

# CD147 promotes IKK/I $\kappa$ B/NF- $\kappa$ B pathway to resist TNF-induced apoptosis in rheumatoid arthritis synovial fibroblasts

Yue Zhai<sup>1,2</sup> · Bo Wu<sup>2</sup> · Jia Li<sup>1</sup> · Xi-ying Yao<sup>2</sup> · Ping Zhu<sup>1</sup> · Zhi-nan Chen<sup>2</sup>

Received: 8 April 2015 / Revised: 23 July 2015 / Accepted: 7 August 2015 / Published online: 23 August 2015  
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## Abstract

TNF is highly expressed in synovial tissue of rheumatoid arthritis (RA) patients, where it induces proinflammatory cytokine secretion. However, in other cases, TNF will cause cell death. Considering the abnormal proliferation and activation of rheumatoid arthritis synovioblasts, the proper rate of synovioblast apoptosis could possibly relieve arthritis. However, the mechanism mediating TNF-induced synovioblast survival versus cell death in RA is not fully understood. Our objective was to study the role of CD147 in TNF downstream pathway preference in RA synovioblasts. We found that overexpressing TNF in synovial tissue did not increase the apoptotic level and, *in vitro*, TNF-induced mild synovioblast apoptosis and promoted IL-6 secretion. CD147, which was highly expressed in rheumatoid arthritis synovial fibroblasts (RASFs), increased the resistance of synovioblasts to apoptosis under TNF stimulation. Downregulating CD147 both increased the apoptotic rate and inhibited I $\kappa$ B kinase (IKK)/I $\kappa$ B/NF- $\kappa$ B pathway-dependent proinflammatory cytokine secretion. Further, we determined that it was the extracellular portion of CD147 and not the intracellular

portion that was responsible for synovioblast apoptosis resistance. CD147 monoclonal antibody inhibited TNF-induced proinflammatory cytokine production but had no effect on apoptotic rates. Thus, our study indicates that CD147 is resistant to TNF-induced apoptosis by promoting IKK/I $\kappa$ B/NF- $\kappa$ B pathway, and the extracellular portion of CD147 is the functional region.

## Key messages

- CD147 inhibits TNF-stimulated RASF apoptosis.
- CD147 knockdown decreases IKK expression and inhibits NF- $\kappa$ B-related cytokine secretion.
- CD147's extracellular portion is responsible for apoptosis resistance.
- CD147 antibody inhibits TNF-related cytokine secretion without additional apoptosis.

**Keywords** Rheumatoid arthritis synovial fibroblast · TNF · NF- $\kappa$ B · CD147 · Apoptosis

✉ Ping Zhu  
zhuping@fmmu.edu.cn

✉ Zhi-nan Chen  
zhinanchen@fmmu.edu.cn

<sup>1</sup> Department of Clinical Immunology, PLA Specialized Research Institute of Rheumatology & Immunology, Xijing Hospital, Fourth Military Medical University, 15 West Changle Road, Xi'an 710032, China

<sup>2</sup> Cell Engineering Research Center & Department of Cell Biology, Fourth Military Medical University, 17 West Changle Road, Xi'an 710032, China

## Abbreviations

RA	Rheumatoid arthritis
OA	Osteoarthritis
TNF	Tumor necrosis factor
I $\kappa$ B	NF-kappa-B inhibitor
IKK	I $\kappa$ B kinase
CTL	Blank control
NC	Negative control
A6	With CD147 shRNA lentivirus transfection
PI	Propidium iodide
EP	Extracellular portion
IP	Intracellular portion

## Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease with severe inflammatory symptoms in joints, mostly in hands, feet, and knees [1]. RA affects approximately 1 % of the population, with high incidence in women and old people [2]. Abnormal accumulation of fibroblasts, macrophages, and lymphocytes in synovial joints finally causes the destruction of the bone and cartilage [3]. Rheumatoid arthritis synovial fibroblasts (RASFs) are regarded as direct inducers of such damage. They receive molecular signals from macrophages and lymphocytes, and they secrete metalloproteinases (MMPs), IL-6, IL-8, and granulocyte–macrophage colony-stimulating factor (GM-CSF) among others [4]. One of the most important upstream signals for RASFs is TNF, a highly expressed cytokine in both serum and synovium of RA patients [5].

TNF is a type II transmembrane homotrimer that is released in a soluble form through proteolytic cleavage by TNF-converting enzyme (TACE) [6]. Mainly secreted by synovial macrophages, TNF is considered to be an important factor in the pathogenesis of rheumatoid arthritis, which causes intractable inflammation in joints. Infliximab, a TNF antibody, has already proved its effectiveness in treating RA patients [7]. However, with different cell types, various activation states, and a variety of microenvironment factors, TNF can contribute to either proinflammatory cytokine secretion or cell death [8]. The TNF receptor is known as one of the death receptors, and it can receive death signals given by TNF ligands and further induce caspase-dependent apoptosis or caspase-independent necroptosis (a type of programmed necrosis) [9]. The apoptotic rate of RASFs under TNF stimulation could be significant, because it could both decrease the number of effective RASFs and inhibit TNF-related immune activation. With two different downstream signaling pathways, NF- $\kappa$ B-dependent or NF- $\kappa$ B-independent cell survival versus caspase-related apoptosis [10], TNF signaling could be influenced by certain other important molecules that help to ‘switch’ between different patterns [11]. Although TNF has been studied for a long time, the mechanism underlying downstream-pattern preference is still unclear. Elucidating the TNF-related cell death pathway in synovioblasts should increase our understanding of RA and may suggest treatments.

CD147, also called extracellular matrix metalloproteinase inducer (EMMPRIN) and leukocyte activation-associated M6 antigen or HAb18G, is a transmembrane glycoprotein [12]. CD147 is a receptor for cyclophilin A (CypA) and PfRh5, and it contributes to tumor metastasis, inflammation, and pathogen (plasmodium falciparum and coronavirus) infection [13–20]. Our previous works have proven that CD147 is up-regulated in RA synoviocytes, including RASFs and macrophage-like cells [21]. CD147 overexpression can increase MMP production and invasiveness in synoviocytes. In addition, CD147 on RASFs induces the upregulation of VEGF

and HIF-1, which further promotes angiogenesis in synovium and causes persistence of synovitis [22]. CD147/HAb18 monoclonal antibody can inhibit cartilage erosion and synovitis in the SCID mouse model of rheumatoid arthritis [23]. However, so far, the role of CD147 in TNF-induced RASF apoptosis has not been studied. It was reported that in hepatocellular carcinoma (HCC), CD147 inhibits HCC cell apoptosis under endoplasmic reticulum (ER) stress [24]. Others have shown that TNF is the key in the cross talk between ER stress and oxidative stress [25]. These data highlight the possibility that CD147 plays a negative role in TNF-related apoptosis.

In our report, we investigated the function of CD147 in TNF signaling pathway preference in RA synovioblasts, and further, we attempted to identify the functional region of CD147 that is responsible for this preference.

