Interleukin 17A Promotes Cell Migration, Enhances anoikis resistance, and Favors Microenvironment for Triple Negative Breast Cancer Tumor Metastasis

Yi-Fang Tsai
Comprehensive Breast Health Center, Department of Surgery, Taipei Veterans General Hospital
Chi-Cheng Huang
Comprehensive Breast Health Center, Department of Surgery, Taipei Veterans General Hospital
Yen-Shu Lin
Comprehensive Breast Health Center, Department of Surgery, Taipei Veterans General Hospital
Chih-Yi Hsu
Department of Pathology, Taipei Veterans General Hospital
Ching-Po Huang
Faculty of Medicine, School of Medicine, National Yang-Ming University
Chun-Yu Liu
Division of Medical Oncology, Department of Oncology, Taipei Veterans General Hospital
Jen-Hwey Chiu (chiujh@ym.edu.tw)
Comprehensive Breast Health Center https://orcid.org/0000-0003-3046-6250
Ling-Ming Tseng
Comprehensive Breast Health Center, Department of Surgery, Taipei Veterans General Hospital

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Abstract

Background: Our previous study demonstrated that overexpression of multiple epidermal growth factor-like domains 11 was involved the recurrence mechanism of triple negative breast cancer (TNBC) via up-regulation of cytokines and chemokines, including IL-17A signaling pathway. However, information concerning the role of IL-17A in tumor behavior or cancer microenvironment remains lacking. The aim of this study was to investigate the role of IL-17A on TNBC recurrent mechanisms, including tumor behavior, circulating tumor cells (CTCs), and cancer microenvironment.

Methods: Using human TNBC MDA-MB-231 and MDA-MB-468 lines, the role of IL17-A was elucidated by knocked down IL-17A (D*IL-17A*) or administration of different dose of IL-17A in the culture medium. Cell proliferation, migration assay, Western blot analysis and Real-time PCR for IL-17A related signaling were evaluated. Three groups of implanted 4T1 cells in BALB/c mice were designed, namely, wild type (WT), D*IL-17A*, and WT + neutralizing IL-17 antibody (WT+Ab). Tumor weight, necrosis area, and the number of CTCs were measured. Immunohistochemistry or Western blot for CD34, CD8, and TGF-b1 were evaluated. Anoikis resistance was analyzed by live/dead stain and flow cytometry. Finally, clinic-pathological correlation between IL-17A expression and patients' outcome such as disease free survival (DFS) and overall survival (OS) was performed by Kaplan-Meier's method.

Results: Our results demonstrated that IL-17A stimulated migratory activity, but not growth rate, of MDA-MB-231/468 cells via increased Src, Rho, and COX2 expression. In vivo, there was an increased necrosis area, a decreased tumor CD34 expression and CTCs in D*IL-17A* group; while there was a decreased tumor CD34 expression, CD8(+) cells, and CTCs, but an increased TGF-b1 expression in WT+Ab group, compared to the WT group. Knocked down-IL-17A also decreased anoikis resistance in human TNBC and murine 4T1 cell lines. Kaplan-Meier's analysis disclosed a negative correlation between tumor IL-17A expression and OS in TNBC patients.

Conclusion: We conclude that IL-17A promotes migratory and angiogenic activity in the tumors, enhances anoikis resistance, and modulates the immune landscape of tumor microenvironment favoring subsequent cancer metastasis. The blockade of IL-17A might provide a co-treatment target to prevent tumor metastasis or recurrence in TNBC patients.

Introduction

Breast cancer is the most common diagnosed female cancer worldwide and ranks as the fourth most important cause of death in Taiwan [1]. Triple negative breast cancer (TNBC) represents 15% − 20% of all newly diagnosed breast cancer patients and is characterized as a large tumor size, frequently diagnosed among younger women, and a poor prognosis with high metastases potential [2–4]. Recent investigations have demonstrated that TNBC may be composed of a number of distinct subtypes [5–7]. However, early recurrent mechanisms as consequences of poor prognosis in TNBC patients remain further elucidation.

IL-17, a proinflammatory cytokine secreted by activated T cells, regulates the activities of MAPKs and NF-κB and increases the expression of IL6 and cyclooxygenase-2 (COX-2) [8]. Overexpression of IL-17A is known to be highly associated with inflammatory diseases such as multiple sclerosis, psoriasis and rheumatoid arthritis [9]. It is generally accepted that chronic inflammation may increase the risk of cancer [10], as the inflammatory response shares various molecules and corresponding signaling pathways with the carcinogenic process. The IL-17 related protumoral effects on cancer initiation are postulated through MAPK and NF-kB recruitment. By contrast, IL-17 cytokines also act as an antitumor role and the same duality makes IL-17 a “double-edged sword” [11, 12]. However, the role of IL-17A on the survival of circulating tumor cells (CTCs) is unclear.

Evidence suggests that poor prognosis in breast cancer might relate to CTCs [13, 14], and the survival of tumor cells in circulation requires three aspects: 1.resistance to anoikis, 2. escape immune surveillance, and 3. being stable under blood shearing force. The resistance to anoikis requires several signaling pathways related to anchorage- independent growth and epithelial–mesenchymal transition, including Akt, TrkB, Src, and so on [15]. Nonetheless, information concerning the role of IL-17A on the survival of CTCs remains to be elucidated.

Our previous results demonstrated that over-expression of MEGF11 in TNBC cells triggered the expression of many cytokines and chemokines, which effectively resulted in a cytokine cascade. Besides, a positive feedback between MEGF11 and IL-17A in TNBC cells was also demonstrated, which might explain the role of MEGF11 in TNBC recurrence [16]. Accordingly, the aim of this study was to investigate the role of IL-17A on TNBC recurrent mechanisms, including tumor behavior, cancer microenvironment, and the survival of CTCs.

Methods

Cell line and reagents

Human TNBC (ER-, HER2 low) cell lines MDA-MB-468 and MDA-MB-231 and the mouse mammary tumor 4T1 cell line [17], obtained from American Type Culture Collection (ATCC, Manassas, VA), were maintained in high glucose MEM, F12 MEM (NO.12400-024,Gibco, NY) and RPMI, respectively. They were supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin and cultured at 37 °C containing 5% CO2. IL-17A (InvivoGen, San Diego, CA) and anti-mouse neutralizing IL-17A antibody (InVivoMab, Bio X Cell, BE0173, Lobanon, NH) were purchased commercially.

Cell growth by trypan blue dye exclusion assay and cell cycle analysis

MDA-MB-468/231 cells were cultured in low serum medium with a cell density (1×10^5 / well) in a 12-well plate, followed by treatment of different doses of IL17A (0-, 1-, 10 ng/mL). After 1, 2, and 3 days of treatment, cells were washed with phosphate-buffered saline (PBS), pH 7.4, and trypsinized with TE buffer (Gibco/Invitrogen, New York). Suspended cells were washed and cell numbers were counted with a

hemocytometer. For cell cycle analysis, cells (2×10^5 / well) were cultured for 24 h in low serum medium (0.1% FBS), followed by another 24 h-culture, and then cells were harvested for cell cycle analysis by flow cytometry. Cell cycles were presented as percentages of cell cycle fraction, namely, sub G0/G1 phase, G0/G1 phase, S phase, and G2/M phase.

