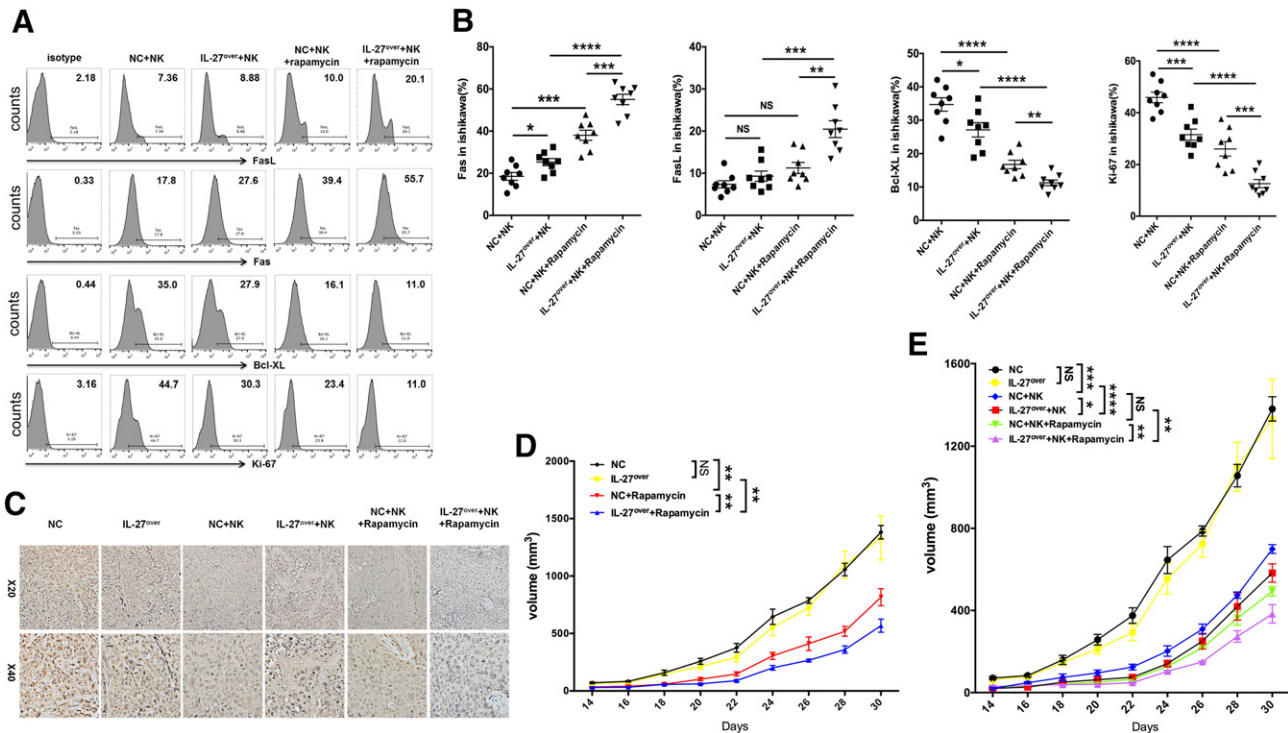
Subsequently, we investigated the effect of rapamycin and IL-27 on UEC. Along with the transfer of human peripheral NK cells, both overexpression of IL-27 and treatment with rapamycin led to the increase of Fas and B-cell lymphoma (Bcl)-xL and the decrease of Ki-67 and PCNA in UECC of cancer lesions from Ishikawa-xenografted nude mice, especially in the combination of rapamycin and IL-27 overexpression group (Figure 5, A-C). However, IL-27 overexpression did not influence the expression of PCNA (Figure 5C) and the tumor volume of UEC (Figure 5D) in the non-NK cells-transferred UEC mice model.

Transferring with NK cells led to the decrease of PCNA expression (Figure 5C) and UEC volume (Figure 5, D and E), and the overexpression of IL-27 and treatment with rapamycin could amplify these effects mediated by NK cells (Figure 5, C-E).