## Materials and methods

### Patients and tissue preparation

Twenty-five RA patients and 14 osteoarthritis (OA) patients were involved in the experiments (informed consent had been obtained). All the patients with RA and OA satisfied the diagnostic criteria of the American College of Rheumatology [26]. The mean age of the RA patients was 48 years, ranging from 39 to 57 years (female/male=1.78); the mean age of the OA patients was 53 years, ranging from 43 to 61 years (female/male=0.75). Synovial tissues were obtained from the RA and OA patients during joint arthroscopy in Xijing Hospital (Xi’an, China). The ethics approval for this study was granted by Ethical Committee of Fourth Military Medical University.

### Cell culture

Primary RA synovial fibroblasts (synoviocyte B) were isolated from synovium of RA patients following a manner previously described [22]. To have pure and biologically functional fibroblasts, the primary fibroblasts used here were at third to fifth passage. RASFs cultured in DMEM high-glucose medium (with 10 % fetal bovine serum) in regular culture conditions.

### shRNA lentivirus

The Trans-Lentiviral pLKO System was used to construct the stable CD147 knockdown synovioblasts RASF A6, and RASF NC was RASF transfected with lentivirus without the CD147 short hairpin RNA (shRNA) sequence. The construction of the lentivirus carrying CD147 shRNA was described previously [27]. Namely, the plasmids of Trans-Lentiviral pLKO System (psPAX2 1.125  $\mu$ g/mL, pMD2G 0.125  $\mu$ g/mL, pLKO A6/NC 1.25  $\mu$ g/mL) were transfected into HEK 293T cells, and the medium (containing lentivirus) of HEK

293T cells was collected and then cultured with RASFs. Puromycin at 4 µg/mL (Sigma, Buchs, Switzerland) was added to the medium for further selection (10 days), and the successfully transfected RASF A6 and RASF NC cells were resistant to puromycin, while the blank control group RASF CTL cells were killed by puromycin at this concentration. To obtain stable cell clones, RASF A6 and RASF NC cells were maintained in medium containing 1 µg/mL puromycin for at least 2 weeks.

### Transfection of CD147 extracellular and intracellular portions into RASF A6

The vector pcDNA 3.1 was used to carry the full length, extracellular portion, and intracellular portion of CD147. The exogenous CD147 has 269 amino acids, in which 0–229 is the extracellular portion and 206–269 is the intracellular portion (both containing transmembrane region). The CD147 shRNA sequence A6 targeted 3'-UTR region of CD147 messenger RNA (mRNA) and will not inhibit the exogenous extracellular portion and intracellular portion of CD147 expression which do not contain 3'-UTR sequence. Transfection followed the instructions for Lipofectamine 2000 (Invitrogen, Basel, Switzerland), and concentration of vectors was 5 µg/mL. Cells were tested and used 36 h after the transfection.

### Immunohistochemistry assay

Synovial tissue immunohistochemical staining was performed using a streptavidin–peroxidase kit (Zymed, San Diego, USA). The primary antibodies were anti-TNF mouse mAb (Proteintech, Wuhan, China, 1:100), anti-CD147 mouse mAb (prepared in our laboratory, 1:200, final concentration 5 µg/mL), and anti-cleaved-caspase 3 (CC3) rabbit polyclonal Ab (Cell Signaling, Danvers, USA, 1:200). After primary antibody incubation (4 °C overnight), sections were reacted with biotin-labeled goat anti-mouse/rabbit IgG, horseradish peroxidase-labeled streptavidin, and diaminobenzidine (Zymed, San Diego, USA), respectively. Then, nuclei were restained with hematoxylin. Irrelevant mouse anti-human IgG (Biolegend, San Diego, USA) and rabbit serum were used as negative controls. The TNF and cleaved-caspase 3 quantitative analysis was performed by Image-Pro Plus Version 6.0 software. Three random fields of each section were visualized. The average number of cleaved-caspase 3-positive cells in three fields was considered to be the value of CC3 positive, and the average integral optical density summary (IOD SUM) was used to measure TNF expression.

### Immunofluorescence microscopy

Cells were attached to glass coverslips for 24 h, and then the coverslips were placed in a 6-well plate. After TNF (R&D

system, Abingdon, UK) treatment for 24 h, the cells were fixed in 4 % formaldehyde in PBS, permeabilized with 0.1 % Triton X-100 for 20 min and blocked with goat serum in PBS for 1 h. The coverslips were then incubated with the CD147 (prepared in our laboratory, 1:200, final concentration 5 µg/mL) and cleaved-caspase 3 (Cell Signaling, Danvers, USA, 1:300) antibodies for 12 h. The cells were washed in PBS, incubated with their corresponding FITC and APC secondary antibodies (Thermo scientific, Rockford, USA, 1:200) for 1 h, and nuclei were stained with DAPI. Finally, the cells were detected with a Nikon fluorescence microscope.

### Flow cytometry assay

CD147 expression in the RASFs was detected with flow cytometry as previously described [21]. To assess apoptosis, TNF-stimulated and unstimulated cells were collected and washed in PBS and stained for annexin V and propidium iodide (PI) with the Annexin V-FITC Apoptosis Detection Kit (Millipore, Boston, USA) following the instructions. The cells were analyzed by FACSCalibur flow cytometry (BD Pharmingen, San Diego, USA). The data were processed using FlowJo software.

### Real-time PCR assay

After different treatments, mRNAs of RASFs were isolated by E.Z.N.A. Total RNA Kit II (OMEGA BioTek, Norcross, USA) in RNase-free conditions. Then, reverse transcription of the mRNA used the TaKaRa PrimeScript<sup>®</sup> RT reagent kit following the manufacturer's instructions. The real-time quantitative RT-PCR 25 µL system included the following: SYBR 10 µL (TaKaRa, Otsu, Japan), primers 1 µL each (forward and reverse primers), cDNA template 3 µL, and ddH<sub>2</sub>O 10 µL. GAPDH mRNA was used to normalize the target mRNA. The relative quantity in each experiment was automatically calculated by comparative quantitation mode of MxPro qPCR system software. At least three independent experiments were performed and analyzed. Relative expression is the ratio to control mRNA. Primer sequences: huGAPDH forward 5'-GCACCGTCAAGGCTGAGAAC-3', huGAPDH reverse 5'-TGGTGAAGACGCCAGTGGGA-3'; huCD147 forward 5'-ACTCCTCACCTGCTCCTTGA-3', huCD147 reverse 5'-GCCTCCATGTTTCAGGTTCTC-3'; huIL-6 forward 5'-CAATAACCACCCCTGACCCAA-3', huIL-6 reverse 5'-TCTGAGGTGCCCATGCTACA-3'; huIL-1β forward 5'-AAACAGATGAAGTGCTCCTTCCAGG-3', huIL-1β reverse 5'-TGGAGAACACCACTTGTGCTCCA-3'; huIL-8 forward 5'-CATACTCCAAACCTTTCCACCCC-3', huIL-8 reverse 5'-TCAGCCCTCTTCAAAAATTCTCCA-3'; huCD147EP forward 5'-CTTGAATGACAGCGCCACAG-3', huCD147EP reverse 5'-GGAGTACTCTCCCACTGGT-3'; and huCD147IP forward 5'-GACGTCCTGGATGATG



ACGA-3', huCD147IP reverse 5'-CGGACGTTCTTGCCTTTGTC-3', huIKK forward 5'-GGAAGTACCTGAA CCAGTTTGAG-3', huIKK reverse 5'-GCAGG ACGATGTTTTCTGGCT-3'.