Cell migration assay

In vitro cell migration of MDA-MB-468/231 cells was performed using a trans-well system (ThinCertTM cell culture inserts, 24 well, 8 μm, Greiner bio-one, Switzerland) [18]. In brief, MDA-MB-468/231 cells were cultured in the upper chamber and different doses of IL-17A (0-, 1-, 10 ng/mL) were administrated in the lower chamber. After 4 h (MDA-MB-231) or 8 h (MDA-MB-468) of culture, the cells on the reverse side of upper chamber membrane were fixed and stained with 2% crystal violet for 10 min, then washed and photographed. The migrating cells were examined under a light microscope and quantified.

Measurement of IL-17A level

IL-17A level in the culture medium was quantified with ELISA method under the guidance of commercially available protocol (Quantikine ELISA Human IL-17A). In brief, MDA-MB-468/231 (4×10^5 / well) cells were cultured for 1 d, followed by administration of different doses (0-, 1-, 10 ng/mL) of IL-17A in low serum medium for 24 h. Then, the culture medium were discarded and cells were cultured for another 48 h, followed by harvesting the cells and the supernatants were collected, centrifuged (300 x g) for 5 min, and the final supernatants were ready for IL-17A measurement.

Western blotting analysis

Cultured cells were lysed in lysing buffer (10 mM Tris pH 7.4, 150 mM KCl, 150 mM KCl, 1% Triton X-100, phasphatase inhibitor and protease inhibitors cocktail (Complete Mini; Roche, Mannheim, Germany). Cell homogenates proteins were quantified using Bradford's method [19]. Thirty microgram of proteins were loaded to 10% SDS-PAGE and transferred to a PVDF membrane, which was blocked with 5% skimmed milk and probed with specific primary antibodies such as IL-17A (#3171, R&D Systems, Inc. Minneapolis, MN), Src (#60521, GeneTex, Inc., Irvine, CA), phosphor-Src (#6943, Cell Signaling Technology, Beverly, MA), COX-2 (#12282, Cell Signaling), phospho-AKT (#9271, Ser473, Cell Signaling), AKT (#9272, Cell Signaling), cdc42 (#2466, Cell Signaling), RhoA (#2117, Cell Signaling), RhoC (#3430, Cell Signaling), and anti-β-actin (#3700S, Cell Signaling). The blots were then washed, soaked with anti-Rabbit IgG HRP-linked secondary antibodies or anti-Mouse IgG HRP-linked secondary antibodies (Cell Signaling Technology, Beverly, MA, USA.) After 1 h hybridization, membranes were washed and developed with an ECL detection kit (Amersham Pharmacia Biotech Inc., NJ) and quantified by Multi-Gauge software analysis (Fuji Photo Film Co, Ltd, Tokyo, Japan).The β-actin was used as internal control in all experiments.

RNA extraction and reverse transcription-PCR

Total RNA were isolated by using a modified single-step guanidinium thiocyanate method [20]. (TRI REAGENT, T-9424, Sigma Chem. Co., St. Louis, MO) Complementary DNA (cDNA) was prepared from the total RNA complied with the First Strand cDNA Synthesis Kit (Invitrogen, CA). The de novo gene synthesis

changed by each treatment group was detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Primers pairs were purchased commercially[16]. The possible contamination of any PCR component was excluded by performing a PCR reaction in the absence of RT product in each set of experiment. Quantification of RNA transcripts was analyzed according to the method described previously [16]. For statistical comparison, the relative expression of specific genes mRNA was normalized to the amount of GAPD in the same RNA extracts. All samples were analyzed in triplication.

Short hairpin RNA (shRNA) transfection

Short hairpin RNA (shRNA) used to silence IL-17A genes was obtained from Academia Sinica. One day after MDA-MB-468/231 lines and murine 4T1 cells were subcultured, they (30–40% confluent) were transfected for 24 h with shRNA against IL-17A gene or non-silencing control using GenePORTER 2 transfection reagent (Genlantis, San Diego, CA) dissolved in Optimem (Invitrogen, CA) at a final concentration of 80 nM. And then, MDA-MB-468/231 and 4T1 cells were recovered for further experiments. After several passages, Δ *IL-17A* cell lines were established by puromycin selection. The transfection efficiency was validated by Western blot analysis.

Measurement of anoikis resistance

Anoikis resistance was evaluated using adherent plate and non-adherent (anoikis) plate commercially (CBA081m Cell Biolabs, San Diego, CA). Briefly, MDA-MB-231, MDA-MB-468, and murine 4T1 cells at a density of 2×10^4/well were cultured in adherent- and anoikis plates, respectively. After cells were cultured for 16 h (4T1 cells), 24 h, 48 h (MDA-MB-231), mixed live (Calcein -AM) and dead (Ethidium homodimer-1) staining solution (L3224, Invitrogen Detection Technologies, Eugene, OR) was added into the cultured medium for 30–45 min, followed by quantification of dead cells with a fluorescent microscopy. For flow cytometry analysis (MDA-MB-468), the protocols were the same as above-mentioned ones except the cell density being 2×10^5/well.

In vivo tumor xenograft

In this study, any protocols that involved experimental mice followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were approved by the Institutional Animal Committee of Taipei Veterans General Hospital (No. 2018-029). Immunodeficient NU-Foxn1nu 8-wk-old mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan, ROC). They were given *ad libitum* access to water and food and maintained in a specific pathogen-free environment with a 12 h dark-light cycle at 22–24 °C with 50% humidity. Wild type (WT) and knocked down IL-17A (Δ*IL-17A*) MDA-MB-231 cells (1×10^7 in 0.1 mL PBS) containing a luciferase gene were injected into back of immunodeficient NU-Foxn1nu mice, which gave rise to a solid tumour that was noticeable around the injection site. At the 2nd, 3rd, and 5th week, the progression of the tumor was visualized using an in vivo imaging system (IVIS).

In vivo metastasis study

Three groups of animal studies were designed, namely, WT, knocked down IL-17A (Δ*IL-17A*), and anti-IL-17A neutralizing antibody (250 μg/100μL, twice a week) (WT + anti-IL-17A antibody). Mouse mammary

4T1 cells (5×10^6 in 0.1 mL PBS) were orthotopically injected into the fat pads (left upper and/or right lower mammary glands) of 8-wk-old female BALB/c mice. The mice were sacrificed at 8 weeks thereafter or when the tumour sizes being more than 2% of the body weight. The tumor sizes and weights were measured, and the circulating tumor cells were isolated as mentioned below.

Selection of circulating mammary breast cancer 4T1 cells

After the anaesthetized 4T1 bearing mice were sacrificed, blood cells were obtained and centrifuged (400 g) in Ficoll-Paque PREMIUM (density: 1.084) (17-5446-02, GE Healthcare Bio-Sciences, Sweden) gradient medium. The peripheral mononuclear cells were subjected to primary culture. After several passages, circulating 4T1 cells were selected using 6-thioguanine (60 μM) (A4882, Sigma-Aldrich, MO, USA) [21], which was followed by quantification with a 2-hydroxyethyl agarose colony assay (A4018, Sigma-Aldrich, MO, USA). A colony was defined as a blue dye-stained group of cells that was ≥ 1 mm.

