Research Square

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IKBKE is a critical mediator of TAM-induced breast cancer resistance to BET inhibition

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Abstract

Background: BET inhibitors (BETi) exhibit a strong anti-tumor activity in triple-negative breast cancer (TNBC). However, BETi resistance has been reported in TNBC. The mechanisms of resistance have not been demonstrated. Tumor-associated macrophages (TAMs) are frequently involved in cancer cells resistance to chemotherapy, also associated with poor prognosis in TNBC. However, the role of TAMs in BETi resistance remains unknown. Method: The expression of RNA was evaluated by qRT-PCR. Protein was detected by Western blot, immunofluorescence and Enzyme linked immunosorbent assay (ELISA). Cell viability was detected by Cell Counting Kit-8 (CCK-8). Apoptosis was evaluated by Annexin V-FITC/PI, TUNEL assays and Hoechst 33258 staining. NF-κB activities were detected by luciferase reporter assays. Mice model of breast cancer was established with subcutaneous xenograft tumor. Detailed mechanistic studies were performed by using inhibitors, RNA interference and DNA transfections. Results: Here, we found that BETi JQ1 and I-BET151 exerted anti-tumor effects in TNBC by decreasing IKBKE expression to attenuate NF-κB signaling. BET inhibition was found to downregulate IKBKE levels through BRD2/E2F1 axis. Moreover, we firstly found that TNBC-stimulated TAMs activated NF-κB signaling by upregulating IKBKE expression to enhance breast cancer cells resistance to BETi. The IKBKE levels were also proved to be higher in clinical TNBC tissues than Non-TNBC tissues, suggesting feedback induction of IKBKE expression by TNBC-stimulated TAMs in TNBC. In addition, the induction of IKBKE by TAMs in TNBC cells was identified to be associated with STAT3 signaling, which was activated by TAM-secreted IL-6 and IL-10. Lastly, the combination of inhibitors of BET and STAT3 exerted a synergistic inhibition effects in TAM-cocultured or TAM CM-treated TNBC cells in vitro and in vivo . Conclusion: Altogether, our findings illustrated TNBC-activated macrophages conferred TNBC cells resistance to BETi via IL-6 or IL-10/STAT3/IKBKE/NF-κB axis. Blockade of IKBKE or double inhibition of BET and STAT3 might be a novel strategy for treatment of TNBC.

Background

Epigenetics alterations encompass DNA hypermethylation and histone modifications, which often play a vital role in tumor development and progression. The epigenetic changes in tumor cells are usually valuable markers for diagnosing cancer risk and progression. The epigenetic regulators bromodomain and extra-terminal domain (BET) family proteins, containing BRD2, BRD3, BRD4 and BRDT, exert a crucial role in regulating gene transcription by binding to acetylated histone or other acetylated transcription factors [1]. Previous studies have demonstrated that BET proteins control cell growth, senescence, apoptosis and differentiation [2]. BET proteins also promote the development of tumors by inducing some oncogenes expression such as C-MYC and FoxM1 [3]. Moreover, a growing body of work has demonstrated BET inhibitors (BETi) including JQ1 exert their therapeutic potential in many cancers by suppressing oncogenic factors expression [4].

Triple negative breast cancer (TNBC) is a heterogeneous disease and the most aggressive subtype of breast cancer. Since lack of effective targeted therapy for TNBC, chemotherapy is still the most commonly treatment. Notably, recent studies have highlighted that BETi may be a promising strategy for

TNBC therapy. For example, the BET inhibitor JQ1 can dually target angiogenesis and the hypoxic response in TNBC [5]. In addition, shu et al showed that TNBC is preferential sensitive to BETi in vitro and in vivo. By constructing the cell lines selected for acquired resistance to BET inhibition, they clarified the mechanisms of BETi resistance in TNBC [6]. Recently, several studies reported that BET inhibitors combined with other agents such as BAZ2/BRD9 inhibitor GSK2801, Bcl-XL inhibitor and vitamin C exert synergistic effect in ducing TNBC cells apoptosis [7–9]. Moreover, currently several clinical trials with BETi have been approved for cancer treatment.

Tumor-associated macrophages (TAMs), the important components of tumor microenvironment, are derived from circulating monocytes or resident tissue macrophages. TAMs promote cancer cell proliferation, migration and invasion. Moreover, it is also reported that TAMs accelerate tumor metastasis, angiogenesis, lymphangiogenesis and immunosuppression [10]. Indeed, clinical data showed that the degree of infiltrating macrophages is positively associated with aggressive behaviors in several types of solid tumor [11]. Additionally, the cancer cells resistance to anti-tumor therapies is closely related to TAMs. For example, TAMs have been involved in cancer cells resistance to platinum-based chemotherapy, anti-VEGF/VEGFR therapy and radiotherapy [10, 12–14]. Importantly, depletion of TAMs or inhibition of the TAMs activation has been proven to be an attractive strategy for anti-tumor therapies.

Several small-molecule inhibitors against BET protein have been developed for the management of TNBC and display promising anticancer activity. However, in clinical trials, cancer cells resistance to BET inhibitors are starting to emerge. The molecular mechanisms responsible for the BET inhibitors resistance have not been elucidated. TAMs have been reported to mediate the resistance of cancer cells to anti-tumor therapies; however the relationship between TAMs and BET inhibitor treatment efficacy in tumor is still obscure. We thus explored whether TAMs can promote the resistance of cancer cells to BET inhibitors. In this study, we found TAMs promoted TNBC cells resistance to BET inhibition in vitro and in vivo. Importantly, we identified potential mechanisms of BET inhibition resistance.

Materials And Methods

Cell culture

The MCF7 and TNBC cell lines MDA-MB-231 (231), MDA-MB-468 (468), BT549 were obtained from the American Type Culture Collection (ATCC). THP1 cells were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF7, BT549 and 231 cells were grown in DMEM medium (Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) at 37°C in 5% CO_2 incubator. THP1 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% FBS at 37°C in 5% CO_2 incubator. MDA-MB-468 cells were grown in Leibovitz L-15 medium (Gibco) supplemented with 10% FBS (Gibco) at 37°C in 100% air incubator. Cells were grown in monolayer and passaged routinely 2–3 times a week. All cell lines were validated by STR fingerprinting and were routinely screened for mycoplasma.

Cell viability and apoptosis assays

Cell viability was assessed using standard Cell Counting Kit-8 (CCK-8) assay as previously described [15]. CCK-8 was purchased from Dojindo (Kumamoto, Japan). Apoptosis assay was conducted by Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) analysis using FACS Calibur^{TM} flow cytometer (BD Biosciences, San Jose, CA, USA) as described previously [16]. Annexin V-FITC/PI detection kits were purchased from Thermo Scientific (Rockford, IL, USA).

Cell morphological assessment

Breast cancer cells MDA-MB 231 cells stably expressed IKBKE were treated with 0.8 μM JQ1 and I-BET151 for 48 h. Then cells were washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature. After 30 min, cells was stained with 5 μg/mL of Hoechst 33258 and observed under fluorescent microscope. The apoptotic cells were identified by fragmented and condensed nuclei.

Nuclear and cytoplasmic fractionation

Cytoplasmic and nuclear extracts were performed using Nuclear-Extract Kit (sigma) following the manufacturer's instructions.

NF-κB luciferase reporter assays

For transient transfection, 231, 468 and BT549 cells were seeded in 24 well plates in DMEM lacking phenol red and supplemented with 5% double charcoal-stripped FCS (DSS). Following seeded for 24 h, cells were transfected using Lipofectamine 3000 reagent (Thermo Scientific, Rockford, IL, USA), with 100 ng of NF-kB luciferase reporter gene vector and renilla luciferase vector, luciferase activities were determined after a further 24 h, using the Dual-Glo Luciferase Assay Kit (Promega, UK). RLTK was transfected to control for transfection efficiency, so firefly luciferase activities were calculated relative to the Renilla luciferase (RLTK) activities. NF-κB luciferase reporter gene vector was a gift from Dr. Francis Chan (University of Massachusetts Medical School, Worcester, MA).

Real-time quantitative reverse transcription PCR

Total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions as described previously [17]. Total RNAs were transcribed into cDNAs using using random primers (Promega, Madison, USA) and the Omniscript RT kit (Qiagen GmbH, Hilden, Germany). Real-time quantitative reverse transcription-PCR (RT-PCR) was performed using an Eppendorf Realplex Mastercycler (Eppendorf, Hamburg, Germany) and Quantitect SYBR Green PCR kit (Qiagen GmbH, Hilden, Germany). The relative levels of mRNA were determined by the 2^{-DDCt} method. Expression levels were normalized to actin. IKBKE: forward primer, 5-ATGAAGCTGCTGGCATCTGA-3; reverse primer, 5-GGACTCTTAGCCGTTCGATGA-3 [18]. β-actin: forward primer 5-TGACGTGGACATCCGCAAAG-3, reverse primer 5-CTGGAAGGTGGACAGCGAGG-3. The PCR array for human NF-κB signaling pathway was purchased from Qiagen (Cat. no. 330231 PAHS-025ZA).

