Breast cancer cell–derived IL-35 promotes tumor progression via induction of IL-35-producing induced regulatory T cells

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Abstract

Interleukin 35 (IL-35) is a potent immunosuppressive cytokine, consisting of an Epstein–Barr virus–induced gene 3 (EBI3) subunit and a p35 subunit. IL-35 is mainly produced by regulatory T and regulatory B cells, and plays a crucial role in the development and prevention of infectious and autoimmune diseases. However, the effect of IL-35 in malignant disease is not well understood. In this study, we demonstrated that breast cancer cells (BCCs) also expressed and secreted IL-35 and higher level of IL-35 in BCCs was closely associated with poor prognosis of patients and was an independent unfavorable prognostic factor for breast cancer. Subsequent study revealed that BCC-derived IL-35 inhibited conventional T (T_{conv}) cell proliferation and further induced suppressed T_{conv} cells into IL-35-producing induced regulatory T (iTr35) cells. Furthermore, BCC-derived IL-35 promoted the secretion of inhibitory cytokine IL-10 and obviously decreased the secretion of Th1-type cytokine IFN-γ and Th17-type cytokine IL-17 in T_{conv} cells. Meanwhile, the expression of inhibitory receptor CD73 was also elevated on the surface of T_{conv} cells following the BCCs’ supernatant treatment. Mechanistically, BCC-derived IL-35 exhausted T_{conv} cells and induced iTr35 by activating transcription factor STAT1/STAT3. Hence, our results indicate functions of BCC-derived IL-35 in promoting tumor progression through proliferation inhibition of tumor-infiltrating T_{conv} cells and induction of iTr35 cells in tumor microenvironment. This study highlights that IL-35 produced by BCCs are a potential therapeutic target for breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths among Chinese women (1). In spite of improvements in surgical and pharmacological strategies, there remains high rates of recurrence and metastases of breast cancer and a major cause of tumor progression is immune suppression in cancer (2,3).

Regulatory T cells (Tregs), a unique subset of CD4+ T cells, have been found to play key roles in maintaining suppressive tumor microenvironment and thus contribute to cancer progression. Although Tregs normally comprise only ~4% of CD4+ T cells in adult peripheral blood, they can make up as much as 20−30% of the total CD4+ T cell population in tumor microenvironment (4,5). Increased numbers of Tregs are correlated with poor prognosis in various types of cancers including breast cancer (6). Two subsets of Tregs have been confirmed: naturally occurring Tregs (nTregs) that occur and develop in the thymus and induced Tregs (iTregs) that generate from natural conventional T (T_{conv}) cells in the periphery. According to the cytokines that induced them, three types of iTregs have been described: iTreg-TGF-β, iTreg-IL-10 and iTreg-IL-35 (iTr35). Immunosuppressive cytokine interleukin-35 (IL-35) induces the conversion of T_{conv} cells into IL-35-producing regulatory T (iTr35) cells.
IL-35, a novel member of IL-12 family, exists as a heterodimer of EB13 (IL-27j) and p35 (IL-12a) (7). IL-35 is mainly produced by Tregs and regulatory B cells and contributes to the immune suppression function of these cells (8,9). Recent studies have found that IL-35 is also expressed in some types of cancer cells, such as pancreatic cancer, colon cancer and hepatocellular carcinoma (10–12). In vivo studies have shown that IL-35 neutralization limits tumor growth in multiple mouse models of human cancer (13). Therefore, IL-35 has emerged as a new biomarker and a potential therapeutic target for cancer. In this study, we demonstrated that breast cancer cells (BCCs) expressed and secreted IL-35, and BCC-derived IL-35 was capable of inhibiting the proliferation of T cells and converting them into iTreg cells, which contributed to the progression of breast cancer and poor prognosis of patients.

Materials and methods

Cell lines and cell culture

Human breast cancer cell lines MDA-MB-231 (231), MCF-7, SKBR-3 and T47D were obtained from the American Type Culture Collection. HEK293T cell line was obtained from Chinese Academy of Sciences Cell Bank. All cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) medium containing 10% fetal bovine serum and 100 U/ml rhIL-2 (PeproTech). For induction of iTreg cells, rhIL-35 (50 or 100 ng/ml; PeproTech) or culture supernatants (SN) of 231 or MCF-7 at a volume 30% of the total culture volume were added to T cell culture system for 5 days.

Breast cancer patients

Eighty-six unrelated Chinese women with breast cancer who underwent lumpectomy or mastectomy were from the Second Hospital of Shandong University and Dongying People’s Hospital of Shandong Province, excluding patients receiving neoadjuvant chemotherapy or neoadjuvant radiotherapy. Clinicopathological characteristics included age of onset, histological grade, pathological tumor, node, metastases (TNM) stage, the status of estrogen receptor, progesterone receptor (PR), human epidermal growth factor receptor 2, lymph node involvement, progression-free survival and overall survival. Human study was approved by the Human Investigation Committee of the Second Hospital of Shandong University and Dongying People’s Hospital of Shandong Province, and informed consent was obtained from each study participant.