Subjects

Under the approval of the Institutional Review Board of Taipei Veterans General Hospital (2013-10-020BC), human tumour tissues from a bio-bank were obtained. From Jan. 2001 to Dec. 2010, eighty three patients diagnosed as female breast cancer in department of pathology, Taipei Veterans General Hospital were enrolled. Records such as oestrogen receptor (ER) status, progestin receptor (PR) status, HER2 status and clinical outcome, including overall survival (OS) and disease-free survival (DFS), were retrospectively reviewed. All data had been collected during clinical care and did not involve direct contact with the patients. Besides, written consent by the study subjects was waived by the Institutional Review Board. Every subject participated in this study (n = 83) was followed up for ≥ 5 years. Patients' outcome was subjected to a Kaplan-Meier survival analysis. ER or PR values of ≥ 1% were defined as positive, while ER or PR values of < 1% were defined as negative.

Immunohistochemical analysis

The protein expression levels of IL-17A from a tissue array (83 tumour samples) were assessed by immunohistochemical staining for IL-17A (Genetex, GTX49102). The results were analysed by one pathologist over a short period of time (2 months). The protein expression of IL-17A was semi-quantified and expressed as (0), < 10%, (1), 11–25%, (2), 26–50%, and (3) > 50% of the tumour cells examined.

On the animal tumour tissues, the protein expression levels of CD34, CD8, and TGF-β1 were assessed by immunohistochemical staining for CD34 (#65867, Genetex, Inc., Irvine, CA) and CD8 (#98941, Cell Signaling, Danvers, MA), and TGF-β1 (#130023, Genetex Inc., Irvine, CA) respectively. The CD8 (+) cells were quantified by counting the cells within an area of 1 mm^2, while the CD34 and TGF-β1 were quantified by Western blot analysis.

Statistic analysis

Results were expressed as the mean ± SEM. Differences between two groups were identified by Mann-Whitney U test or Student t test. Differences between groups on each time point or each dose were

identified by one-way ANOVA, followed by Dunnet's post hoc test. Statistical comparison between two independent variables was determined by Two-way ANOVA with Bonnforonni post-hoc to correct multiple comparisons. A *p*-value < 0.05 is considered statistically significant compared to vehicle or no treatment group.

DFS was defined as the time between initial breast cancer diagnosis and the date of recurrence as confirmed by pathology or an imaging study. OS was calculated from the time of initial breast cancer diagnosis to the date of death or last visit in outpatient clinic. The Kaplan–Meier method was used to estimate the cumulative incidence of RFS and OS, and log-rank tests were then used for the various comparisons (GraphPad Prism 5).

Results

Role of IL-17A in tumor behavior

Knocked-down IL-17A did not affect cell growth in human TNBC cell lines.

After IL-17A gene was silenced in TNBC MDA-MB-231 and MDA-MB-468 lines, there was no significant change of growth rate in Δ*IL-17A* group compared to the WT group, both in cell lines studies (Fig. 1a) and in tumor-bearing nude mice studies (Fig. 1b).

IL-17A did not affect cell growth but promoted cell migration in TNBC lines

When IL-17A (0-, 1-, 10 ng/mL) was administrated in the culture medium of MDA-MB-231 and MDA-MB-468, there was an increased cell number in 1- or 2-, but not 3-d culture (Fig. 2a, 2-way ANOVA). Cell cycle analysis demonstrated no significant change of cell cycle fractions such as G0G1- or S + G2M phase (Fig. 2b, one way ANOVA).

By Western blot analysis (Fig. 2c), the results showed that IL-17A dose-dependently increased p-Src, but not AKT, ERK, CDK4, and cyclin D1 protein expression in both TNBC lines (Fig. 2d) .By contrast, IL-17A (0-, 1-, 10 ng/mL) dose-dependently increased migratory activity (Fig. 3a) via activation of Rho A, CDC42, COX2, but not Rho C, in MDA-MB-231, while activation of Rho A, COX2, but not Rho C and CDC42, in MDA-MB-468 line (Fig. 3b).

IL-17A up-regulated gene expression of pro-inflammatory cytokines and chemokines

When IL-17A was administrated in the culture medium of TNBC cells, there was an increased mRNA transcripts of pro-inflammatory cytokines (TNF-α, IL-1β) and chemokines (CXCL2, CCL20) and COX2 in MDA-MB-231 and MDA-MB-468 (Fig. 3c). Of note, IL-17A up-regulated IL-17A gene expression both at

transcript level and protein level (Fig. 3d). These data suggest that IL-17A has autoregulation activity and enhances inflammatory cytokines and chemokines cascades.

In vivo metastasis study

Role of IL-17A on the tumor behaviour

Using murine mammary 4T1 cell line as in vivo metastatic model, three groups of animal studies were designed, namely, WT, knocked down IL-17A (Δ*IL-17A*), and WT + anti-IL-17A neutralizing antibody (WT + Ab). The roles of IL-17A in tumors (Δ*IL-17A* group) and around tumor microenvironment (WT + Ab group) were compared to the WT group. The results showed that there was a significant decreased tumor volume and an increased tumor necrosis area (Fig. 4a) in Δ*IL-17A* group compared to the WT group. To investigate the role of IL-17A on angiogenesis in vivo, CD34 expression by immunohistochemistry and Western blot was performed. The results demonstrated that there was a significant decreased CD34 expression (Fig. 4b) in Δ*IL-17A* and WT + Ab groups compared to the WT group.

Role of IL-17A on tumor microenvironment

When dissecting the tumors away from the mice chest wall, it was surprising to notice that the tumors of the WT + Ab group, which were no smaller in size than those of the WT group, were easily freed from adjacent chest wall without any adhesion nor increased vascularity (Fig. 4a). To investigate the role of IL-17A on immune landscape *in vivo*, CD8 and TGF-β1 expressions by immunohistochemistry and TGF-β1 expression by Western blot were performed. The results demonstrated that there was a significant decreased CD8 (+) T cells (Fig. 5a), and also decreased COX2 but increased TGF-β1 expression in Δ*IL-17A* and WT + Ab groups compared to the WT group (Fig. 5b).

Role of IL-17A on anoikis resistance in circulating tumor cells (CTCs)

The CTCs obtained from the blood of sacrificed mice were isolated, subcultured, and selected by colony assay. The results disclosed that there were significant decreased CTCs colonies in both Δ*IL-17A* group and WT + Ab group compared to the WT group (Fig. 6a). Using anoikis plate as *in vitro* model, the results showed that there was a decreased anoikis resistance via caspase 3 activation (Fig. 6b) in Δ*IL-17A* 4T1 group compared to the WT 4T1 group. Similarly, there was a decreased anoikis resistance (Fig. 6c) in Δ*IL-17A* MDA-MB-231/468 group compared to the WT MDA-MB-231/468 group. These data suggest that IL-17A plays important roles in maintaining CTCs survival against anoikis. Clinically, the expression of IL-17A correlated negatively with patients' OS (Fig. 7a), but a trend with patients' DFS (Fig. 7b).

Discussion:

Using paired (non-recurrent and recurrent) resected tumor tissues obtained from breast cancer patients, we have identified a novel gene, MEGF11, is highly associated with tumor recurrence in TNBC patients. Besides, Overexpression of MEGF11 gene up-regulates IL-17A gene expression in TNBC MDA-MB-231 and

MDA-MB-468 cells. Although IL-17A is well known to be highly associated with inflammatory diseases such as rheumatoid arthritis and psoriasis [9], the role of IL-17A in TNBC recurrence remains further elucidation. In this study, we have demonstrated that IL-17A promotes migration-promoting activity in the tumors and changes the immune landscape favoring tumor microenvironment for cancer metastasis.