Western blot analysis

Cell extracts were prepared using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid, 0.02% sodium azide, 1% NP-40, 2.0 mg/mL aprotinin, 1 mM phenylmethylsulfonylfluoride). The cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C, and then were collected. The protein concentration was determined by Bradford dye method [19]. Equal amounts (30 to 50 μg) of protein were subjected to electrophoresis and run in 6-12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE). Then proteins were transferred to PVDF membranes (Millipore, Darmstadt, Germany) for antibody blotting. The membranes were blocked with 5% BSA or non-fat milk for 1 h at room temperature, and then incubated with IKBa, p-p65, p65, IKBKE, Flag, STAT3, p-STAT3 and Actin antibodies. IKBa, p-p65, p65, STAT3, p-STAT3 were purchased from Cell Signaling Technologies (Massachusetts, USA). And the IKBKE, BRD2 and E2F1 antibodies were purchased from Abcam (Cambridge, U.K). HA and Flag antibody was purchased from Sigma (St. Louis, MO). β-actin (Actin) antibody was purchased from Santa Cruz Biochemical (Santa Cruz, CA). Subsequently, the membranes were incubated with a HRP-conjugated secondary antibody (Protein Tech Group, Chicago, IL) at room temperature for 1 h. The signals were stimulated with Enhanced Chemiluminescence Substrate (GE Healthcare; Munich, Germany), according to the manufacturer's instructions.

Immunofluorescence

Immunofluorescence was performed as previously described [20]. In brief, cells were fixed with 4% paraformaldehyde for 10 min, penetrated with 0.2% Triton-X 100 for 15 min at 4 °C. And then cells were washed twice and blocked with 3% BSA for 1 h at room temperature. Subsequently, cells were incubated with indicated antibodies (diluted 1:400 in blocking buffer) overnight at 4 °C. And then cells were washed three times for 5 min in PBS at room temperature, and then incubated for 2 h in dark with Alexa Fluor secondary antibody (diluted 1:200 in blocking buffer; Invitrogen). The anti-fade reagent without DAPI was used to mount slides. Images were acquired with a laser scanning confocal microscope (LSM510 Meta; Zeiss).

TUNEL assay

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling (TUNEL) assay was performed by using TUNEL Apoptosis Assay Kit (Abcam, UK), according to the manufacturer's instructions.

Plasmids and siRNAs transfection.

The IKBKE expression vector pcDNA3-IKBKE-flag (#26201) and the E2F1 expression vector E2F1-HA (#24225) were obtained from Addgene. BRD2-HA vector were obtained from OriGene Technologies Company. Overexpression and RNA interference were performed by transfecting overexpression vectors and siRNA with lipofectamine 3000 (Invitrogen, USA) respectively, according to the manufacturer's instructions as previously described [21]. One day before transfection, cells were plated on a 35-mm culture dish in complete growth medium. After cell density was 50–60% confluence, and then cells were

transfected with overexpression vectors or siRNAs. Briefly, cells were incubated with 1 mL of siRNA mixture including 50 nM siRNA and 5 μL lipofectamine 3000, or 2.5μg vector and 6 μL lipofectamine 3000. After 8 h of transfection, 1 mL of complete growth medium was added. After 48 h transfection, experiments were conducted. Protein levels were analyzed by Western blot. The siRNAs against BRD2 and E2F1 were purchased from Santa Cruz Co.. The negative control (NC) siRNA and siRNAs against IKBKE were synthesized from Shanghai GenePharma Co.. For IKBKE siRNA: 5'-GAGCTATCTCACCAGCTCC-3' [22].

Statistics

All experiments were repeated three times and were expressed as mean ± SD. *P* values were calculated using student's *t* test and *P* value < 0.05 was considered significant. Statistical analysis was analyzed using the Statistical Package for Social Sciences (SPSS) software (version 20.0).

Results

BET inhibition suppresses cells growth and induces cells apoptosis in TNBC by attenuating NF-κB signaling

To determine the effects of BET inhibition on human TNBC cell viability and apoptosis, MDA-MB-231 (231), MDA-MB-468 (468) and BT549 were exposed to different concentrations of BET inhibitors JQ1 and I-BET151 for 48 h. And then, cell viability and apoptosis were detected by CCK-8 and Annexin-V/PI assay, respectively. As shown in Fig. 1A and B, both JQ1 and I-BET151 caused a concentration dependent inhibition of cell viability and induction of cell apoptosis in all three cell lines. Additionally, to examine the possible role of BET inhibitors in NF-κB activation in TNBC, NF-κB activity assays were measured with a dual-luciferase reporter system. As shown in Fig. 1C, both BET inhibitors obviously decreased the NF-κB luciferase reporter activity in a dose-dependent manner in three TNBC cells. In 231 cells, we showed that both JQ1 and I-BET151 decreased the NF-κB luciferase reporter activity in a time-dependent manner (Fig. 1D).

To further confirm attenuation of NF-κB signaling by BET inhibition, we detected the expression of p-p65 (active form of NF-κB subunit p65) and NF-κB upstream regulators IKBα in TNBC cells treated by BET inhibitor by Western blot assay. As shown in Fig. 1E and G, JQ1 and I-BET151 significantly increased IKBα levels and decreased p-p65 expression. The nuclear translocation of NF-κB p65 represents the induction of NF-κB signal activation. By extracting nuclear protein for Western blot assay, we found that JQ1 and I-BET151 significantly decreased p65 expression in nuclear and increased p65 expression in cytoplasm (Fig. 1F and H). These data suggested that BET inhibitors suppressed cells growth and induced cells apoptosis in TNBC by attenuating NF-κB signaling.

BET inhibition attenuates NF-κB signaling in TNBC by decreasing IKBKE expression

To explore the molecular mechanism of NF-κB signaling inhibition by BET inhibitor, we performed PCR array to detect the mRNA levels of NF-κB cytoplasmic sequestering molecule including BCL3, CHUK (IKKα), IKBKB (IKKβ), IKBKE, IKBKG, NFKBIA (IKBα, MAD3), NFKBIB (TRIP9), NFKBIE. As shown in Fig. 2A, we found BET inhibitors JQ1 and I-BET151 observably decreased the IKBKE mRNA levels in 231 cells. To simultaneously correlate the fold change on gene expression induced by JQ1 and the statistical significance at the global level, volcano plot was delineated by log2-fold change as x axis and a log10 *P* value as y axis. As shown in Fig. 2B, volcano plot showed the difference of gene expression in different dose JQ1 treated groups. The four red dots represented the selected IKBKE gene in four doses of JQ1 treated groups. These results showed IKBKE was the most significant change gene regulated by JQ1 in 231 cells.

To further confirm expression of IKBKE regulated by BET inhibition, we treated 231 and BT549 cells with JQ1 and I-BET151. IKBKE mRNA and protein levels were detected by RT-PCR and Western blot respectively. As shown in Fig. 2C, D and E, F, JQ1 and I-BET151 markedly reduced IKBKE levels in a concentration demand manner. In addition, we also showed overexpression of IKBKE obviously blocked the suppression of NF-κB signal by BET inhibitor in TNBC cells. As shown in Fig. 2G and H, in 231 and BT549 cells transfected IKBKE overexpression vector, the IKBα expression induced by BET inhibitors was clearly downregulated, while p-p65 expression inhibited by BET inhibitors was clearly upregulated. Above data showed BET inhibition decreased IKBKE expression, and thus attenuating NF-κB signaling in TNBC.

IKBKE prevents cells apoptosis and growth arrest by BET inhibition in TNBC

To determine if IKBKE could prevent the cytotoxic effects of BET inhibitor, 231 and BT549 cells with IKBKE overexpression were treated with JQ1 and I-BET151 for 48 h. The overexpressed IKBKE in TNBC cells was displayed in Fig. 3A. And then cell viability and apoptosis were determined by CCK-8 assay, hoechst 33258 staining and Annexin V-FITC and PI staining respectively. By Hoechst staining, we found BET inhibitors induced nuclear condensation and fragmentation in 231 cells (Fig. 3B). By CCK-8 assay, IKBKE was also found to significantly inhibit the cytotoxic effects of JQ1 and I-BET151 in two types of TNBC cells (Fig. 3C and D). Additionally, Fig. 3E-I indicated that two BET inhibitors induced cell apoptosis was significantly attenuated by IKBKE in both 231 and BT549 cells.