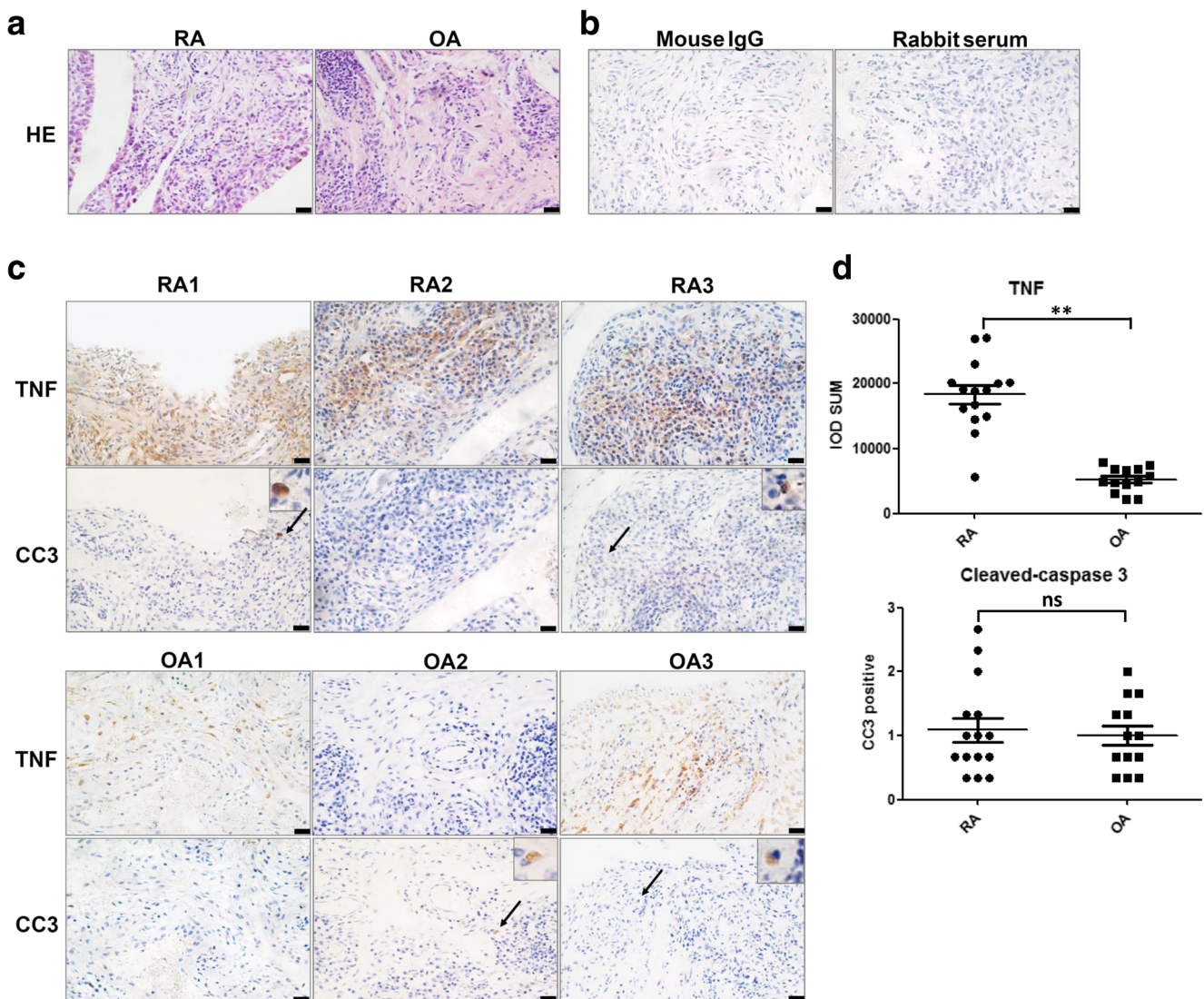
### Cytokine ELISA

Serum-free medium from RASF samples was collected and centrifuged at 10,000g for 5 min. IL-6 release from RASFs into the culture medium was directly measured with a Human IL-6 ELISA Kit and Human IL-1 $\beta$  ELISA Kit (DAKEWE, Shenzhen, China) according to the manufacturer's instruction. Optical density was determined with a Bio-Rad Microplate reader, and absorption was measured at 450 nm. A standard

curve for each measurement was established using a cytokine standard provided by the ELISA Kit.

### Western blot assay

RASF CTL, RASF NC, and RASF A6 cells treated with TNF or TPCK were collected using 100  $\mu$ L RIPA (Biotime, Shanghai, China) with protease inhibitor and phenylmethanesulfonyl fluoride (PMSF, Biotime, Shanghai, China) on ice. After 20 min 13,000g centrifugation at 4  $^{\circ}$ C, supernatant protein concentrations were determined by bicinchoninic acid (Thermo scientific, Rockford, USA) protein assay. A 10 % SDS-PAGE gel was used to separate proteins, and the proteins were transferred to PVDF membrane. Blots were probed for 12 h at 4  $^{\circ}$ C with primary antibodies diluted in



**Fig. 1** TNF and cleaved-caspase 3 expression in synovial tissues of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). **a** HE staining of RA and OA synovial sections. **b** Mouse IgG and rabbit serum were used as isotype controls. **c** Representative sections of RA1,

RA2, RA3, OA1, OA2, and OA3 synovial tissue specimens stained for TNF and cleaved-caspase 3. Positive cleaved-caspase 3 staining (black arrow) indicated apoptotic cells. **d** Statistical results of TNF and cleaved-caspase 3 stains. Scale bar=20  $\mu$ m. \*\* $p$ <0.01/2. ns not significant



Tris-buffered saline (TBS) containing 5 % nonfat milk (1:2000 dilution for CD147 antibody (final concentration 1 μg/mL), 1:50 dilution for β-tubulin antibody (final concentration 1 μg/mL), 1:1000 dilution for RelA, NF-κBI (p50), IκB, and IKK antibodies (Proteintech, Wuhan, China). Then, secondary antibodies (goat anti-mouse antibody, goat anti-rabbit antibody, 1:3000, Thermo scientific, Rockford, USA) were incubated with the PVDF membrane for 1 h at room temperature. The visualization was realized with an enhanced chemiluminescence (ECL)/Western blotting detection system.