While knocked down IL-17A group was designed to investigate the role of IL-17A in tumor behavior, the WT + anti-IL17A antibody group was designed to elucidate that in tumor microenvironment. Tumor necrosis, a form of cell death, is commonly associated with hypoxia, rapidly growing, and aggressive forms of breast cancer [22]. There is evidence that angiogenesis is essential for tumor growth and metastasis [23]. and IL-17A promotes tumor angiogenesis via stimulation of endothelial fatty acid β-oxidation [24]. Using CD34 as a marker for hematopoietic stem cells [25], our results showing a significantly increased necrosis area and a decreased CD34 expression in Δ*IL-17A* group compared to the WT group suggest that IL-17A enhances angiogenesis and hence against ischemic necrosis in rapid progress tumor tissue.

By contrast, when dissecting the tumors away from the mice chest wall, tumors of the WT + Ab group were easily detached from adjacent chest wall without any adhesion nor increased vascularity (Fig. 5A). A decreased CD34 expression level supports the speculation of decreased angiogenesis in this group. Recent investigations suggest that the immune system plays a dual role in tumor progression, namely, inhibiting or promoting tumor expansion. For example, TGF-β1 produced during chronic inflammation actively promotes growth and metastasis [10, 26]. Besides, the TGF-β signaling pathway plays key roles in the early development of Treg cells in the thymus [27], in maintaining the homeostasis of peripheral CD4 + T cells via by increasing IL-7Rα expression [28], and in controlling $CD8^+$ T-cell homeostasis [29]. Of note, several lines of evidence demonstrate the opposing effects of TGF-β on Th17 cells, namely, the immunoregulatory or pathogenic role of the Th17 cells in clinical settings [30, 31].

Our results that there was a decreased CD8(+) cells and an increased TGF-β expression in WT + Ab (anti-IL-17A) group lead us to speculate that TGF-β plays an immunosuppressive role leading to immune escape of the implanted 4T1 tumors, which is in agreement with previous investigation [32].

The presence of circulating tumor cells (CTCs) is well known as a mechanism of tumor metastasis or cancer recurrence [33]. Several hypotheses about CTCs evading immune surveillance have been proposed, such as adhered to platelet and myeloid-derived suppressor cells [34, 35], or mediated by PD-L1 [36]. Our results that there was significantly decreased CTCs colony number in Δ*IL-17A* and WT + anti-IL17A Ab groups compared to the WT group demonstrated a negative correlation of IL-17A expression and CTCs in the blood, which is consistent with previous findings observed in colon cancer [37].

There is consensus that persistent chronic inflammation increases the risk of cancer [10] and necrosis has proinlfammatory and tumor promoting potential [38]. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is well demonstrated to decrease incidence and mortality of many cancers [39]. IL-17E/IL-17E receptor axis may underlie TNBC resistance to EGFR inhibitors and suggest that inhibiting IL-17E or

its receptor in combination with EGFR inhibitor administration may improve TNBC management [40]. Recent studies suggest that IL-17A inhibitor might play important role in immune therapies for psoriasis and ankylosing spondylitis patients [41].

Conclusion

Our results demonstrate that IL-17A stimulates migratory activity of TNBC cells and modulates the immune landscape of tumor microenvironment favoring subsequent cancer metastasis. The blockade of IL-17A might provide a co-treatment target to prevent tumor metastasis or recurrence in TNBC patients.

Declarations

Ethics approval and consent to participate: Study protocols involving experimental mice followed ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were approved by the Institutional Animal Committee of and Taipei Veterans General Hospital (No. 2018-029).

The human study for tumour tissue utilization from the bio-bank was approved by the Institutional Review Board of Taipei Veterans General Hospital (# 2013-10-020BC).

Consent of publication: Not applicable

Data availability: All data generated or analysed during this study are included in this published article (and its Supplementary Information files)

Competing interest: We declare that we have no conflicts of interest, including financial and non-financial interests such as the following items.

- Unpaid membership in a government or non-governmental organization
- Unpaid membership in an advocacy or lobbying organization
- Unpaid advisory position in a commercial organization
- Writing or consulting for an educational company
- Acting as an expert witness

Author contributions:

Chiu, JH formed the idea. Tseng, LM and Chiu, JH contributed equally in this manuscript. Tseng, LM supervised the experiments. Liu, CY, Tseng, LM, Tsai, YF, Huang CC and Lin YS provided clinical samples and data. Huang, CP performed the experiments. Hsu, CY read the pathology slices.