BET inhibition downregulates IKBKE levels through BRD2/E2F1 axis

Previous studies have demonstrated that IKBKE is transcriptionally upregulated by E2F1 [23]. Therefore, we investigated whether BET inhibition downregulates E2F1 levels. Our results showed that both BET inhibitors JQ1 and I-BET151 decreased E2F1 protein expression and E2F1 mRNA levels in 231 cells (Fig. 4A and B). To directly verify that IKBKE induced by E2F1, we used siRNA to knock down the E2F1 expression and detected the IKBKE expression in 231 cells. As shown in Fig. 4C, E2F1 siRNA significantly decreased IKBKE expression. To further determine that BET inhibition attenuated IKBKE levels was involved in repressing E2F1, 231 cells were transfected E2F1-HA overexpression constructs, subsequently cells were treated with BET inhibitor JQ1. Results showed that overexpression of E2F1 promoted the

IKBKE expression (Fig. 4D). Moreover, attenuation of IKBKE by JQ1 was remarkably rescued by E2F1-HA overexpression vectors (Fig. 4D).

The BET protein BRD2 has been linked to promote E2F1 transcription [24]. To investigate whether BET inhibitor suppressed IKBKE levels through inhibiting BRD2/E2F1 pathway. The BRD2 was depleted by transfecting siRNA in 231 cells. We found BRD2 siRNA significantly decreased both E2F1 and IKBKE expression (Fig. 4E). Furthermore, attenuation of E2F1 and IKBKE by JQ1 was remarkably rescued by BRD2-HA overexpression vectors (Fig. 4F). To further examine the association between BRD2, E2F1 and IKBKE, the mRNA levels of these three genes in 1104 breast cancer tissues were extracted from TCGA database. By Pearson correlation analysis, we found significantly positive correlations between E2F1 and IKBKE expression ($r = 0.15$, $P < 0.001$; Fig. 4G), between E2F1 and BRD2 expression ($r = 0.11$, $P < 0.001$; Fig. 4H).

TNBC-stimulated TAMs activate NF-κB signaling to enhance breast cancer resistance to BET inhibition

Previous studies have showed that TAMs are associated with chemoresistance in breast cancer [10]. We thus supposed that the TAMs might be associated with the TNBC cells resistance to BET inhibitor. To assess potential effects of macrophage on the BET inhibitor resistance of TNBC, we mimicked *in vivo* model by co-culturing TNBC cells with macrophages derived from PBMC or THP1 cells. Subsequently, these TNBC cells were subjected to BET inhibitors treatment and then to perform drug sensitivity and apoptosis analysis. As shown in Fig. 5A, the IC_{50} of JQ1 in TNBC cells co-cultured with PBMC-TAMs was significantly higher than those of their corresponding control cells. Moreover, apoptosis assay showed that the ratios of apoptosis induced by BET inhibitors were significantly lower in 231 and BT549 cells co-cultured with PBMC- or THP1-derived macrophages than control cells (Fig. 5B-F). These results suggested TAMs promoted TNBC cells resistance to BET inhibitor.

To further determine TAMs increased JQ1 resistance in TNBC, we performed tumor formation assay in BalB/C nude mice. TAMs were induced from THP1 cells and then co-cultured with 231 cells for 72 h *in vitro*. Subsequently, 231 cells mixed with THP1 were injected into flanks of nude mice. Treatment schedule was shown in Fig. 5J. We found the tumors of the 231 cells mixed with THP1 cells were more resistant to JQ1 therapy than those tumors of the 231 alone (Fig. 5G and I). In addition, no significant loss in body weight was observed in these mice treated with JQ1 (Fig. 5H), suggesting the side effects of JQ1 were minimal *in vivo*.

In addition, THP1-derived macrophages were also co-cultured with non-TNBC cell line MCF7. Subsequently, these macrophages were used to co-culture with 231 cells. After 72 h, 231 cells were treated with different dose of JQ1 for 48 h. And then, cell apoptosis was evaluated. Results showed macrophages co-cultured by MCF7 failed to decrease the cytotoxic effects of JQ1 in 231 cells (Fig. S1).

Since TNBC cells apoptosis induced by BET inhibitor was associated with suppression of NF-κB activity, we thus explored whether BET inhibitors reduced NF-κB activity to provoke cells apoptosis. By immunofluorescence assay, we showed the translocation of NF-κB p65 from the cytoplasm to the

nucleus was notably blocked by both JQ1 and I-BET151 in 231 cells. However, the levels of NF-κB p65 in the nucleus were obviously increased in 231 cells treated with PBMC-TAM-CM or co-cultured with PBMC-TAMs. Furthermore, the reduction of p65 levels in nucleus by BET inhibitors was significantly blocked by PBMC-TAM-CM or PBMC-TAMs (Fig. 5K). Besides, by Western blot, we also found that PBMC-TAMs or PBMC-TAM-CM attenuated the upregulation of IKBα levels and downregulation of p-p65 expression elicited by BET inhibitors (Fig. 5L).

TNBC-treated TAMs promote IKBKE expression to prevent BET inhibitor-induced apoptosis by activating STAT3 signaling

Above data have indicated that BET inhibitor induced TNBC cell apoptosis was associated with inhibition of IKBKE/NF-κB signal. Moreover, TAMs have been shown to activate NF-κB signaling in breast cancer cells in this study. We thus explored whether TAMs regulated IKBKE expression of TNBC cells. The results in Fig. 6A indicated that TNBC cells-treated TAMs or TAM-CM significantly increased IKBKE protein levels, while BET inhibitors restrained IKBKE expression in 231 cells. Moreover, the reduction of IKBKE by BET inhibitors in 231 cells was obviously alleviated by TAMs or TAM-CM (Fig. 6A). By RT-PCR assay, we showed that the mRNA levels of IKBKE were observably higher in 231 cells co-cultured with TNBC cells-activated TAMs than those cells co-cultured with Non-TNBC cells MCF7-activated TAMs (Fig. S2A). Similarly, in 231 cells treated with TNBC cells-activated TAMs CM, the mRNA levels of IKBKE were significantly higher (Fig. S2B).

Clinical studies have found that a large number of macrophages are infiltrated into the tumor tissue in breast cancer [25]. Thus, we speculated that TAMs in TNBC induced higher levels of IKBKE of TNBC cells relative to TAMs in Non-TNBC. By RT-PCR analysis, IKBKE expression was performed in 19 TNBC tissues and 45 Non-TNBC tissues from breast cancer patients. Results indicated that the IKBKE levels were higher in TNBC tissues than Non-TNBC tissues (Fig. 6B). In addition, IKBKE expression was analysed in 93 TNBC tissues and 898 Non-TNBC tissues from breast cancer patients in TCGA data sets. We found the IKBKE levels were also higher in TNBC tissues than Non-TNBC tissues (Fig. 6C).

Previous study has reported that IKBKE expression was regulated by STAT3 signal [26]. To determine the mechanism by which TAMs regulated IKBKE expression, we used STAT3 inhibitor STAT3-IN-1 to treat TNBC cells. Western blot analysis indicated induction of IKBKE levels by TAM-CM were strikingly inhibited by STAT3-IN-1 (Fig. 6D). To investigate whether STAT3 inhibition influenced TAM-mediated TNBC cells resistance to BET inhibitor, we performed cell apoptosis and viability assay in 231 and BT549 cells. As shown in Fig. 6E and F, STAT3-IN-1 synergistically enhanced JQ1-induced cytotoxic effects in both 231 and BT549 cells. Moreover, STAT3-IN-1 completely abrogated the resistance to BET inhibitors induced by TAMs or TAM-CM in TNBC cells. In addition, to more directly assess the role of IKBKE in TAM-induced BET inhibitor resistance, cell apoptosis and viability were evaluated in JQ1-treated TNBC cells with siRNA-mediated knockdown of IKBKE. As shown in Fig. 6G-I, knockdown of IKBKE significantly enhanced cell apoptosis induced by JQ1 treatment in TNBC cells. Importantly, depletion of IKBKE significantly prevented the resistance to BET inhibitors induced by TAMs or TAM-CM in TNBC cells.

To test whether STAT3-IN-1 combined JQ1 was an effective strategy to overcome TAM-induced JQ1 resistance *in vivo*, we further assessed the antitumor activity of the two drugs in xenografts established in nude mice implanted with 231 mixed THP1 cells. Treatment schedule was shown in Fig. 6M. As shown in Fig. 6J, either JQ1 or STAT3-IN-1 alone inhibited the tumors growth; the two drugs in combination displayed a much stronger antitumor effects in the xenograft tumor models ($P < 0.001$). By TUNEL assay, we detect the apoptotic cells in tumor tissues treated by JQ1 and STAT3-IN-1 alone or in combination. Fig. 6L showed that the numbers of apoptotic cells in the tumors treated by JQ1 and STAT3-IN-1 in combination were significantly higher than other group. This result also suggested that JQ1 combined with STAT3-IN-1 displayed a much stronger antitumor effects *in vivo*. The body weight of mice recorded throughout the experiments was shown in Fig. 6K. Results showed no significant loss in body weight was found in the mice treated with the two drugs alone or in combination, suggesting the side effects were minimal *in vivo*.

TAMs promote IKBKE expression in TNBC by secreting IL-6 and IL-10.