**Statistical analysis**

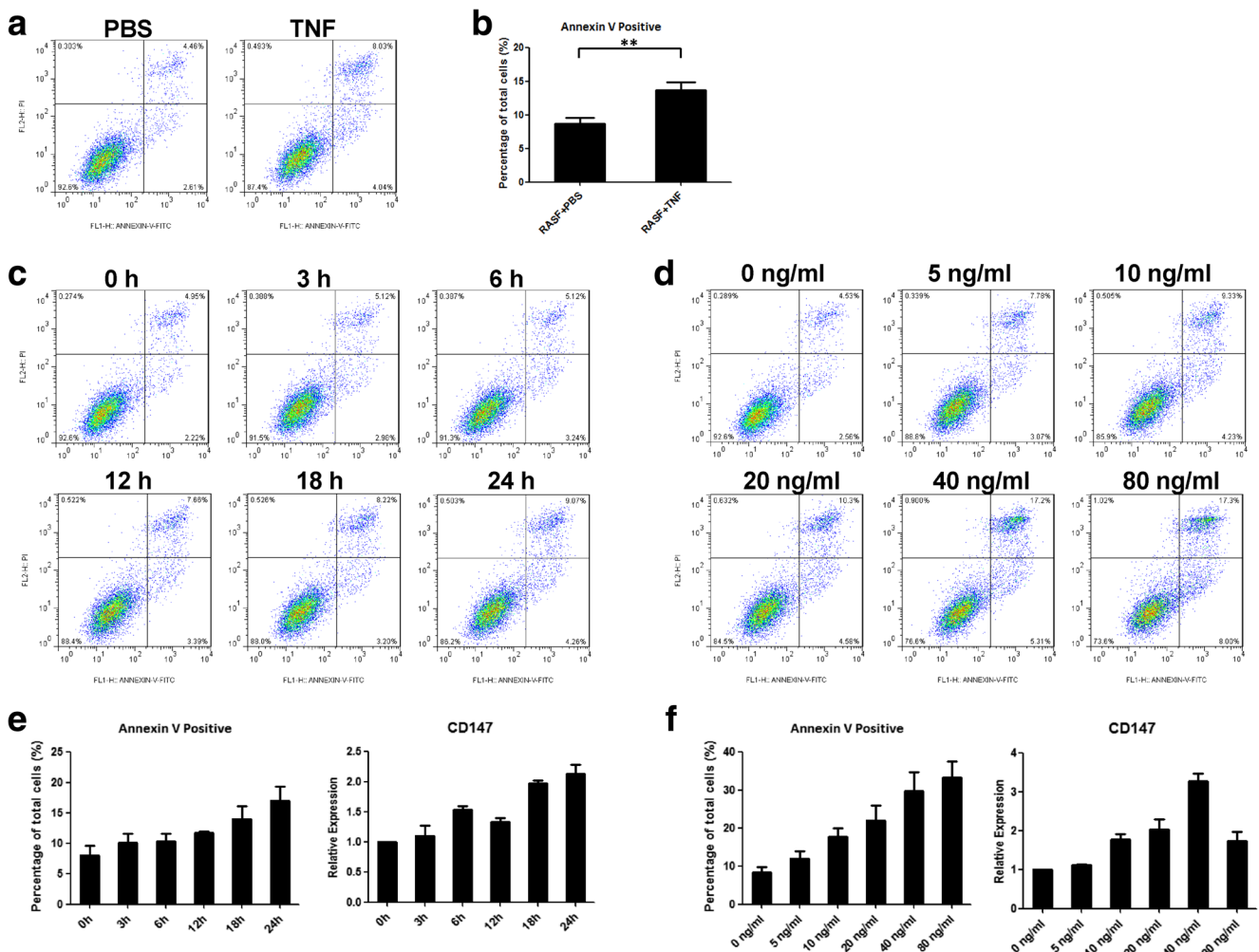
The experiments were performed independently at least three times. The data were expressed as the mean value±SD. Unpaired and paired *t* tests or one-way analysis of variance

followed by Dunnett’s posttest (for subgroup analyses) was used. All of the analyses were made using the SPSS 19 statistical software package, and the statistical images were processed by GraphPad Prism 5.0 software. Bonferroni’s correction was applied for the number of hypotheses made (*n*), and differences were considered to be statistically significant when  $p < 0.05/n$ .

**Results**

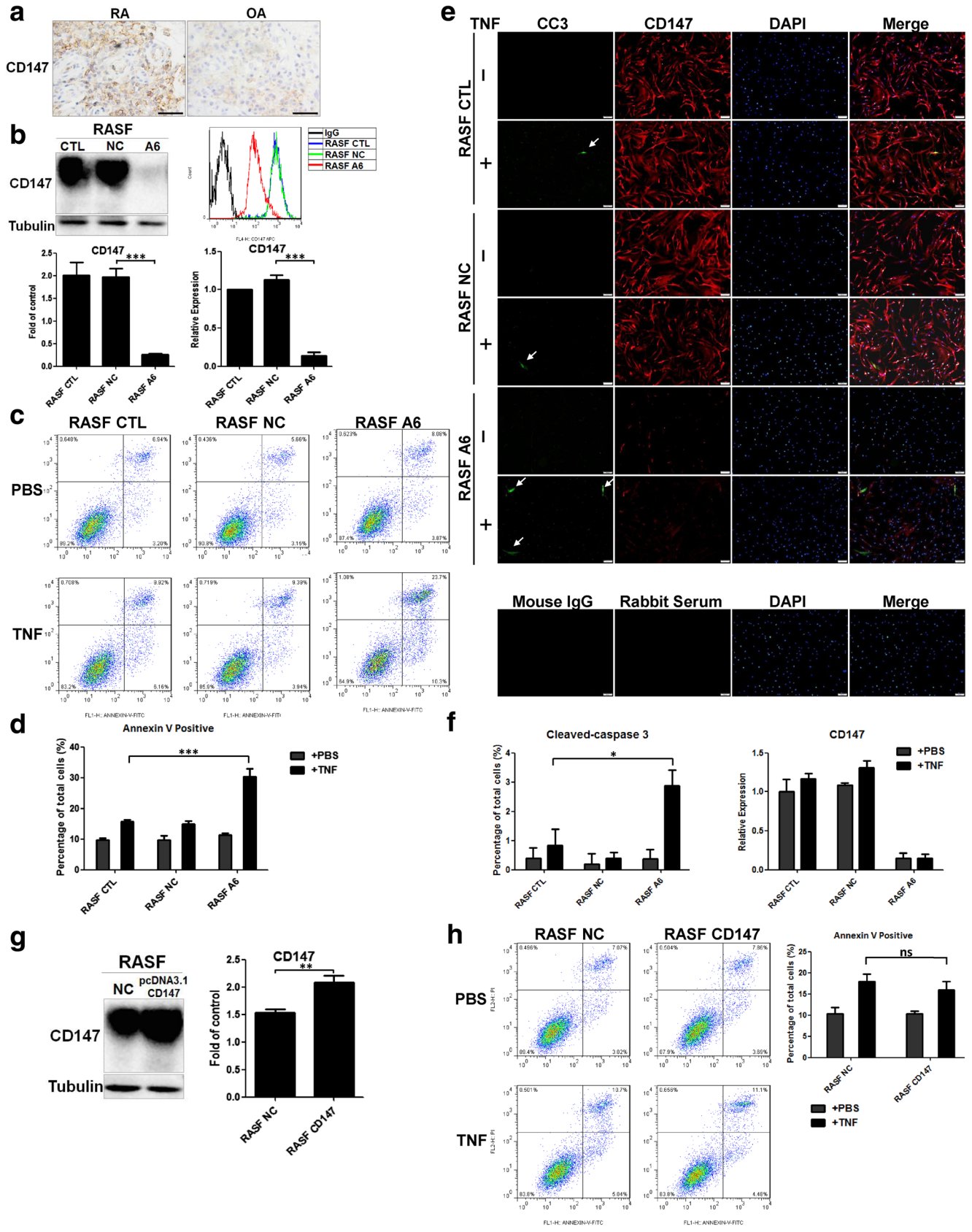
**Cells in RA synovium are resistant to TNF-induced apoptosis**