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Figures

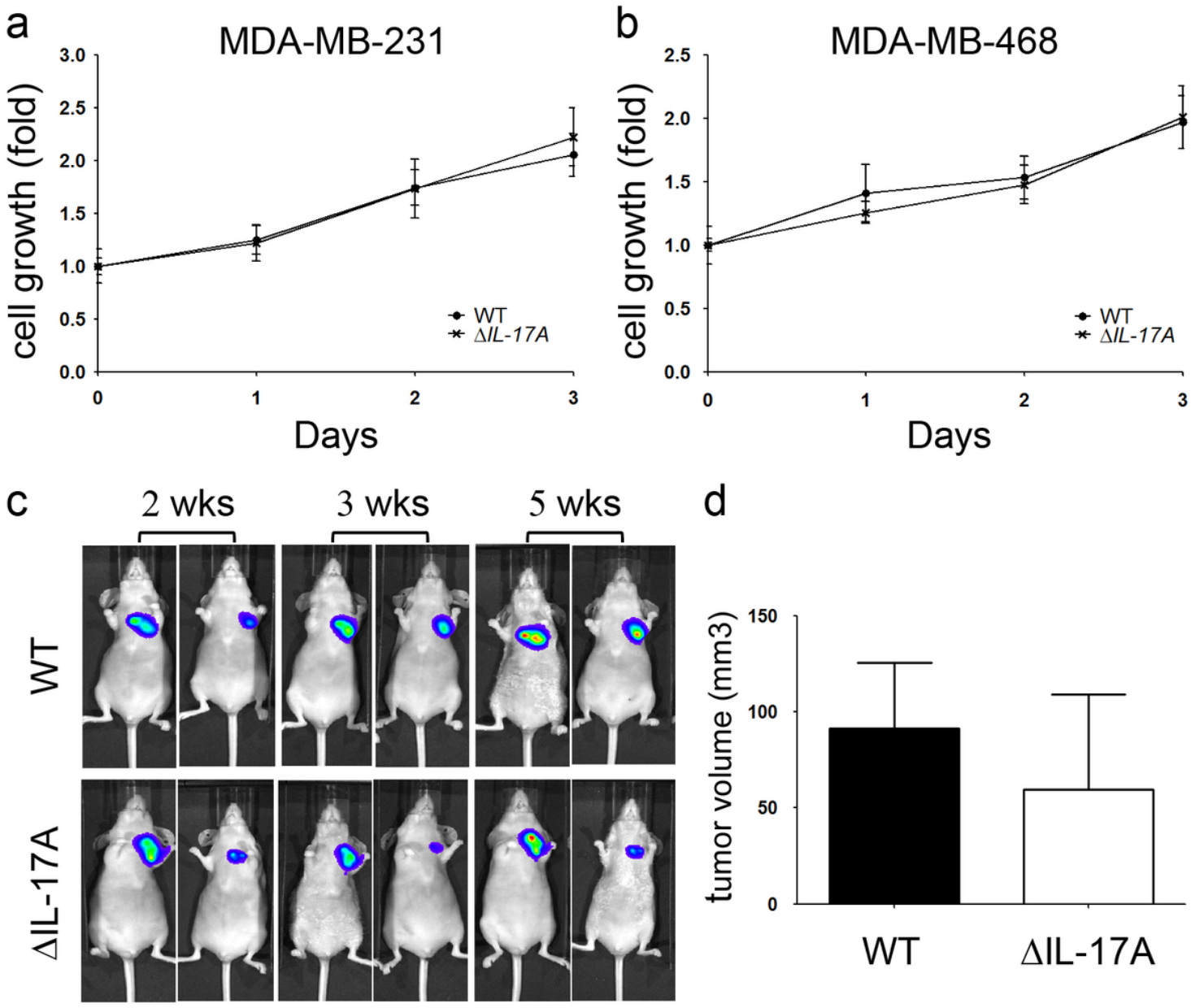

Figure 1

The role of IL-17A on cell growth in MDA-MB-231 and MDA-MB-468 cell lines. After IL-17A was knocked down in MDA-MB-231 and MDA-MB-468 cells (a), cell proliferation was evaluated by trypan blue exclusion assay. Wild type (WT) and knocked down IL-17A (ΔIL-17A) MDA-MB-231 cells (1x107 in 0.1 mL PBS) containing a luciferase gene were injected into back of immunodeficient NU-Foxn1nu mice. At the 2nd, 3rd, and 5th week, the progression of the tumors was visualized using an in vivo imaging system (IVIS) and tumour volume was quantified (b). Two-way ANOVA and Mann-Whitney U test were used for statistical analysis for cell growth in vitro and in vivo, respectively.

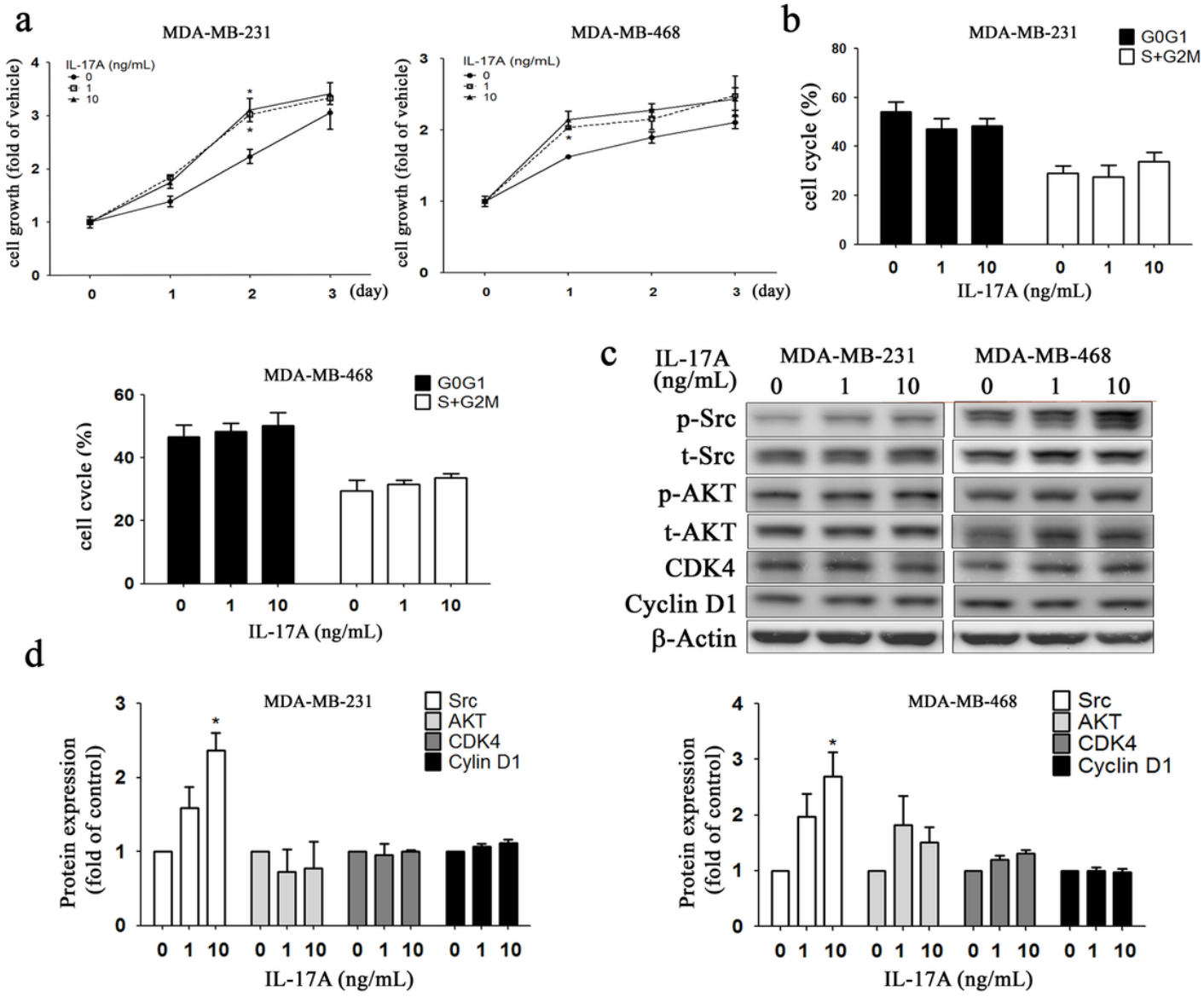

Figure 2

Effects of exogenously administration of IL-17A on cell growth in MDA-MB-231 and MDA-MB-468 cell lines. MDA-MB-468/231 cells were cultured in low serum medium with a cell density (1 x 104 / well), followed by treatment of different doses of IL17A (0-, 1-, 10 ng/mL). After 1, 2, and 3 days of treatment, cell proliferation rate was evaluated by trypan blue assay (a). For cell cycle analysis, cells (2 x 105 / well) were cultured for 24 h in low serum medium, followed by another 24 h-culture, and then cells were harvested for cell cycle analysis (b). Cell cycles were presented as percentages of cell cycle fraction, namely, sub G0/G1 phase, G0/G1 phase, S phase, and G2/M phase. Growth related signalling proteins expression, such as Src, AKT, CDK4, and cyclinD1, were analyzed by Western blot (c) and quantified (d). Asterisk indicates a p value < 0.05 (one-way ANOVA).