STAT3 activation is responsible for IL-6 and IL-10. Therefore, we supposed the induction of IKBKE by TAMs in TNBC cells was relevant to IL-6 and IL-10 secreted by TAMs. The IL-6 and IL-10 in TAMs and breast cancer condition media were detected by ELISA assay. Fig. S3 indicated the levels of two interleukins were obviously higher in 231- and BT549-treated TAMs CM relative to others CM from MCF7-treated TAMs, THP1 macrophage and breast cancer cells. Subsequently, the IL-6 and IL-10 in TAMs CM was deprived by incubating with IL-6 and IL-10 neutralizing antibody alone or in combination. And then TAMs CM was used to treat 231 and BT549 cells. Subsequently, IKBKE protein levels were detected by Western blot in TNBC cells. As shown in Fig. 7A and B, IL-6 and IL-10 antibodies alone sharply decreased IKBKE levels in both TNBC cells respectively. Moreover, IL-6 combined with IL-10 antibodies synergistically inhibited IKBKE expression. Additionally, by RT-PCR experiment, we also showed that IL-6 and IL-10 antibodies significantly reduced IKBKE mRNA levels in both TNBC cells treated with TAMs CM. IKBKE mRNA levels was synergistically inhibited by IL-6 and IL-10 antibodies in combination (Fig. S4A and B).

More importantly, we also validated the IKBKE expression was induced by IL-6 and IL-10 in TNBC cells. As shown in Fig. 7C-F, IL-6 and IL-10 markedly increased IKBKE protein levels in both TNBC cells in concentration-dependent manner. Similarly, by RT-PCR assay, we also observed IKBKE mRNA levels were increased by IL-6 and IL-10 in dose-dependent manner in 231 and BT549 cells (Fig. S4C-F).

Discussion

Recent data in pre-clinical studies revealed that BET inhibitors exert potential anti-tumor effects in non-small cell lung cancer cells [27], mixed-lineage leukemia gene-fused leukemia [28], and other solid tumors including pancreatic ductal adenocarcinoma [29], prostate cancer [30]. In several subtypes of breast cancer, BET inhibitors have been shown to display a strong anti-tumor effect. In BRCA1-deficient breast cancer, BET inhibitors induce DNA damage and kill cancer cells by elevating cellular oxidative stress [31].

In TNBC, BET inhibitors exert anti-tumor functions by blocking cell proliferation, inducing cell apoptosis and inhibiting angiogenesis [5, 7, 32-34]. Moreover, BET inhibitors have been reported to enhance the effect of immunotherapy by suppressing transcriptional expression of MYC in TNBC [35]. In the current study, we showed that BET inhibitors JQ1 and I-BET151 displayed anti-tumor effects in TNBC by inducing cell apoptosis and inhibiting cell viability. NF-κB signaling was involved in the anti-tumor effects of the BET inhibitors. Furthermore, we found BET inhibitors suppressed NF-κB signaling pathway by downregulating IKBKE levels. Importantly, TNBC-stimulated TAMs conversely activated NF-κB signaling in TNBC cell to facilitate cancer cells resistance to BET inhibition. We further showed that activated NF-κB signaling by TAMs was associated with induction of IKBKE. Lastly, induction of IKBKE in TNBC cell was confirmed to be regulated by STAT3 signaling which be activated by TAM-secreted IL-6 and IL-10 (Fig. 8).

The BET protein BRD4 can specifically bind to acetylated lysine-310 of RelA subunit of NF-κB, and have been demonstrated to be a coactivator of NF-κB [36]. Zou *et al* also found BET inhibitor JQ1 restrains NF-κB activation in lung cancer cell by directly disrupting the bind of acetylated RelA and BRD4 [37]. These studies suggested BET inhibitor exerts anti-tumor effects is associated with inhibition of NF-κB activation. Moreover, recent study has shown that BET inhibitors induce cancer cell apoptosis and growth arrest by attenuating NF-κB signaling in myeloproliferative neoplasm [38]. Here, we found a novel molecular mechanism of NF-κB activation regulated by BET protein. We showed BET inhibitors suppressed IKBKE expression and thus inhibited NF-κB activation in TNBC. However, the molecular mechanism by which BET inhibitors downregulated the expression of IKBKE remained to be elucidated.

As an oncogene, IKBKE is implicated in tumorigenesis, tumor progression and poor prognosis in many cancer types. In breast cancer, deregulation of IKBKE is involved in the regulation of tumor development. For example, by integrative genomic approaches, Boehm *et al* found breast cancer cells harbor IKBKE amplifications, which is associated with tumorigenesis [39]. Several recent studies also showed that IKBKE induces tumorigenesis and cancer stem cell phenotype in TNBC [40, 41]. In addition, IKBKE has been reported to promote cell proliferation and attenuate cell senescence in HER2 positive breast cancer [42]. IKBKE has also been found to be linked closely to cell death resistance. Boehm *et al* confirmed suppression of IKBKE expression induces breast cancer cell death by inhibiting NF-κB activation [39]. In this study, we presented similar findings that suppression of IKBKE by BET inhibitors induced cell apoptosis by inhibiting NF-κB activation. Additionally, Lafont *et al* found that IKBKE prevents RIPK1-dependent cell death induced by tumor necrosis factor by phosphorylating the kinase RIPK1 in the TNFR1 signalling complex [43]. All these studies and our results suggested IKBKE was a promising drug target for the treatment of breast cancer.

High macrophage infiltration has been shown to be associated with poor prognosis in some human cancer including breast, colon, pancreatic and cervical cancer [10]. The pro-tumor role of TAMs in solid tumor is further demonstrated *in vitro* and *in vivo* studies. For example, TAMs promote cancer cells migration and invasion by secreting IL-6 to regulate cancer cells epithelial-mesenchymal transition in colorectal cancer. In addition, TAMs play an important role in the chemoresistance of solid cancer. For example, TAM-secreted YWHAZ/14-3-3 protein zeta/delta enhances pancreatic cancer cells resistance to

gemcitabine [44]. In this study, we identified a previously unrecognized role of TAMs in TNBC cells resistance to BET inhibitor. Mechanismly, it was shown that TAMs secreted IL-6 and IL-10 in a paracrine manner to activate STAT3 signal in TNBC cells. And then, STAT3 transcriptionally upregulated IKBKE expression to promote BET inhibitor resistance. Consistent with our results, previous studies also showed that IL-6 and IL-10 were vital mediators of anti-tumor therapy resistance by TAMs. For example, M2 macrophage-derived IL-6 confers chemoresistance in colorectal cancer [45]. In breast cancer, TAM-secreted IL-6 enhances cancer cells resistance to hedgehog inhibition [46]. IL-10 derived from TAMs was reported to enhance breast cancer cells resistance to paclitaxel by upregulating Bcl-2 expression [47].

Notably, the macrophages treated by epithelial-like breast cancer cells MCF7 did not enhance TNBC cells resistance to BET inhibitor. Further, compared with TAMs treated with TNBC cells 231 and BT549, MCF7-induced macrophages produced lower levels of IL-6 and IL-10, and induced lower levels of IKBKE in TNBC cells. These results suggested that low levels of IL-6 and IL-10 produced by MCF7-treated TAMs did not significantly increased IKBKE expression, and thus was not sufficient to enhance TNBC cells resistance to BET inhibitor. Moreover, we also firstly reported that the IKBKE levels were also higher in TNBC tissues than Non-TNBC tissues. These clinical data implied TAMs induced higher IKBKE expression of cancer cells in TNBC tissues relative to Non-TNBC tissues. However, we could not exclude another possibility that the IKBKE expression of TNBC cells was higher than that of Non-TNBC cells in the absence of macrophages stimulation. Above results suggested there was significantly different function between TNBC- and Non-TNBC-activated macrophages. This raised the possibility that the difference between some factors secreted by MCF7 and TNBC cells affected the status of macrophages. Previous study demonstrated that pro-tumor function of macrophages was induced by high levels of GM-CSF and lactate produced by TNBC cells. However, epithelial-like breast cancer cell lines produced a small amount of GM-CSF and lactate, and thus failed to activate macrophages [48].

In the current study, we also indicated that STAT3 inhibitor combined with BET inhibitor JQ1 exerted synergistically anti-tumor effects in TNBC. It has been reported that STAT3 signaling is persistent activation in breast cancer, and promotes cancer stem cell self-renewal and confers resistance to apoptosis [49]. Many recent studies have shown that STAT3-targeted therapy is a very promising treatment for TNBC [50]. These studies and our data provided preclinical evidence that the combination of STAT3 inhibitor and BET inhibitor was a feasible treatment for TNBC, which warranted further investigation in a clinical setting.

Conclusions

In summary, our study suggested TAMs played an important role in TNBC cells resistance to BET inhibitor. TAM-targeted inhibition might represent a promising therapeutic strategy to improve efficacy of BET inhibitor in TNBC.