To study apoptosis in RA patients’ synovium, we first monitored TNF expression in RA synovium tissue. HE staining



**Fig. 2** TNF could induce apoptosis in synovioblasts. **a** After treating synovioblasts with 10 ng/mL TNF for 24 h, the annexin V-positive apoptotic rate increased slightly. **b** Statistical results of TNF-induced apoptosis. **c** Representative results indicating apoptosis under TNF stimulation at different time points (0, 3, 6, 12, 18, and 24 h). **d** Representative results indicating RASF apoptosis with different

concentration of TNF stimulation (0, 5, 10, 20, 40, and 80 ng/mL). **e** Statistical results of annexin V positivity rates and CD147 expression after treating with TNF for different times. **f** Statistical results of annexin V positivity rates and CD147 expression with different concentration of TNF stimulation. \*\* $p < 0.01/2$



◀ **Fig. 3** CD147 downregulation facilitated TNF-induced synovioblast apoptosis. **a** CD147 was overexpressed on RA synoviocytes (RA,  $n=4$ ; OA,  $n=4$ ), scale bar=20  $\mu\text{m}$ . **b** Lentivirus transfection successfully knocked down CD147 mRNA and protein expression (approximately 10 % of original expression) as proven by real-time PCR, western blot, and flow cytometry. **c, d** Representative flow cytometry results and statistical analysis of TNF-induced (10 ng/mL, 24 h) apoptosis in RASF CTL, RASF NC, and RASF A6 cells. Annexin V-positive dots indicating that CD147 assisted synovioblast apoptosis resistance under TNF stimulation. **e, f** Representative immunofluorescence pictures and statistical analysis of TNF-induced (10 ng/mL, 24 h) apoptosis (CC3 positive, white arrow) and CD147 expression, scale bar=100  $\mu\text{m}$ . **g** After transfection of full-length CD147, the expression of CD147 in RASFs was measured by western blot. **h** Representative flow cytometry results and statistical analysis of TNF-induced (10 ng/mL, 24 h) apoptosis in RASF NC and RASF CD147. \* $p<0.05/n$ ; \*\* $p<0.01/n$ ; \*\*\* $p<0.001/n$ . ns not significant

displayed pathologic changes in RA and OA synovial tissues (Fig. 1a). For immunohistochemistry, irrelevant mouse anti-human IgG and rabbit serum were used as negative controls (Fig. 1b). Sections from different patients showed that TNF was overexpressed in RA synovium but remained relatively low level in OA (Fig. 1c, d). Staining for cleaved-caspase 3 (CC3) revealed no obvious differences between RA and OA synovium tissues (Fig. 1c, d), indicating that overexpressed TNF in RA synovium did not cause extra apoptosis.

### TNF induces mild RASF apoptosis

In vitro, we examined TNF-induced synovioblast apoptosis. After treating with TNF (10 ng/mL) for 24 h, apoptotic RASFs (annexin V positive) increased to 1.5 times their normal level (Fig. 2a, b). Then, we assessed apoptosis in RASFs treated with TNF over a time course. The rates of apoptotic RASFs fluctuated at the beginning and reached their maximum value at 24 h (Fig. 2c). As the TNF concentration increased (from 0, 5, 10, 20, 40, to 80 ng/mL), the apoptotic RASFs accordingly increased (Fig. 2d). Real-time PCR results demonstrated that CD147 mRNA expression changed after TNF treatment and were highest at 24 h (Fig. 2e, f).

### CD147 assists synovioblast apoptosis resistance under TNF stimulation

To further analyze the mechanism of TNF-related synovioblast apoptosis resistance, we detected CD147 expression in RA and OA synovial tissue (Fig. 3a). Immunohistochemical staining revealed upregulated CD147 in RA synovium. We used lentivirus to knockdown CD147 expression in the RASFs. Western blot and flow cytometry results indicated that the lentivirus disabled CD147 protein expression (RASF A6) and real-time PCR showed the downregulation of CD147 mRNA (RASF A6) (Fig. 3a). RASF NC refers to lentivirus transfection without inhibition of CD147

expression, and RASF CTL represents RASF without lentivirus transfection.

We next treated RASF CTL, NC, and A6 cells with TNF at 10 ng/mL for 24 h. CD147 downregulation (RASF A6) significantly enhanced TNF-induced apoptosis, whereas for RASF CTL and RASF NC cells, apoptosis remained at a relatively low rate (Fig. 3c, d). An immunofluorescence assay indicated that CD147 fluorescence was markedly lower in RASF A6 and CC3 stain illustrated that CD147 downregulation significantly enhanced TNF-induced apoptosis (Fig. 3e, f).

What's more, we transfected full-length CD147 pcDNA 3.1 (RASF CD147) and negative control (RASF NC) plasmids to primary RASFs, and western blot results indicated the successful CD147 overexpression (Fig. 3g). After transfection, TNF-induced apoptosis was not significantly different between RASF NC and RASF CD147 (Fig. 3h).