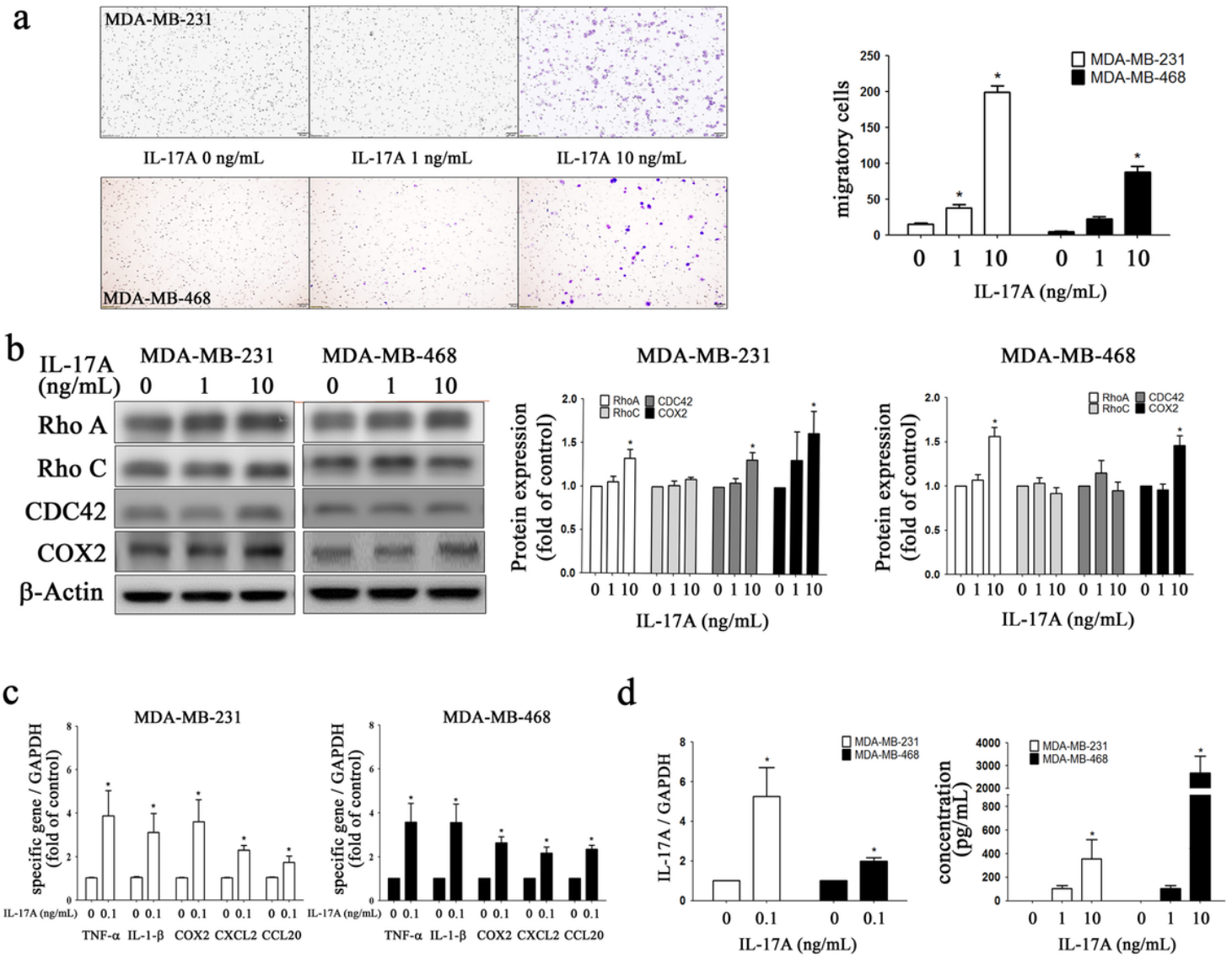

Figure 3

Effects of exogenously administration of IL-17A on cell migration, gene expression of proinflammatory cytokines and chemokines in MDA-MB-231 and MDA-MB-468 cell lines. MDA-MB-468/231 cells were cultured in a trans-well system as described in Method, followed by administration of different doses of IL17A (0-, 1-, 10 ng/mL) in the lower chamber. After 4-8 h, the migratory cells were photographed, quantified (one-way ANOVA) (a). For Western blot analysis, cells (1 x 106 / well) were cultured for 24 h in low serum medium, followed by another 24 h-culture, and then cells were harvested and probed with specific antibodies, quantified (one-way ANOVA) (b). mRNA transcripts of pro-inflammatory cytokines (TNF-α, IL-1β) and chemokines (CXCL2, CCL20) and COX2 were analyzed with real-time PCR (Mann-Whitney U test) (c). IL-17A gene expression including mRNA and protein level (d) were quantified by real-time PCR and ELISA assay (one-way ANOVA), respectively. Asterisk indicates a p value < 0.05.