Supporting Materials And Methods

Additional Materials and Methods are described in Additional file 1 (Supporting Materials and Methods), including the reagents and drugs treatment, generation of macrophage conditioned media, co-culture system with macrophages and breast cancer cells, tissues specimen information and TCGA database analysis, enzyme linked immunosorbent assay (ELISA), xenografted breast cancer model in mice.

Declarations

Acknowledgments

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Authors' contributions

Study conception and design: ZZZ and QMW; data analysis: JHQ, YBC and YJM; cell and molecular experiment: JHQ, YBC, LNW, HJ, TH and HLL; animal experiment: HJ and YJM; manuscript drafting: ZZZ, JHQ, YBC and YJM; manuscript revising: ZZZ, YBC, JC and YCS. All authors reviewed and approved the final manuscript.

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Availability of data and materials

The data used or analyzed during this study were included in this article. Please contact the corresponding author for all data requests.

Ethics approval

All of the procedures of animal experiments were approved by the Ethics Committee of Zhengzhou University and performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines (http://www.aaalac.org). Informed consent was obtained from all participants included in this study according to the committee regulations.

Consent for publication

All authors agreed with the content of the manuscript.

Competing interests

The authors declared that they have no competing interests.

Abbreviations

BET: bromodomain and extra-terminal domain; BETi: BET inhibitors; TNBC: triple negative breast cancer; TAMs: tumor-associated macrophages; DMSO: dimethyl sulfoxide; CM: conditioned media; PBMC: peripheral blood mononuclear cells; ELISA: enzyme linked immunosorbent assay; DMEM: Dulbecco's Modified Eagle's Media; PBS: phosphate buffer saline; PVDF: polyvinylidene fluoride; RT-PCR: quantitative real-time polymerase chain reaction; IL-6: interleukin 6; IL-10: interleukin 10.

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Figures

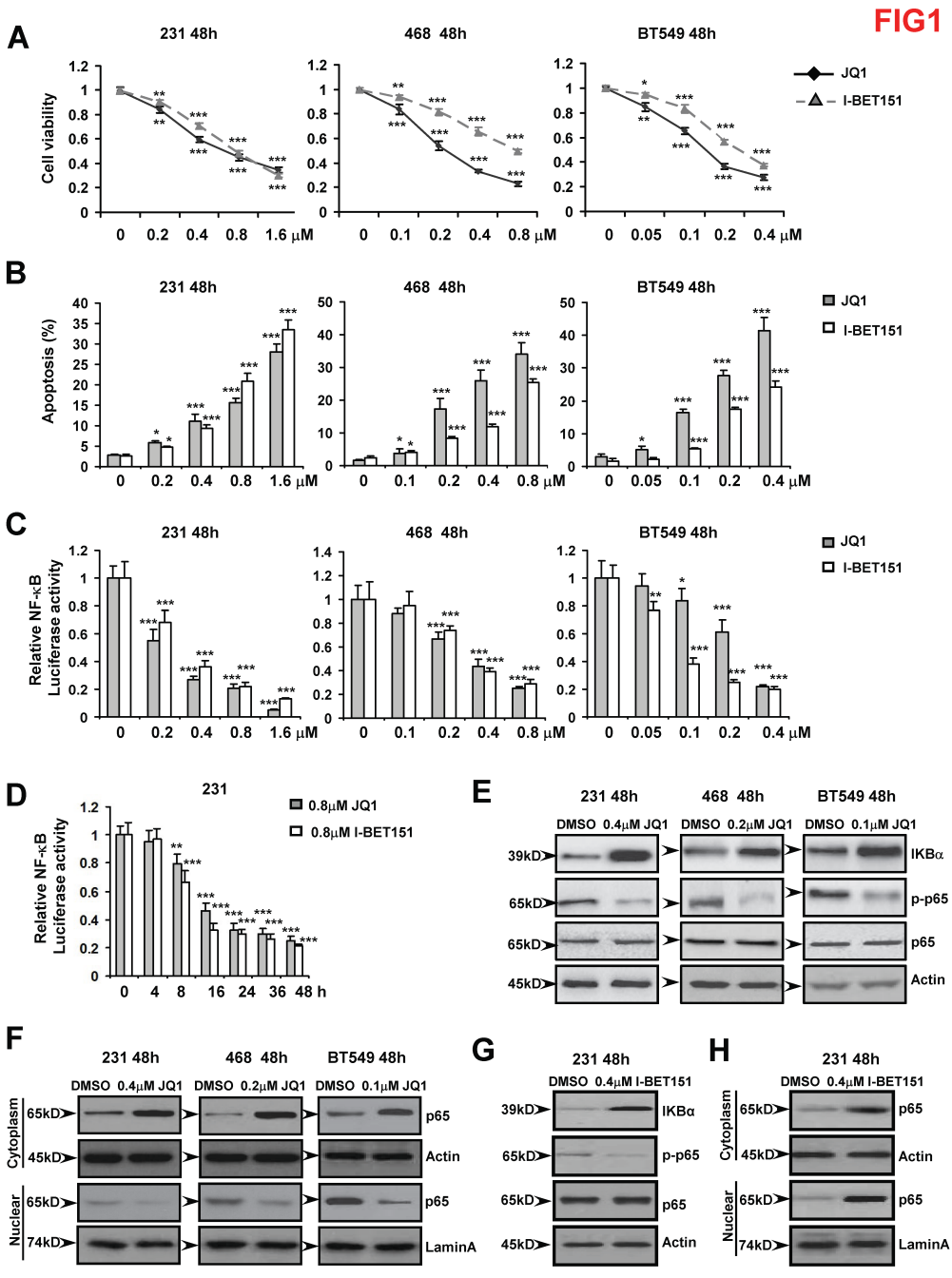

Figure 1

BET inhibition suppresses cells growth and induces cells apoptosis in TNBC by attenuating NF-κB signaling. (A and B) TNBC cells MDA-MB-231 (231), MDA-MB-468 (468) and BT549 cells were treated with indicated dose of JQ1 and I-BET151 for 48 h, and cell viability and apoptosis were detected by CCK-8 and Annexin V-FITC/PI staining assay. (C and D) TNBC cells 231, 468 and BT549 were transfected with the NF-κB reporter gene vector, and then were treated with indicated dose of JQ1 and I-BET151 for 48 h or

different time, relative NF-κB luciferase acitivity was detected with the dual luciferase reporter assay system. (E-H) Western blot analyses with indicated antibodies were used to detect the protein from whole cells protein extracts in TNBC cells treated with indicated dose of JQ1 (E and G). Cytoplasm and nuclear proteins were extracted from 231, 468 and BT549 cells treated by indicated dose of JQ1 or I-BET151. The expression of proteins was detected with indicated antibodies by Western blot (F and H). Actin was used as the loading control of whole protein and cytoplasm protein. LaminA was used as the loading control of nuclear protein. Data represented the mean ± S.D. of at least three times biological replicates. **P < 0.01. ***P < 0.001.