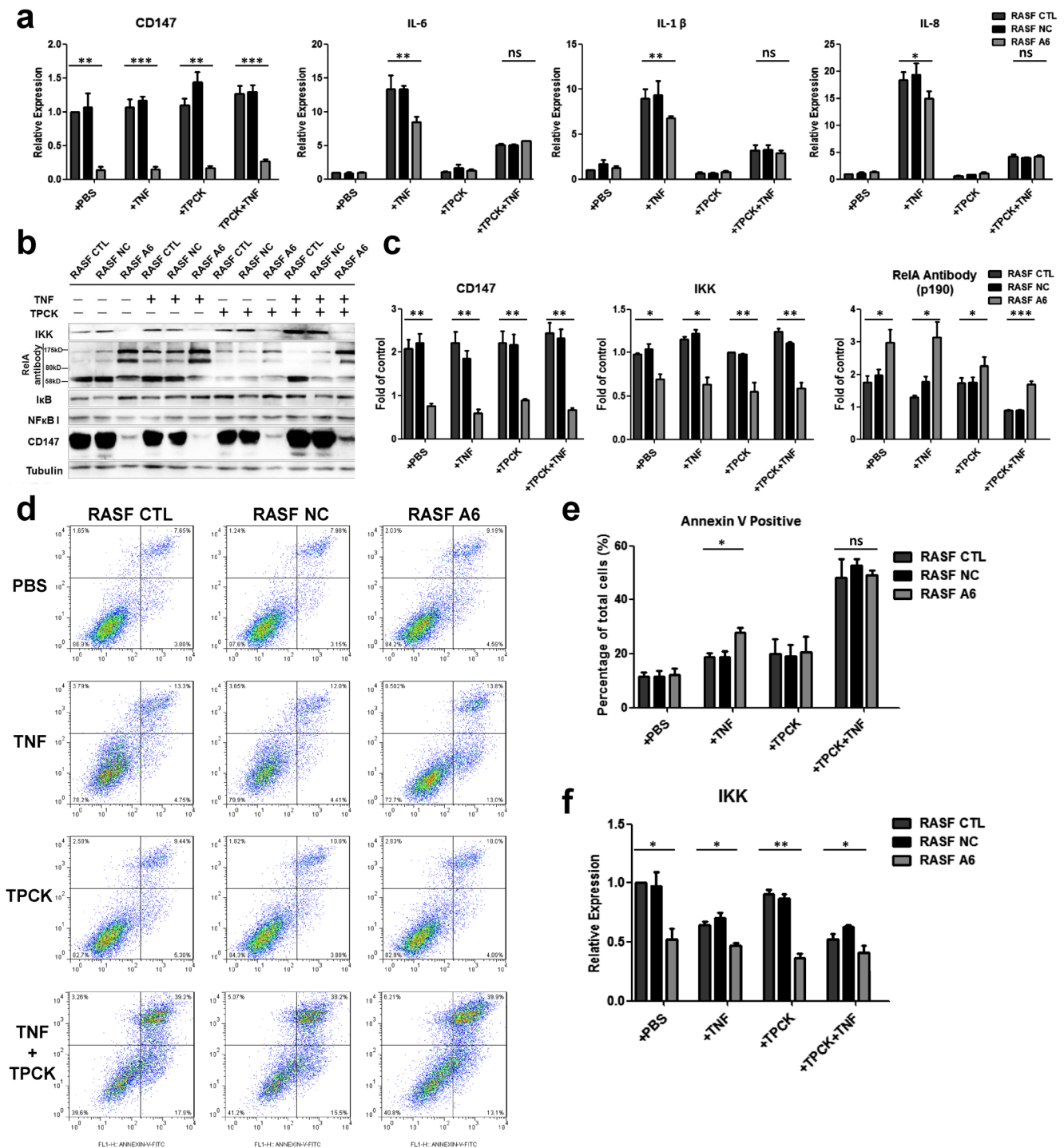
### Downregulated CD147 inhibits NF- $\kappa$ B pathway-dependent proinflammatory cytokine secretion

After testing CD147's contribution to TNF-induced apoptosis resistance, we further examined the relationship between cytokine expression and CD147 expression in TNF-treated and *N-p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK, a NF- $\kappa$ B inhibitor, final concentration 50  $\mu\text{M}$ , Sigma, Buchs, Switzerland)-treated synovioblasts. Real-time PCR results demonstrated that CD147 expression decreased after lentivirus transfection, and CD147 downregulation (A6 group) inhibited IL-6, IL-8, and IL-1 $\beta$  mRNA expression after TNF treatment while revealed no obvious difference when treating with both TNF and the NF- $\kappa$ B inhibitor TPCK (Fig. 4a). Western blot assay demonstrated that downregulated CD147 decreased I $\kappa$ B kinase (IKK) protein level. Meanwhile, using RelA antibody, we found three main bands around 65 kD (RelA), 115 kD (RelA-NF $\kappa$ B I complex), and 160 kD (I $\kappa$ B-RelA-NF $\kappa$ B I complex). In RASF A6, 160 kD (I $\kappa$ B-RelA-NF $\kappa$ B I complex) band was much stronger than RASF CTL and RASF NC, which indicated that CD147 probably played a role in collapse of I $\kappa$ B-RelA-NF $\kappa$ B I complex (Fig. 4b, c). In addition, CD147 downregulation (RASF A6) significantly enhanced TNF-induced apoptosis; however, downregulated CD147 made no difference in apoptotic rate when using both TNF and the NF- $\kappa$ B inhibitor TPCK (Fig. 4d, e). IKK mRNA expression was decreased in A6 group, which illustrated that CD147 positively regulated IKK transcription (Fig. 4f).

### The extracellular portion of CD147 is responsible for synovioblast apoptosis resistance

After transfecting the extracellular or intracellular portion (EP or IP) of CD147 into the RASF A6 (CD147 knockdown) cells (Fig. 5a), real-time PCR results indicated that compared to the





**Fig. 4** CD147 knockdown inhibited NF- $\kappa$ B pathway activation under TNF stimulation. **a** Relative CD147, IL-6, IL-1 $\beta$ , and IL-8 mRNA expression was measured by real-time PCR under TNF and/or TPCK stimulation. **b, c** Representative results and statistical analysis of IKK, NF- $\kappa$ B (p65, p50, and relative complex), I $\kappa$ B, and CD147 protein

expression by western blot assay under TNF and/or TPCK stimulation. **d, e** Representative results and statistical analysis of apoptosis induced by TNF and/or TPCK. **f** Relative IKK mRNA expression was measured by real-time PCR under TNF and/or TPCK stimulation. \* $p < 0.05/n$ ; \*\* $p < 0.01/n$ ; \*\*\* $p < 0.001/n$ . ns not significant

RASF A6 group, RASF A6EP cells only expressed the CD147 extracellular portion mRNA, whereas the RASF A6IP group only expressed the CD147 intracellular portion mRNA. Western bolt and flow cytometry results indicated that the

extracellular portion of CD147 successfully expressed on cell membrane (Fig. 5b); however, owing to the lack of proper antibody, we did not get the proof of intracellular portion of CD147 protein in RASF A6IP. TNF-induced apoptosis was

reduced in the RASF A6EP group but not in RASF A6IP group (Fig. 5c, d), which suggested that the extracellular portion of CD147 was responsible for the TNF-induced apoptosis resistance of RASFs.