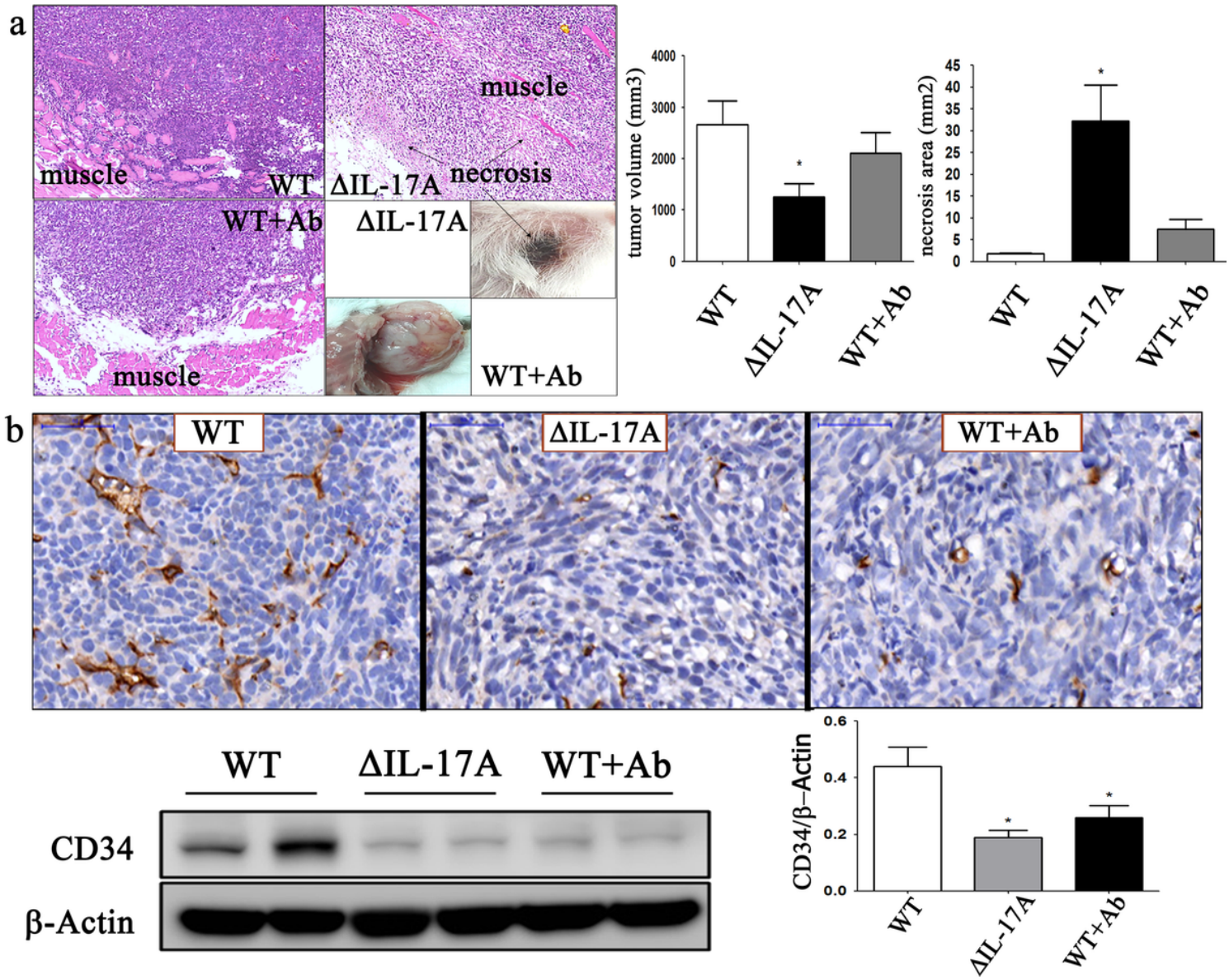

Figure 4

The role of IL-17A on in vivo implanted 4T1 cells in BALB/c mice. Three groups of 4T1 –bearing BALB/c mice were designed, namely, wild type (WT), IL-17A, and WT + neutralizing IL-17 antibody (WT+Ab). After tumor cells were implanted. Neutralizing IL-17A antibody (250 μg/100μL) was injected, twice a week, intraperitoneally in WT+Ab group. After 4 weeks after tumor cells implantation, animals were sacrificed under adequate anesthetized. Tumor weight and necrosis area were photographed and measured (a). Immunohistochemistry and Western blot for CD34 expression was quantified (b). Bar indicated 50 μm. Asterisk indicates a p value < 0.05, Mann-Whitney U test.

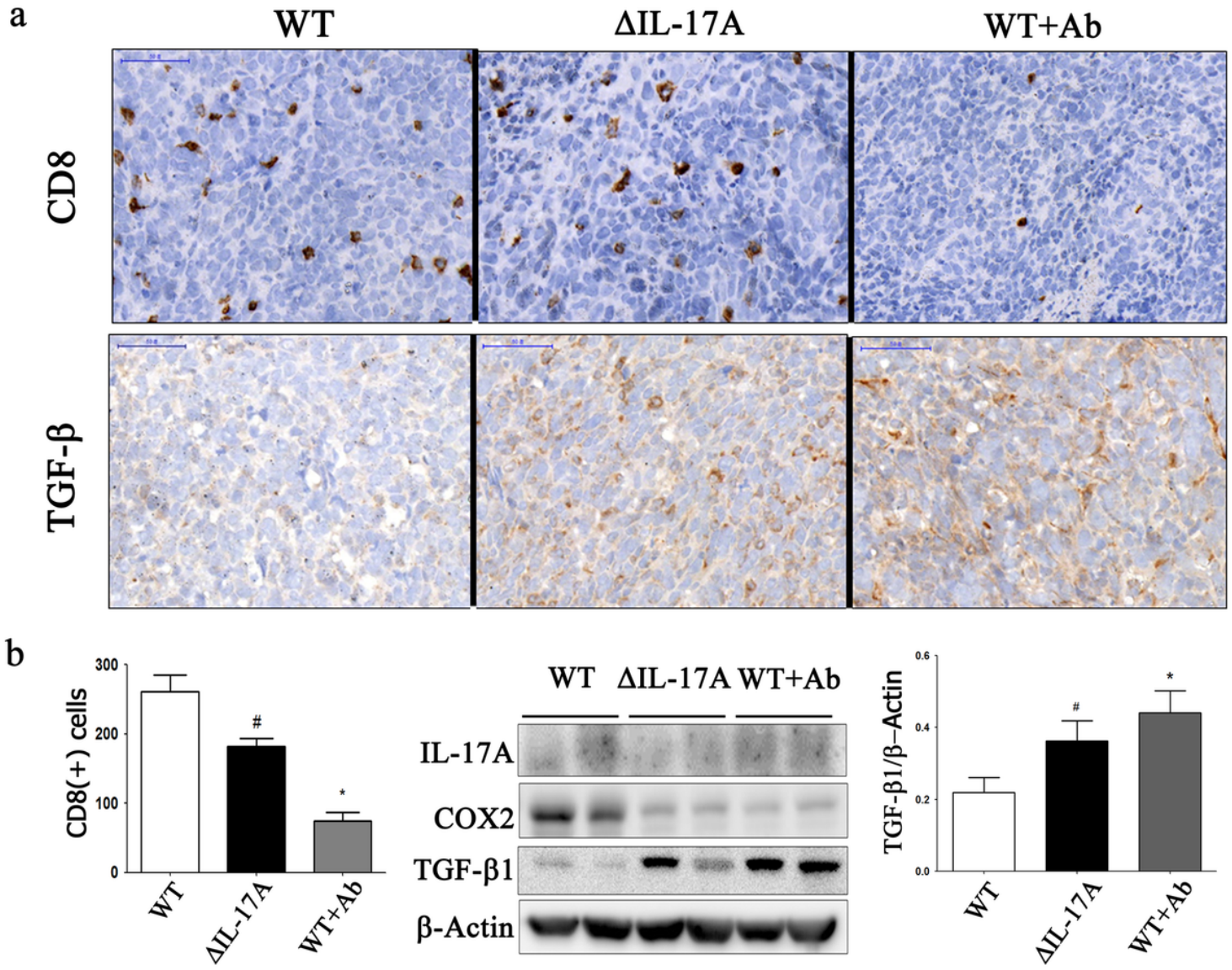

Figure 5

The role of IL-17A on CD8 and TGF-β1 4T1 cells-implanted BALB/c mice. Three groups of 4T1 –bearing BALB/c mice were designed, namely, wild type (WT), ΔIL-17A, and WT + neutralizing IL-17 antibody (WT+Ab). After tumor cells were implanted. Neutralizing IL-17A antibody (250 μg/100μL) was injected, twice a week, intraperitoneally in WT+Ab group. After 4 weeks after tumor cells implantation, animals were sacrificed under adequate anesthetized. CD8 and TGF-β1 expression were performed by immunohistochemistry (a) or/and Western blot (b). Bar indicated 50 μm. *, p value < 0.05, Mann-Whitney U test, compared to the WT group; #, p<0.05, Student t test, compared to the WT group.