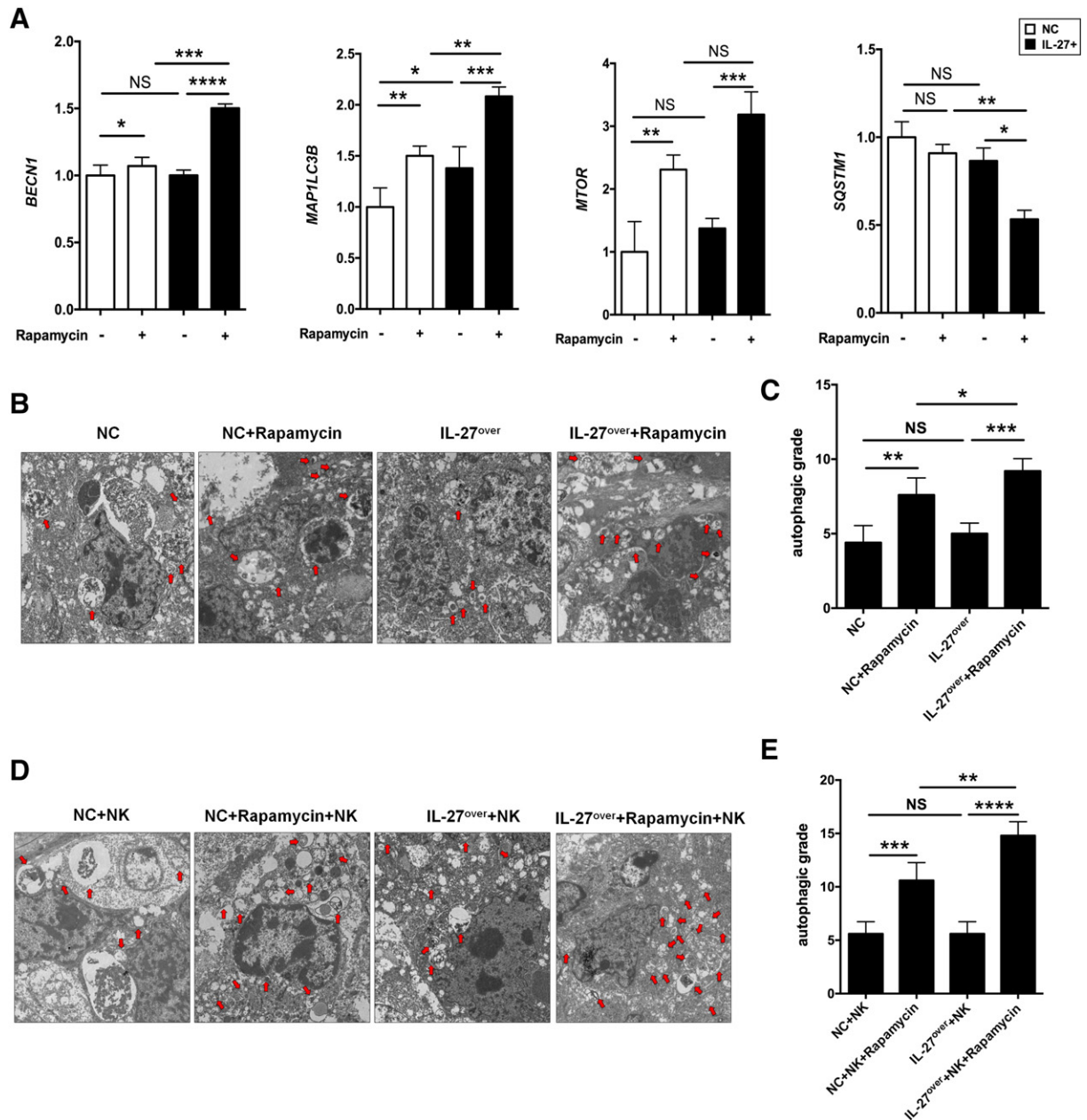
Taken together, these results suggest that rapamycin enhances cytotoxic activity of NK cells and further mediates anti-UEC activation and restricts the progression of UEC partly by upregulating the expression of IL-27 from UECC and the expression of IL-27R on NK cells.