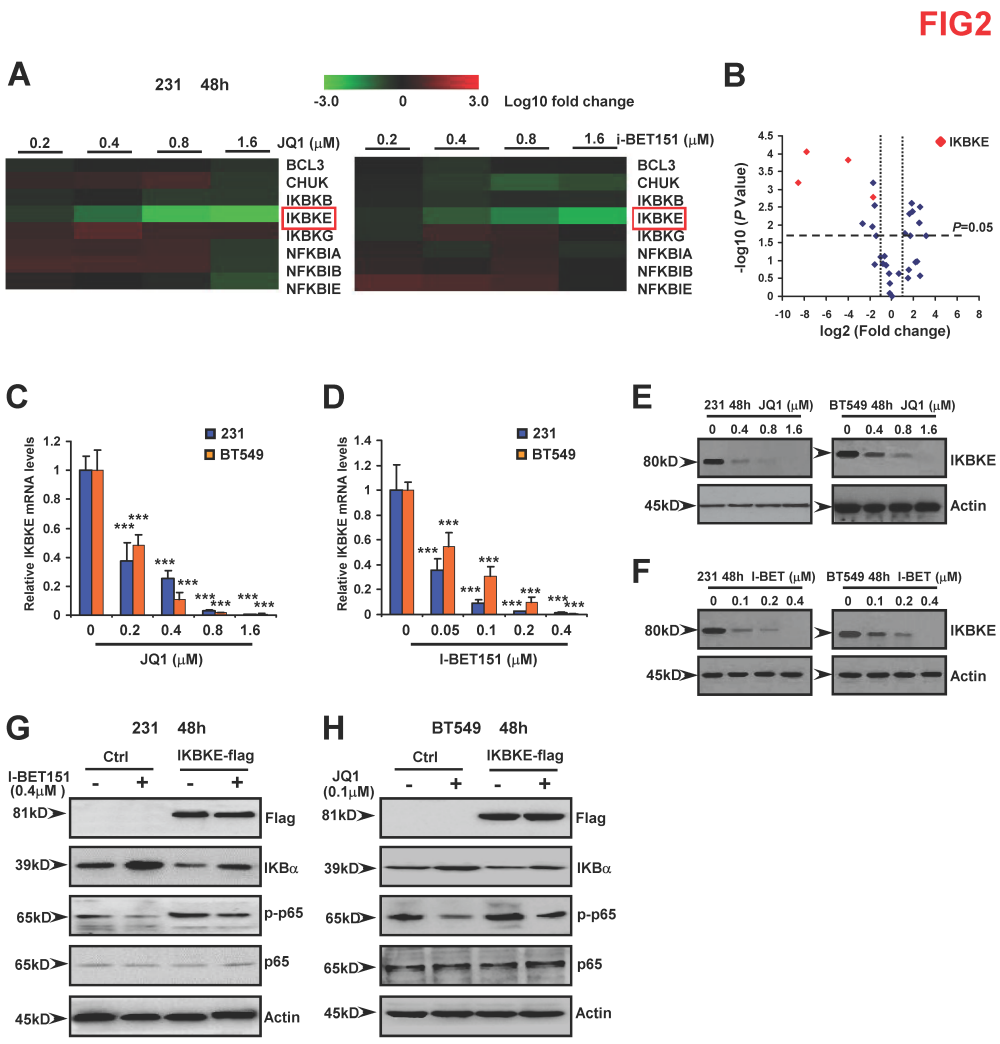

Figure 2

BET inhibition attenuates NF-κB signaling in TNBC by decreasing IKBKE expression. (A and B) 231 cells were treated with indicated dose of JQ1 and I-BET-151 (I-BET). Human NF-κB signaling pathway gene PCR array was used to identify potential upstream target genes of NF-κB inhibited by BET inhibitor. The expressions of genes were shown in the heatmap (A). Volcano map indicated the relationship between the observed fold change in gene expression and the P value significance of such changes in cells treated

with different dose of inhibitors. The dotted lines represented the P=0.05, and solid line represented the 2 fold-change cut-offs, the red dots represented the selected IKBKE gene (B). (C-F) 231 and BT549 cells were treated by indicated dose of JQ1 or IBET-151 (I-BET) for 48 h. The mRNA levels of IKBKE were analyzed by RT-PCR (C and D) and Western blot (E and F). (G and H) 231 and BT549 cells were transfected with IKBKE overexpression vector (IKBKE-flag). After 8 h, cells were treated with indicated dose of JQ1 and I-BET-151 for 48 h. The expression of proteins was detected with indicated antibodies by Western blot. Actin was used as the loading control. Data represented the mean ± S.D. of at least three times biological replicates. ***P < 0.001.

FIG3

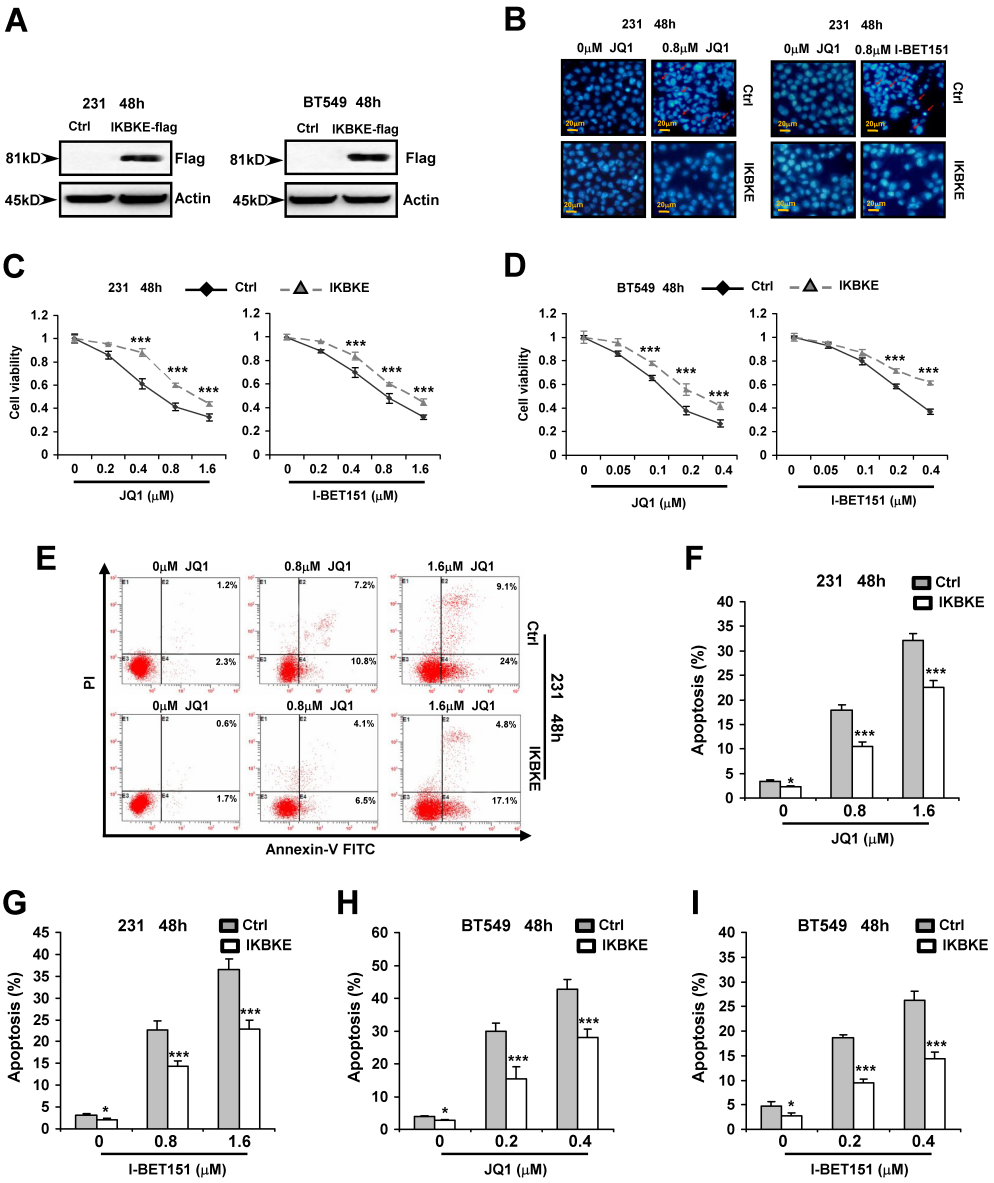

Figure 3

IKBKE prevents cells apoptosis and growth arrest by BET inhibition in TNBC. (A-D) MDA-MB-231 (231) and BT549 cells transfected stably with IKBKE overexpression vector were treated with indicated dose of JQ1 and I-BET151 for 48 h, cell apoptosis were examined by hoechst 33258 staining (B), and cell viability was detected by CCK-8 assay (C and D). The expression of proteins was detected with indicated antibodies by Western blot. Actin was used as the loading control (A). (E-I) 231 and BT549 cells

transfected with IKBKE overexpression vector were treated with indicated dose of JQ1 and I-BET151 for 48 h, and cell apoptosis was analysed by Annexin V-FITC/PI staining. Data represented the mean ± S.D. of at least three times biological replicates. *P < 0.05. **P < 0.01. ***P < 0.001.

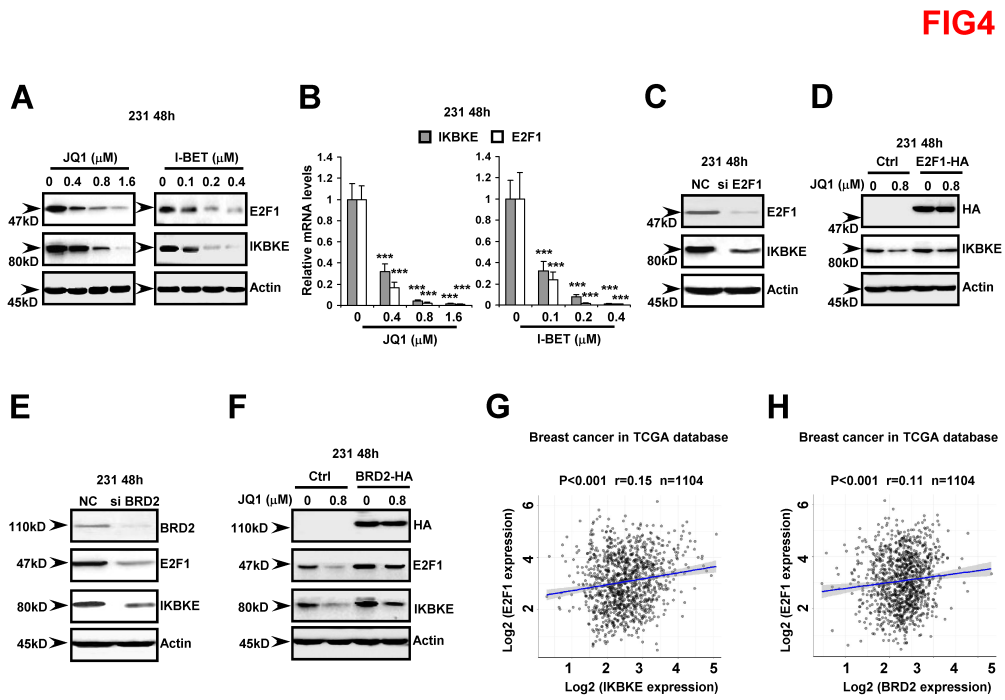

Figure 4

BET inhibition downregulates IKBKE levels through BRD2/E2F1 axis (A and B) MDA-MB-231 (231) cells were treated with indicated dose of JQ1 and I-BET151 for 48 h, the expression of proteins was detected