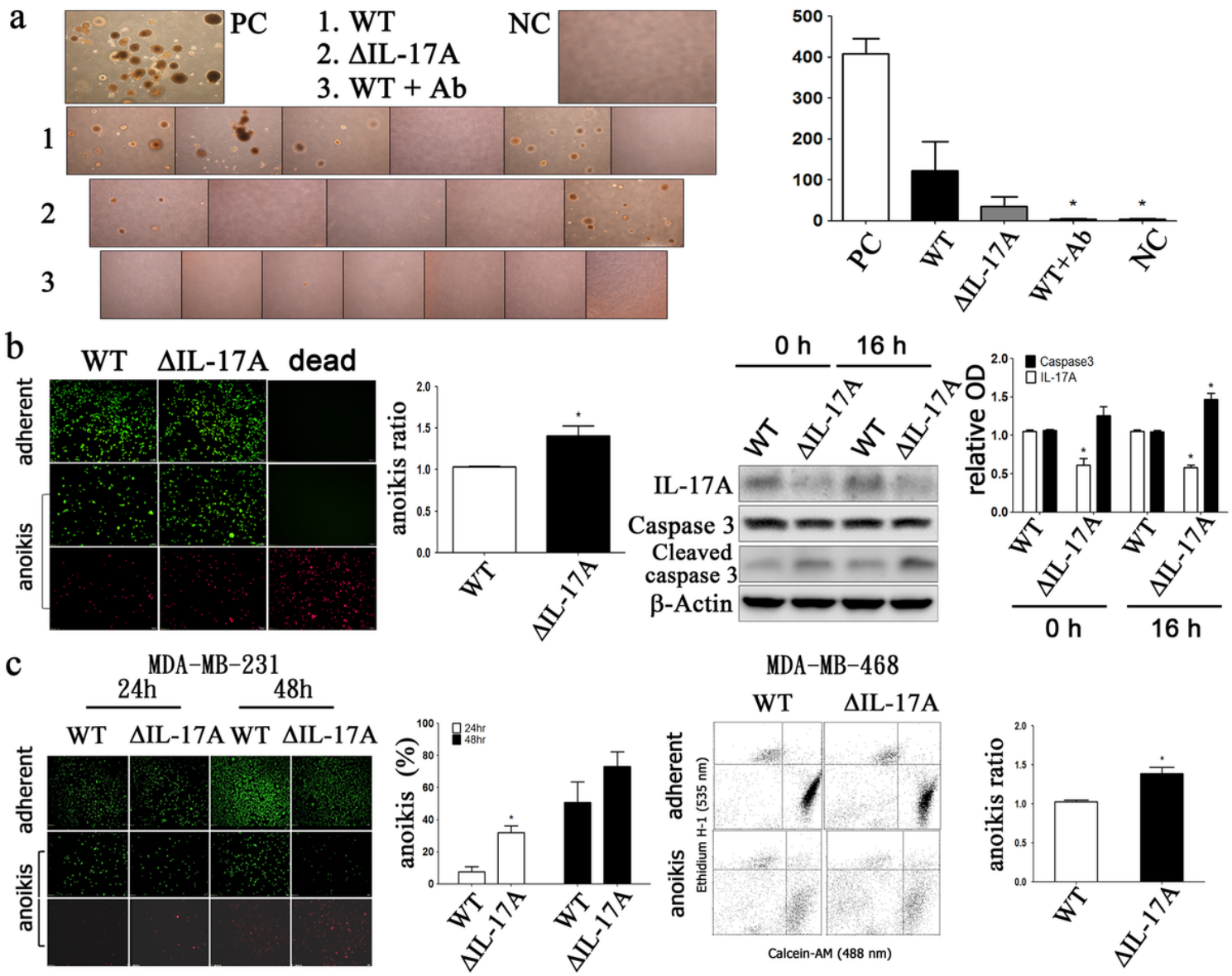

Figure 6

The role of IL-17A on circulating tumor cells (CTCs) and anoikis resistance in 4T1 cells-implanted BALB/c mice. Three groups of 4T1 –bearing BALB/c mice were designed, namely, wild type (WT), IL-17A, and WT + neutralizing IL-17 antibody (WT+Ab). After tumor cells were implanted. Neutralizing IL-17A antibody (250 μg/100μL) was injected, twice a week, intraperitoneally in WT+Ab group. After 4 weeks after tumor cells implantation, animals were sacrificed under adequate anesthetized. CTCs were isolated, sub-cultured, selected with 6-thioquanine (60 M), and ready for quantification with a 2-hydroxyethyl agarose colony assay (a). For anoikis assay, WT and IL-17A 4T1 cells (b) or MDA-MB-231/MDA-MB-468 cells (c) were seeded onto adherent and anoikis (non-adherent) plate for 24 h, followed by stained with Calcein-AM and Ethidium homodimer-1 (live and dead stain) solutions and quantified by fluorescence microscopy (MDA-MB-231), flow cytometry (MDA-MB-468) or Western blot analysis as described in Methods. Relative optic density (O.D.) indicated IL-17A/β-Actin or Caspase (cleaved/total) ratio. Asterisk indicated a p value < 0.05, compared to the WT group at the same time point (Mann-Whitney U test).

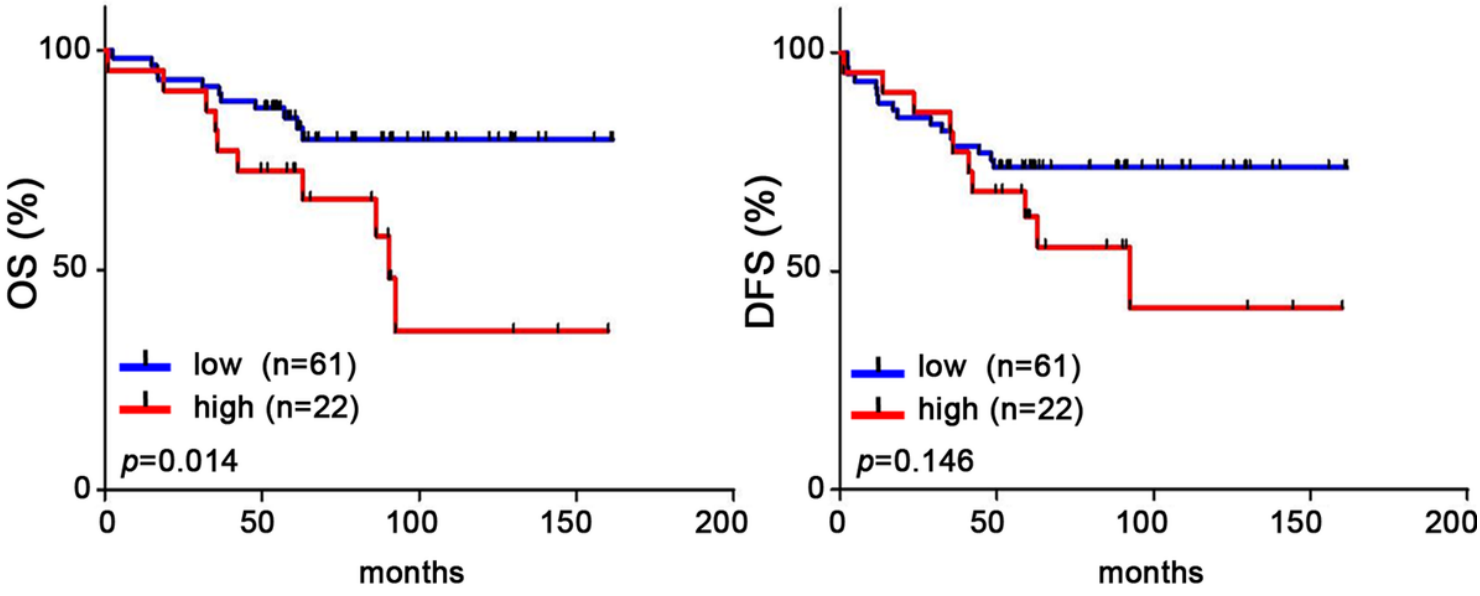

Figure 7

Clinicopathologic correlation between IL-17A expression and patients' prognosis. IL-17A protein expression by immunohistochemistry was correlated with patient survival, including overall survival (OS, a) and disease-free survival (DFS, b). The protein expression of IL-17A was semi-quantified and expressed as (0), <10%, (1), 11-25%, (2), 26-50%, and (3) >50% of tumour cells. The MEGF11 expression level was defined as low (≤ 60%, n=61) and high (> 60%, n=22). Asterisk indicated a p value < 0.05 by Kaplan-Meier survival analysis.