**IL-27 Improves Rapamycin-Induced Autophagy of UECC**

In view of the important role of rapamycin in promotion of cell autophagy [21], we investigated whether rapamycin regulated the levels of autophagy-related genes and autophagy in UEC and, if so, whether this effect was dependent on IL-27. As a “platform protein,” Beclin-1 in humans is encoded by the *BECN1* gene and is essential in the induction process of autophagy [22], which provides a framework for other autophagy-related (Atg) proteins and class III phosphoinositide 3-kinase (Vps34), which initiate macroautophagic activity together. Microtubule-associated protein 1A/1B light chain 3B (hereafter referred to as LC3) is a protein that in humans is encoded by the *MAP1LC3B* gene. LC3 is the most widely used marker of autophagosomes [23]. mTOR is a kinase that in humans is encoded by the *MTOR* gene, which is a major repressor of autophagy [24,25]. Sequestosome 1 is a protein encoded by the *SQSTM1* gene. Also known as the ubiquitin-binding protein p62, the level of p62 is accumulated when autophagy is inhibited [26]. As expected, rapamycin resulted in the elevation of autophagy-related genes *BECN1*, *MAP1LC3B*, and *MTOR* (Figure 6A) and autophagy levels



**Figure 5.** Rapamycin/IL-27 restricts the growth of UEC cells by increasing cytotoxicity of NK cells. (A, B) The expression of Fas, FasL, Bcl-XL, and Ki-67 in Ishikawa cells from the tumor of xenografted nude mice ( $n = 8$  mice/group) as described in Figure 3G was analyzed by FCM. (C) IHC was used to evaluate the expression of PCNA in tumors of xenografted nude mice ( $n = 8$  mice/group) as described in Figure 3G. (D, E) The xenografted nude mice ( $n = 8$  mice/group) were described in Figure 3G, and the tumor volume was measured every other day. Data are expressed as mean  $\pm$  SEM. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , and \*\*\*\* $P < .0001$ .