with indicated antibodies by Western blot. Actin was used as the loading control (A). The expression of mRNA was detected by RT-PCR. Data represented the mean ± S.D. of at least three times biological replicates. ***P < 0.001 (B). (C and E) 231 cells were transfected with E2F1 siRNA (C) or BRD2 siRNA (E) for 48 h, the expression of proteins was detected with indicated antibodies by Western blot. Actin was used as the loading control. (D and F) 231 cells were transfected with E2F1-HA overexpression vectors (D) or BRD2-HA overexpression vectors (F). After 8 h, cells were treated with indicated dose of JQ1 for 48 h, the expression of proteins was detected with indicated antibodies by Western blot. Actin was used as the loading control. (G and H) The mRNA expression of BRD2, E2F1 and IKBKE genes in 1104 breast cancer tissues were extracted from TCGA database. Their expression correlations were analyzed by pearson correlation coefficient and t-test.

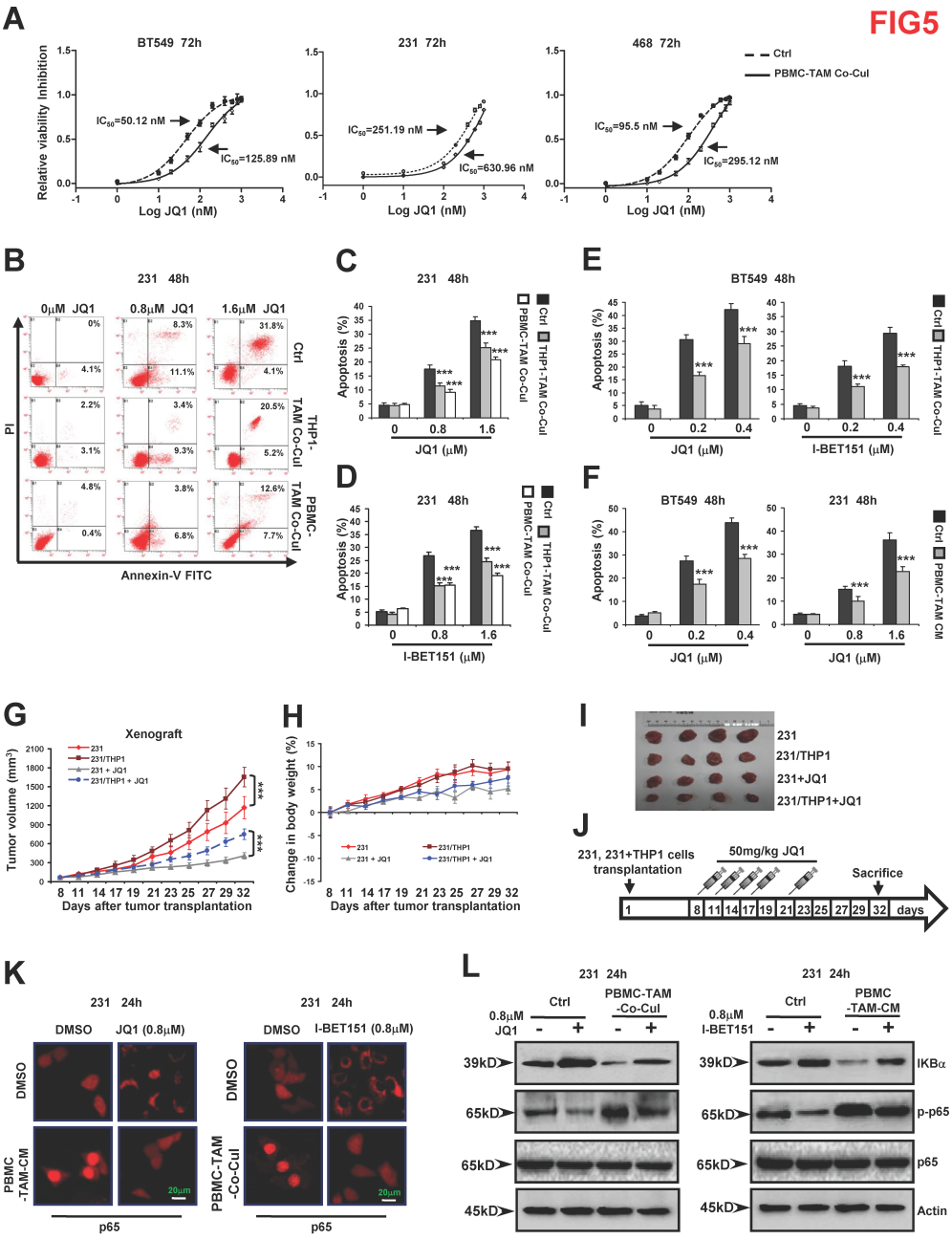

Figure 5

TNBC-activated TAMs induce NF-κB signaling to enhance breast cancer cells resistance to BET inhibition. (A) BT549, 231 and 468 cells were co-cultured with human PBMC-derived macrophages. And then cells were treated with indicated dose of JQ1 for 72 h, and cell viability was detected by CCK-8 assay. IC50 was labelled on the curve. (B-F) BT549 and 231 cells were co-cultured with THP1 or human PBMC-derived macrophages. And then cells were treated with indicated dose of JQ1 or I-BET151 for 48 h, and cell

apoptosis was analysed by Annexin V-FITC/PI staining. (G-J) TAM (THP1) and 231 cells were mixed and inoculated subcutaneously in nude mice. JQ1 displayed significantly antitumor efficacy in xenograft tumor models TAM (THP1) prevented BET inhibitor-reduced tumor growth in xenograft tumor models. The volume of xenograft was calculated by a caliper every 2-3 days (n = 8 mice per group) (G). Change in mice body weight was dispalued (H). Representative images of 4 xenograft tumors from different groups of nude mice following indicated treatment (n = 8 mice per group) (I). Treatment schedule (J). (K and L) 231 cells were co-cultured with human PBMC-derived macrophages. Cells were treated with indicated dose of JQ1 and I-BET151 for 24 h. The p65 protein was detected by immunofluorescence and Western blot assay. Data represented the mean ± S.D. of at least three times biological replicates. ***P < 0.001.