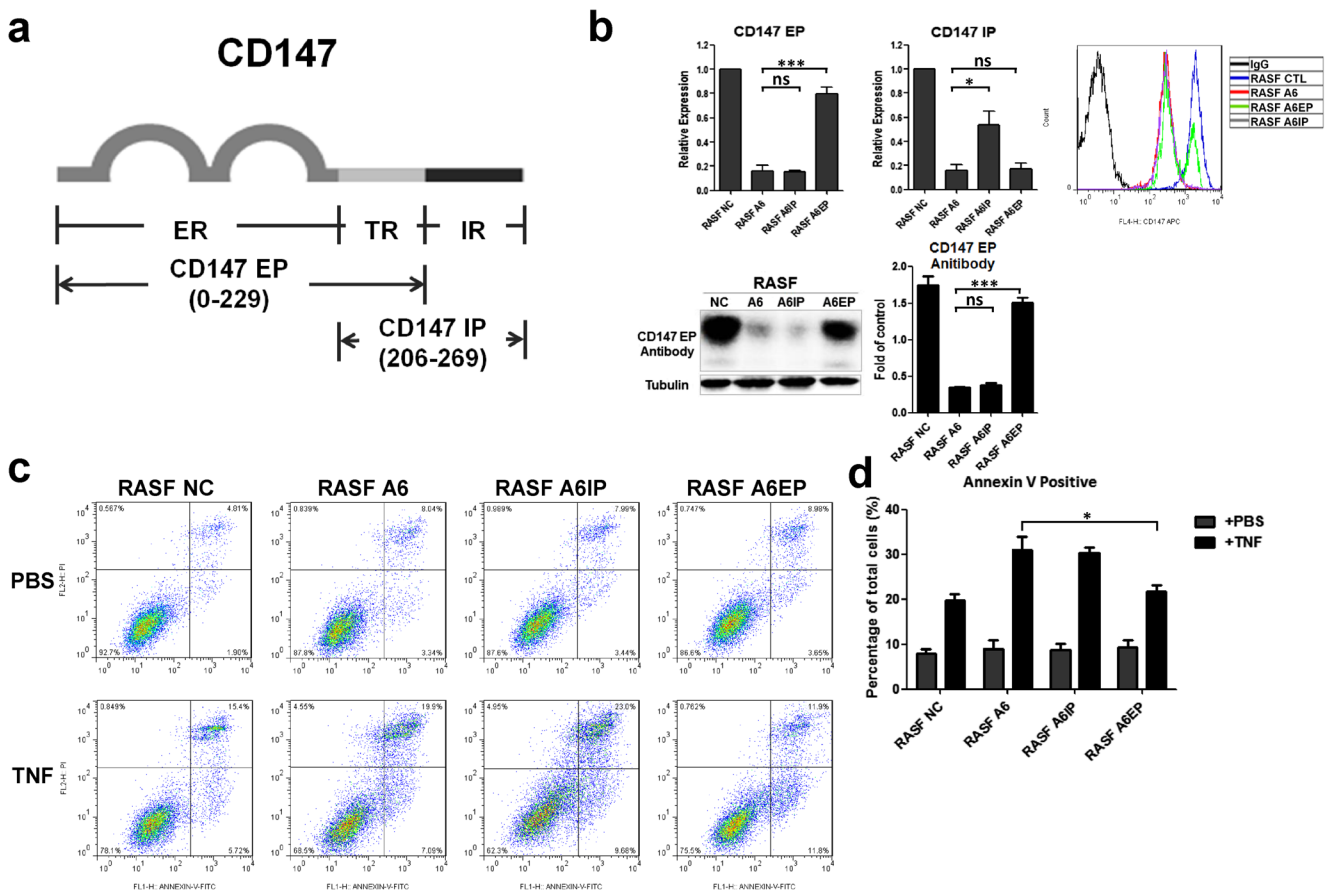
### Decreased level of proinflammatory cytokines in CD147 antibody-treated RASFs

The CD147 monoclonal antibody 5A12 (prepared in our laboratory) was previously shown to inhibit proinflammatory cytokine secretion in T lymphocytes. We added 5A12 mAb (20 μg/mL) to TNF-treated RASFs to assess the role of the antibody in both apoptosis and cytokine secretion. Apoptotic RASFs increased after the TNF treatment, but there was no obvious change after the 5A12 mAb was added (Fig. 6a, b). Real-time PCR data indicated a negative role of 5A12 mAb in TNF-induced IL-6, IL-1β, and IL-8 mRNA expression

(Fig. 6c), and ELISA results supported the inhibitory effect of 5A12 on IL-6 and IL-1β secretion (Fig. 6d).

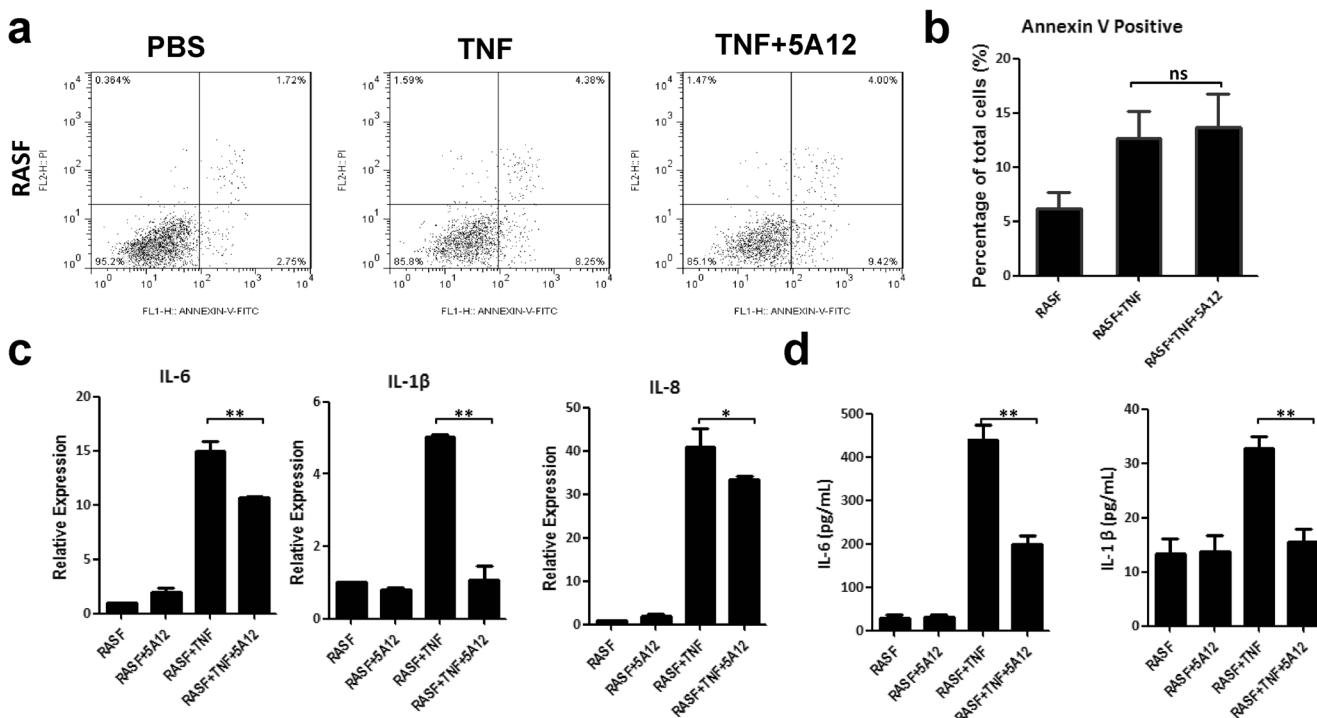
### Discussion

TNF overexpression is a significant signature of RA. Detected in serum and synovial fluid, TNF is responsible for the immune activation of lymphocytes (both B and T lymphocytes), fibroblasts, endothelium cells, and macrophages through promoting the secretion of cytokines, such as IL-6 and IL-1β, which in turn cause intractable inflammation [28]. Infliximab, a TNF antibody, has already proven its effectiveness in treating RA patients. However, in other cases, TNF is also known as an inducer of apoptosis and necroptosis. Apoptosis is dominant in physiological cell death, which plays an important role in individual development [29] and in responding to mild stress. In contrast, necroptosis, a