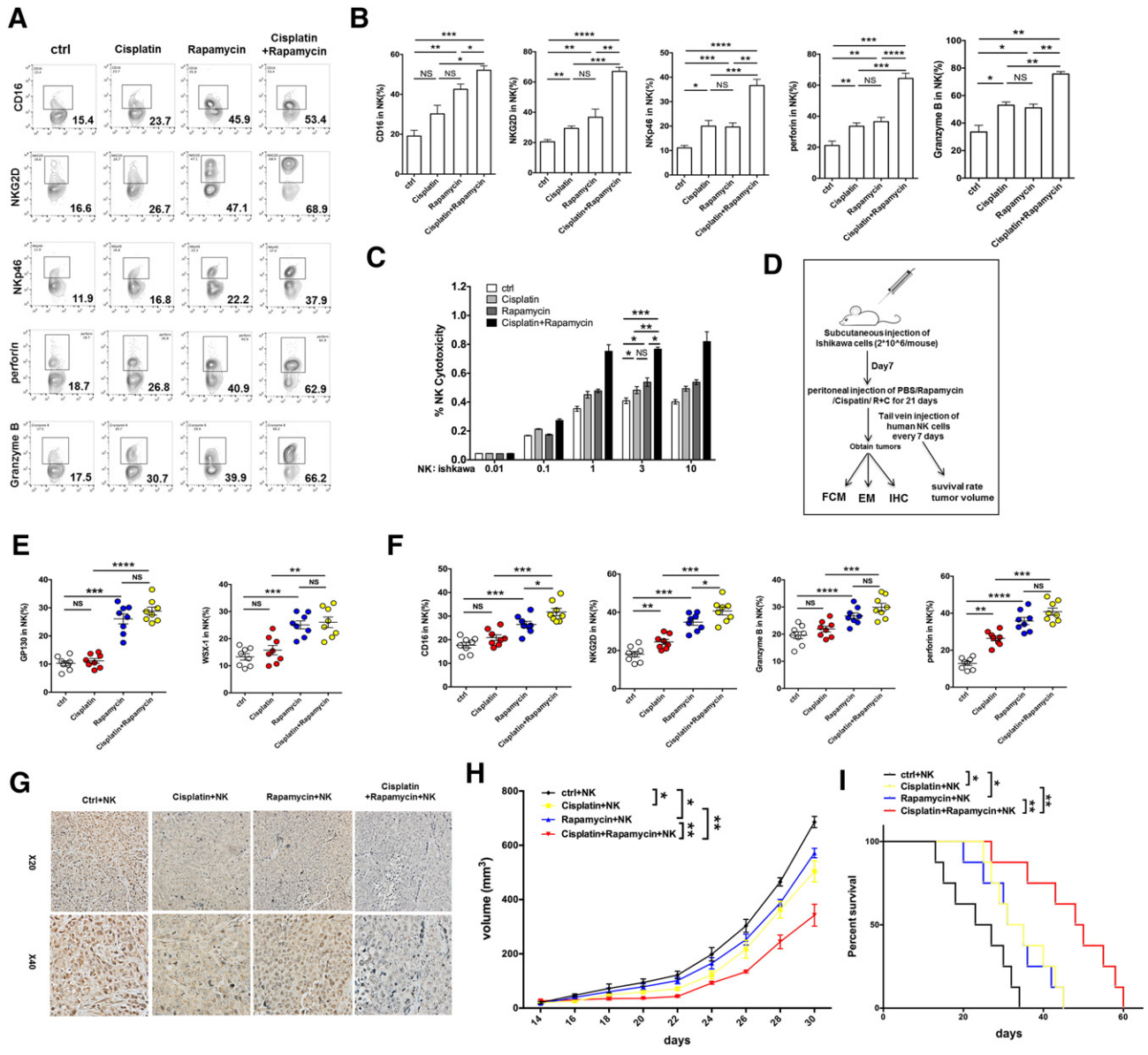
**Figure 6.** IL-27 improves rapamycin-induced autophagy of UICC. (A) The transcription level of *BECN*, *MAP1LC3B*, *MTOR*, and *SQSTM1* in Ishikawa cells treated as described in Figure 4A was analyzed by RT-PCR. (B, C) TEM was used to analyze the autophagy levels of cancer lesions from Ishikawa (NC or IL-27<sup>over</sup>)-xenografted nude mice ( $n = 8$  mice/group), which were peritoneally injected with or without rapamycin and (D, E) transferred with peripheral human NK cells. The autophagy grade was based on the average number of autophagic vacuoles and autolysosome/per cell visible by TEM. Data are expressed as mean  $\pm$  SEM. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , and \*\*\*\* $P < .0001$ .

(Figure 6, B and C) in cancer lesions from Ishikawa-xenografted nude mice; however, we had not observed the change of autophagy-related genes and autophagy levels after overexpression of IL-27 in UEC (Figure 6, A-C). Only the combination of IL-27 and rapamycin downregulated the transcription level of *SQSTM1* (Figure 6A). Additionally, regardless of whether NK cells were present, IL-27 improved the regulation of rapamycin on *BECN*, *MAP1LC3B*, *MTOR*, and autophagy levels in UEC (Figure 6, A-E). These data suggest that IL-27 enhances rapamycin-mediated autophagy activation in UEC. However, the induction of autophagy by rapamycin is not necessarily dependent on IL-27.