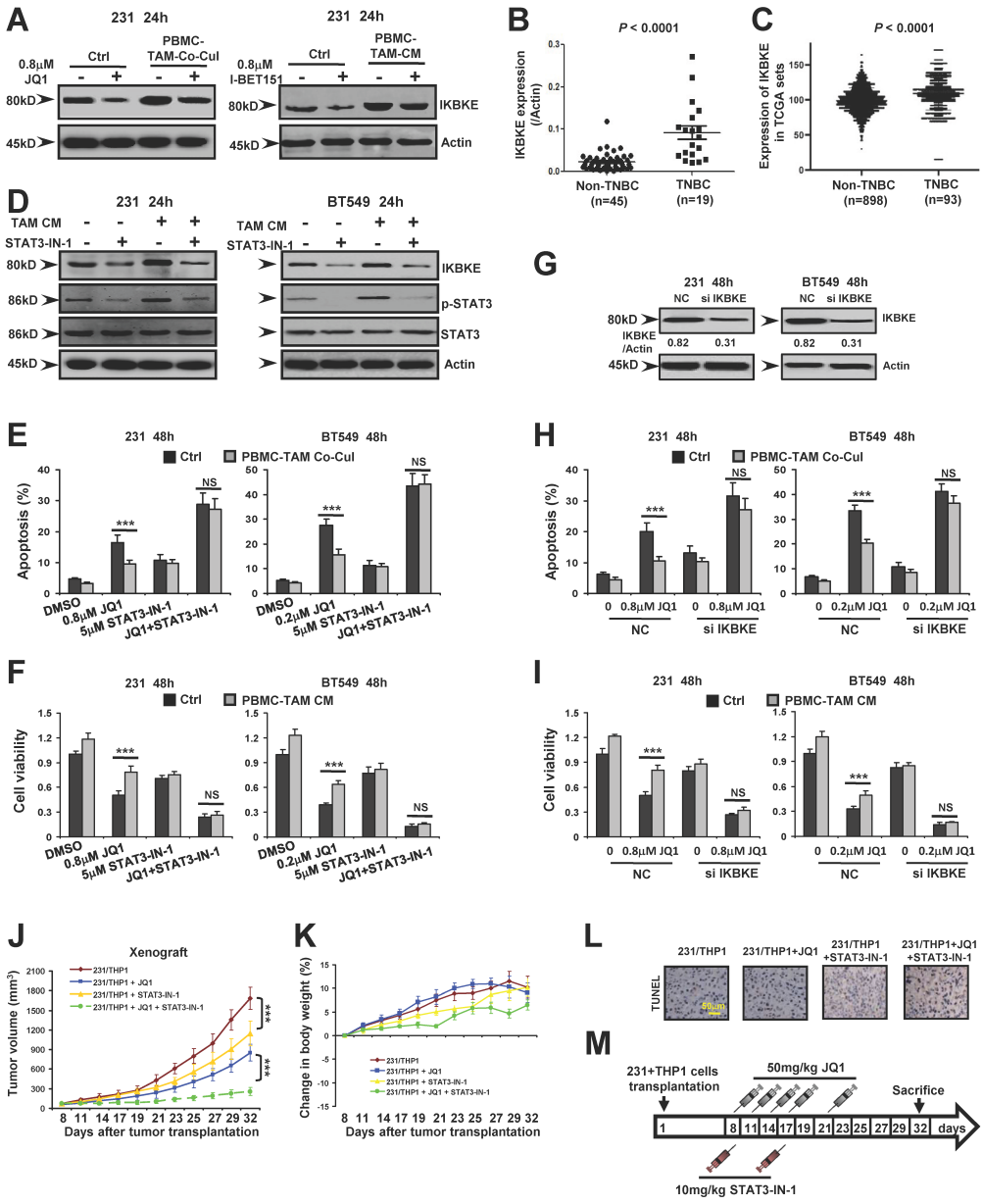

FIG6

Figure 6

TNBC-treated TAMs promote IKBKE expression to prevent BET inhibitor-induced apoptosis by activating STAT3 signaling. (A and D) BT549 and 231 cells were co-cultured with human PBMC-derived macrophages or treated with PBMC-derived macrophages condition media (TAM CM). And then cells were treated with indicated dose of JQ1 or I-BET151, STAT3-IN-1 for 24 h. The expression of proteins was detected with indicated antibodies by Western blot. Actin was used as the loading control. (B and C) The

IKBKE levels were higher in TNBC tissues than Non-TNBC tissues. By RT-PCR analysis, relative IKBKE expression was performed in 19 TNBC tissues and 45 Non-TNBC tissues from breast cancer patients (B). IKBKE expression was analysed in 93 TNBC tissues and 898 Non-TNBC tissues from breast cancer patients in TCGA data sets (C). (E and F) BT549 and 231 cells were co-cultured with human PBMC-derived macrophages or treated with PBMC-derived macrophages condition media (TAM CM). And then cells were treated with indicated dose of JQ1 and STAT3-IN-1 alone or in combination for 48 h. Cell apoptosis and viability were analysed by Annexin V-FITC/PI staining and CCK-8 assay respectively. (G-I) BT549 and 231 cells were transfected with IKBKE siRNA (si IKBKE), and then cells were co-cultured with human PBMC-derived macrophages (H) or treated with PBMC-derived macrophages condition media (TAM CM) (I). And then cells were treated with indicated dose of JQ1 for 48 h. The knockdown effects on IKBKE were confirmed by Western blot analysis (G). Data represented the mean ± S.D. of at least three times biological replicates. (J-M) TAM (THP1) and 231 cells were mixed and inoculated subcutaneously in nude mice. (J) STAT3 inhibitor STAT3-IN-1 significantly enhanced JQ1-induced antitumor efficacy in xenograft tumor models. (K) Average body weight changes were measured over the course of the study. (L) A TUNEL assay was used to detect the apoptotic cells in these tumor tissues. Representative images represent one of three replicates. Bars were showed on the images. (M) Treatment schedule. Data were shown as mean ± S.D. (n = 8 per group). *P < 0.05; **P < 0.01; ***P < 0.001 vs. control.

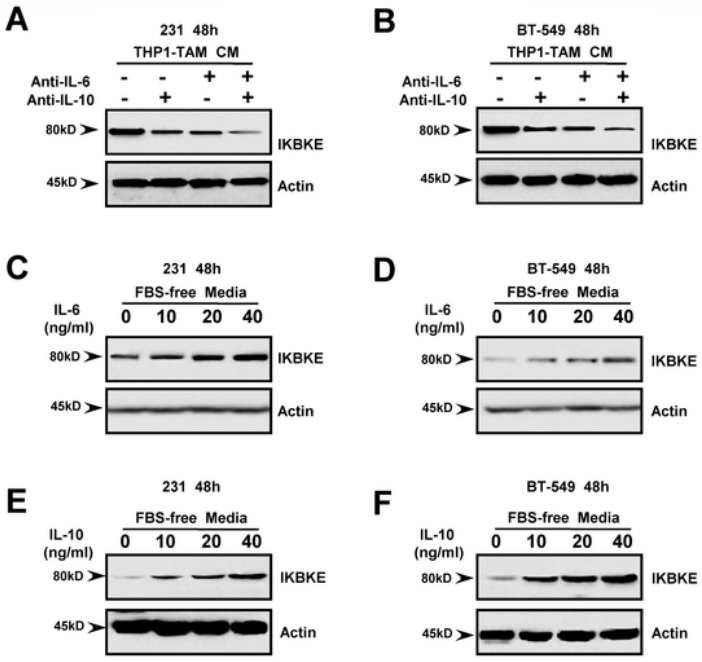

Figure 7

TAMs promote IKBKE expression in TNBC by secreting IL-6 and IL-10. (A and B) PBMC-derived macrophages condition media (TAM CM) was pre-treated with 2.5 μg/ml of anti-IL-6 and anti-IL-10 neutralizing antibody or human IgG isotype control for 1 h. Subsequently, BT549 and 231 cells were incubated with TAM CM for 48 h. The expression of IKBKE was detected by Western blot. (C-F) BT549 and 231 cells were incubated with FBS-free growth media with indicated dose of IL-6 and IL-10 for 48 h.

The expression of IKBKE was detected by Western blot. Actin was used as the loading control. Data were representative of at least three times biological replicates.

FIG8

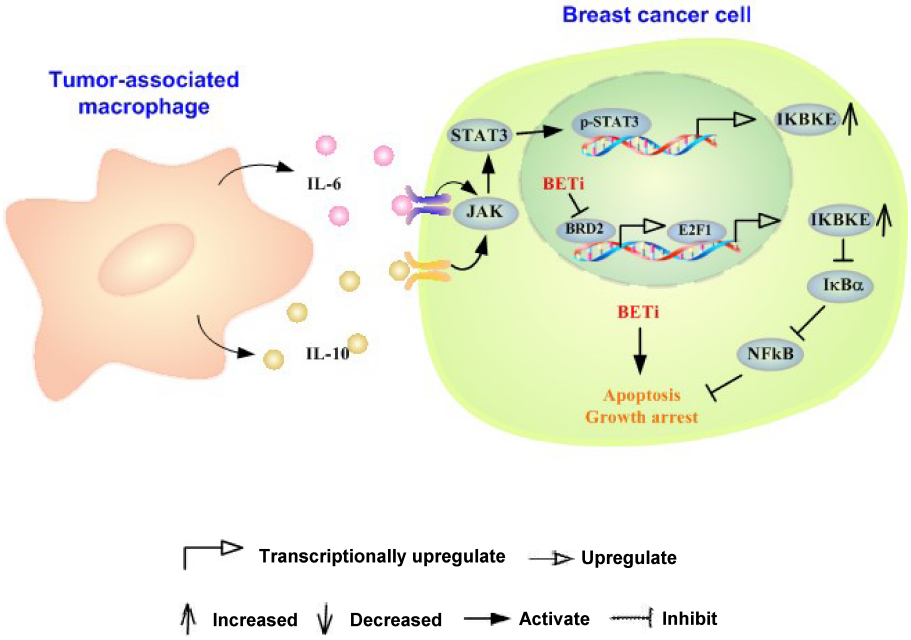

Figure 8

Model of macrophage-derived IL-6 and IL-10 inducing TNBC cells resistance to BET inhibition. BET inhibition induced TNBC cell apoptosis and growth arrest, and downregulated IKBKE expression through suppressing BRD2/E2F1 signal axis. Tumor-associated macrophage (TAM) secreted IL-6 and IL-10 to

activate JAK-STAT3 signal in TNBC cell. Activated STAT3 transcriptionally upregulated IKBKE levels. IKBKE stimulated IKBα/NF-κB signal and inhibited cell apoptosis and growth arrest.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FIGS2.tif
- supplementary.doc
- FIGS1.tif
- FIGS4.tif
- FIGS3.tif