**Fig. 5** The extracellular portion of CD147 is responsible for synovioblast resistance to TNF-induced apoptosis. **a** Diagram of exogenously expressed CD147. *ER* extracellular region (0–206), *TR* transmembrane region (206–229), *IR* intracellular region (229–269). CD147 EP denotes the extracellular portion of CD147 (including transmembrane region); CD147 IP denotes the intracellular portion of CD147 (including transmembrane region). Genes for the exogenous proteins were carried by pcDNA 3.1 vectors. **b** Successful expression of CD147 EP and CD147 IP mRNAs in the RASF A6 cell line was verified by real-time PCR.

RASF A6EP denotes the extracellular portion of CD147 expressed in RASF A6; RASF A6IP denotes the intracellular portion of CD147 expressed in RASF A6. Western blot and flow cytometry results for CD147 EP protein expression. **c, d** Representative results and statistical analysis flow cytometry assay illustrated that only the extracellular portion of CD147 could reverse the resistance to TNF-stimulated apoptosis of RASF A6 cells. \* $p < 0.05/n$ ; \*\* $p < 0.01/n$ ; \*\*\* $p < 0.001/n$ . *ns* not significant



**Fig. 6** In RASFs, CD147 monoclonal antibody 5A12 inhibits TNF-induced proinflammatory cytokine production but does not influence apoptotic rates. **a** Representative data of TNF-induced apoptosis, which could not be amplified by 5A12 mAb. **b** Statistic analysis of TNF-induced apoptosis illustrating that 5A12 mAb did not affect TNF-

induced cell apoptosis. **c** Inhibitory role of 5A12 mAb in TNF-related cytokine production. **d** ELISA results showed that IL-6 and IL-1 $\beta$  secretion was lower in the TNF+5A12 group than in the TNF group. \* $p < 0.05/n$ ; \*\* $p < 0.01/n$ . ns not significant

programmed type of necrosis, is more common under pathological conditions [30]. Unlike necrosis, apoptotic cells produce apoptotic bodies that can be removed quickly without further damage.

In the synovium of RA patients, high expression of TNF greatly enhances the potential for TNF-related synovioblast apoptosis. The proper degree of RASF apoptosis, for one thing, can alleviate the bone and cartilage erosion by reducing the number of fibroblasts; for another, it can consume more TNF, which would otherwise cause extra immune responses. In our study, we found that TNF expression was not correlated with apoptotic rates between RA and OA synovial tissues, both of which remained at a relatively low level, and TNF only caused mild apoptosis in vitro. The key question is which TNF signaling pattern is preferred in RA synovium, the NF- $\kappa$ B-dependent (or NF- $\kappa$ B-independent) cell survival pathway or the caspase-related apoptosis pathway. The factors contributing to TNF signaling pattern preference include the concentration of TNF, the time course of TNF stimulation [31], and the expression of other important molecules. The high level and long duration of TNF expression in serum and synovium of RA patients have been proven in clinical studies [32–34]; nevertheless, little is known about the vital molecules that can switch TNF signaling between the different patterns.

CD147 is a transmembrane protein originally found in hepatocytes. As a cancer biomarker, CD147 facilitates MMP production and increases the invasiveness of hepatocellular carcinoma [35–37]. Similarly, in RA synoviocytes, CD147 promotes the erosion of the bone and cartilage. Moreover, the overexpression of CD147 in RASFs results in angiogenesis in synovium. Even though there is no indication of CD147 in TNF-stimulated RASFs, it has been confirmed that CD147 inhibits HCC cell apoptosis under ER stress. On the other hand, TNF is significant in ER stress, suggesting that CD147 may be vital to protecting cells from TNF-stimulated apoptosis.

Our results indicate for the first time that CD147 is one of the molecules causing the NF- $\kappa$ B-dependent cell survival pathway to be preferred over the apoptosis pathway under TNF activation, which finally causes the apoptosis resistance of RASF. Though previous study had testified the NF- $\kappa$ B activating role of recombinant human CD147 (as an exogenous stimulation) in cardiomyocytes [38], we for the first time revealed that CD147 downregulation in RASF could inhibit the IKK transcription and protein expression, which demonstrated that CD147 could be a direct transcription factor of IKK or a regulator of other IKK transcription factors. The possible mechanism of such inflammatory activation and apoptosis resistance is CD147 that increases IKK transcription



and protein expression, so that I $\kappa$ B-RelA-NF $\kappa$ B I complex is easier to be collapsed under TNF stimulation. As a result, in RA synovium, where there is a high level of TNF, the NF- $\kappa$ B pathway of RASF is activated; meanwhile, the apoptosis pathway is blocked.

As for the region of CD147 that functions in RASFs TNF-related inflammatory activation and apoptosis resistance, we find that the extracellular portion plays a major role. Blocking the function of CD147's extracellular portion by using the CD147 monoclonal antibody 5A12 failed to alleviate TNF-related apoptosis resistance, indicating that it is not the antigen-antibody binding site that contributes to the resistance. However, the direct molecule interacting with CD147 extracellular portion and mechanism of IKK transcriptional activation is still unknown. In our research, the intracellular portion of CD147 is not related to synovioblasts apoptosis resistance. Although CD147 intracellular portion is detected in mRNA level, we could not exclude the possibility of unsuccessful protein expression. CD147 IP is a 7-kD exogenous protein, which can be easily degraded and difficult to be detected. Therefore, the concrete mechanism of intracellular portion of CD147 still needs further exploration.

In summary, for the first time, CD147 has been proven to be responsible for the apoptosis resistance of RASFs under TNF stimulation. Our study clarifies one of the mechanisms of TNF-related RA pathogenesis, and it sheds light on a potential RA treatment targeting CD147-related inflammatory activation and apoptosis resistance.

**Acknowledgments** We thank Ling Li for generously giving CD147 shRNA-related lentiviral plasmids. This research was supported by the National Basic Research Program of China (2015CB553704) and the National Science and Technology Major Projects of New Drugs (2012ZX09103301-026).

**Conflict of interest** The authors declare no conflict of interest.

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