#### Rapamycin Combined with Cisplatin Exerts an Anti-UEC Effect by IL-27-Mediated NK Cell's Activation

To further identify the potential synergistic anti-UEC effect of rapamycin/IL-27 and cisplatin, we collected Ishikawa cells after treatment with or without cisplatin and found that the level of IL-27 in these two groups of Ishikawa cells had no significant difference (data not shown). Similarly with rapamycin, cisplatin-pretreated Ishikawa cells upregulated the expression of NKG2D, NKp46, NKp44, Granzyme B, and IFN- $\gamma$  in co-cultured NK cells (Figure 7, A and B; Supplementary Figure 2). Compared with rapamycin alone or cisplatin alone, the combination of rapamycin and cisplatin had a





**Figure 7.** Rapamycin combined with cisplatin exerts an anti-UEC effect by IL-27-mediated NK cell's activation. (A, B) After co-culture with rapamycin (100 nM) and/or cisplatin (10  $\mu$ M)-pretreated Ishikawa cells, the expression of KIR2DL1, KIR3DL1, NKG2D, CD16, NKP46, Granzyme B, and perforin in NK cells ( $n = 6$ ) was analyzed by FCM. (C) NK cells ( $n = 6$ ) were treated as described Figure 7A and then co-cultured with fresh Ishikawa cells for the cytotoxicity assay at different E/T ratios (1:100, 1:10, 1:1, 3:1, or 10:1) for 3 hours. (D-I) Ishikawa-xenografted nude mice were intraperitoneally injected with rapamycin (2 mg/kg) and/or cisplatin (2 mg/kg) or PBS for 3 weeks. PKH67-labeled human peripheral NK cells were then adoptively transferred to these Ishikawa-xenografted nude mice ( $n = 8$  mice/group) every 7 days for the first 2 weeks. After 3 weeks, the tumors were obtained for FCM (E, F) to analyze the expression of GP130, WSX-1, NKG2D, CD16, Granzyme B, and perforin in PKH67-labeled NK cells, and IHC (G) to evaluate the expression of PCNA in cancer lesions. (H) In addition, the tumor volume was measured every other day. (I) Kaplan-Meier survival curves of these Ishikawa-xenografted nude mice. Data are expressed as mean  $\pm$  SEM. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .01$ , \*\*\*\* $P < .001$ , and \*\*\*\*\* $P < .0001$ .

stronger regulation on NK cell function-related molecules (Figure 7, A and B; Supplementary Figure 2) and cytotoxic activity to UECC (Figure 7C). The results of trials *in vivo* (Figure 7D) showed that cisplatin also had no regulatory effect on IL-27R on NK cells, which is different with rapamycin (Figure 7E). In addition, we found that the combination of rapamycin and cisplatin led to the highest level of NK cells activation (Figure 7F) and UEC autophagy (Supplementary Figure 3), the lowest expression of PCNA in UEC (Figure 7G), the minimum of UEC volume (Figure 7H), and the longest survival rate of Ishikawa-xenografted nude mice (Figure 7I). These findings

provide evidence of a synergistic anti-UEC effect between rapamycin and cisplatin; what is different is that the roles of the former and latter are independent and partly dependent on IL-27, respectively.

**Discussion**

Rapamycin acts as a specific inhibitor of mTOR, a serine/threonine kinase that appears to be downstream of the phosphoinositide 3-kinase/Akt signal pathway [27]. mTOR exists in two complexes, that is, mTOR complex 1 and 2 (mTORC1 and mTORC2). A complex of rapamycin and the FK506 binding protein 12 can bind to

mTOR1 and inhibit its activity for controlling cell responses to environmental cues [28–30]. The signaling of mTOR modulates the activation of two major downstream components: eIF4E-binding protein 1 and p70S6K [31,32]. mTOR plays a key role in regulating the transcription initiation of many genes that are related to the process of oncogenic transformation and cancer progression [33,34]. Rapamycin is also a lipophilic macrolide antibiotic that was initially developed as a fungicide and immunosuppressant [35]. For example, rapamycin can inhibit myeloid DC differentiation, maturation, and function [6,36]. However, the role and mechanism of rapamycin in immune regulation of the UEC microenvironment were not well understood. Here, we found that rapamycin could enhance the killing activation of NK cells to UEC possibly through upregulation of IL-27 expression.

The level of IL-27 was gradually decreased along with the decrease of the differentiated degree of UEC, suggesting that the level of IL-27 may be a good prognosis marker for UEC patients. Our and other studies have shown that the inflammatory environment also leads to the accumulation of IL-27 [19,37]. TGF- $\beta$  downregulates IL-27 production of monocytes [19]. In addition, estrogen can stimulate IL-27 secretion by monocytes [19]. Therefore, the low expression of IL-27 in KLE cells may be caused by the negative expression of estrogen receptor  $\alpha$  [38]. The endocrine immune microenvironment of UEC should be involved in modulating IL-27 expression in UEC, and the detailed role and mechanism for these processes require further studies. Rapamycin not only upregulated the expression of IL-27 in UECC but also increased the expression of IL-27Rs (WSX-1 and gp130) on NK cells. Interestingly, overexpression of IL-27 also enhanced gp130 level of UECC through an unknown mechanism.

In our current study, IL-27 could not directly inhibit the proliferation, apoptosis, and growth of UEC. We found that IL-27 secreted by UECC could activate the cytotoxic activity of NK cells *in vitro* and *in vivo*; this effect should be attributed to the upregulation of CD16, NKG2D, NKp46, perforin, and Granzyme B and the downregulation of KIR3DL1 and KIR2DL1. This regulatory effect of IL-27 on NK cells partly echoed a previous report [37]. IL-27 has been shown to have antiproliferative activities that inhibit the growth and metastasis of murine melanoma, and the activation of signal transducers and activators of transcription-1 signaling is essential for the antiproliferative effects mediated by IL-27 [39]. However, apart from minor direct effects on the proliferation and survival of tumor cells, the major antitumor role of IL-27 had been reported to rely on antiangiogenic effects on surrounding endothelial cells and fibroblasts [18,40,41]. In addition, several studies have reported that IL-27 inhibits tumor growth and metastasis through CD8<sup>+</sup> T cells, NK cells, and DCs [42–44]. Therefore, the regulatory effect of IL-27 on UEC is possibly dependent on other immune cells with a different molecular mechanism.

Autophagy has opposing, context-dependent roles in cancer, and interventions to both stimulate and inhibit autophagy have been proposed as cancer therapies [45]. Contrary to previous report [46,47], cisplatin can induce the autophagy of UEC with the transfer of NK cells *in vivo*. Rapamycin also promoted UEC autophagy regardless of the presence of NK cells. Sharma et al. reported that IL-27 inhibited IFN- $\gamma$ -induced autophagy in macrophages [48]. Here, we had not observed the regulation of IL-27 on UEC autophagy; however, IL-27 could amplify the induction of autophagy triggered by rapamycin. The specific mechanism needs further study.

In addition to synergistic effects on autophagy induction, cisplatin and rapamycin also had a synergistic effect on activation of NK cells and suppression of UEC progression. These effects of cisplatin were independent on IL-27.

Collectively, based on our findings and other reports, it can be concluded that rapamycin can enhance the cytotoxic activity of NK cells and restrict the progression of UEC by upregulating IL-27 and IL-27Rs expression, and furthermore synergistically act with cisplatin as an anti-UEC. Moreover, rapamycin possibly modifies inflammatory conditions and/or promotes anti-UEC responses by IL-27-mediated immune regulation. Therefore, rapamycin should be proposed as a combination therapy for UEC for cooperating with traditional cytotoxic agents (*e.g.*, cisplatin), especially in patients with abnormally low expression of IL-27 or poorly differentiated UEC.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neo.2017.11.003>.

### Disclosure of Potential Conflicts of Interest

The authors declare no financial or commercial conflict of financial interests.

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