Isolation of Tconv cells

Human peripheral blood mononuclear cells from leukocyte-enriched buffy coats were separated on a Ficoll (Sigma) gradient and Tconv cells (CD4+CD25-CD45RO+) were purified by negative selection using a Naive CD4+ T Cell Isolation Kit (Miltenyi Biotec, Germany). The purity was >97% as confirmed by flow cytometry.

Immunohistochemistry

The immunohistochemistry (IHC) was used to detect Epstein-Barr virus-induced gene 3 (EB13) and p35 expression in breast cancer tissues. In brief, the paraffin-embedded tissue sections were deparaffinized followed by microwave treatment in ethylenediaminetetraacetic acid solution for 15 min. After being treated with 3% hydrogen peroxide and blocked with 10% goat serum, the consecutive tissue sections were probed with following antibodies, including anti-EB13 (5 μg/ml; Novus Biologicals) and anti-p35 (5 μg/ml; R&D Systems, Minneapolis, MN), at 4°C overnight. Then, tissue sections were incubated with the biotinylated secondary antibody, followed by performing the chromogenic reaction using a DAB Substrate Kit. The images were captured using an Olympus microscope.

All the slides were independently analyzed by two pathologists. The intensity of the staining was evaluated using the following criteria: 0, negative; 1, low; 2, medium and 3, high. The extent of staining was scored by using the following criteria: 0, 0% stained; 1, 1–25% stained; 2, 26–50% stained and 3, 51–100% stained. Five random fields (×20 magnification) were evaluated under a light microscope. The final scores were calculated by multiplying intensity and extent scores, and the samples were divided into four grades: 0, negative; 1, 1–2, low staining (+); 3–5, medium staining (+++) and 6–9, high staining (+++). The following criteria were used to quantify the expression levels of IL-35 in breast cancer tissues: high expression, both EB13 and p35 were scored as ++/+++; low expression, other than high expression.

Induction of iTreg cells

Tconv cells were activated with CD3/CD28 T cell activator (12 μg/ml; STEMCELL Technologies, Canada) and cultured in RPMI 1640 medium containing 10% fetal bovine serum and 100 U/ml rhIL-2 (PeproTech). For induction of iTreg cells, rhIL-35 (50 or 100 ng/ml; PeproTech) or culture supernatants (SN) of 231 or MCF-7 at a volume 30% of the total culture volume were added to T cell culture system for 5 days.

Cell Counting Kit-8 assay

The direct effect of BCC-derived IL-35 on Tconv cells growth was measured by Cell Counting Kit-8 (CCK-8) assay. Briefly, Tconv cells were seeded in triplicate into 96-well plates at 1 × 10⁴ cells per well with rhIL-35, or BCCs’ SN in the presence of a neutralizing anti-IL-35 monoclonal antibody (mAb) (10 μg/ml, clone 27537; R&D Systems) or not for 5 days and the fresh conditional medium was added every 2 days (14). After treatment, 10% of CCK-8 buffer (Dojindo, Kumamoto, Japan) was added to culture medium for the final 2 h at 37°C until visible color conversion occurred. The absorbance value was detected at 450 nm wavelength by a Microplate Reader (Bio-Rad, Hercules, CA). Results were representative of three independent experiments.

RNA extraction and real-time quantitative reverse transcription PCR

Total RNA was extracted from the BCCs or T cells with TRIzol reagent (Invitrogen) in accordance with the manufacturer’s protocol. Equal amounts of total RNA from each sample were then reverse-transcribed into cDNA using a Revert Ace kit (Toyobo, 538100). The following sequence-specific primers were used for PCR amplification: (i) the internal control GAPDH gene: forward, 5′-GGT GGT GCT TTC TCA GAA CAG-3′, reverse, 5′-GTT TGT GTA GGC AAA TTA TCT GTG-3′; (ii) ebi3 gene: forward, 5′-GCA GAC GCC AAC GTT CTA-3′, reverse, 5′-CCA TGG AGA ACA GGT CAT-3′; (iii) p35 gene: forward, 5′-CTT CCA TCA CTC CCA AAA C-3′, reverse, 5′-TGT CTC GCC TCC TGG AGT AT-3′. The primers used in figure 11 and J were as follows: (i) ebi3 gene: forward, 5′-TTC ATT GCC ACG TAC AGG CT-3′, reverse, 5′-GGA TGA GGT GGT GGC TTC AA-3′; (ii) p35 gene: forward, 5′-TCT CCA TGG TTC AAC TTA AAG AGG-3′. Amplification cycle conditions were set up as follows: 95°C for 60 s, followed by 40 cycles of 94°C for 5 s, 60°C for 10 s and 72°C for 15 s, with final extension of 45 s at 72°C. Data were normalized to the internal control and relative expression levels were evaluated using the 2⁻ΔΔCT method. All experiments were conducted in triplicate.

Flow cytometry and intracellular staining

For cell-surface markers, T cells were harvested and incubated with antibodies, including FITC-CD3, CD73-PE, LAG3-PE, CTLA-4-PE or appropriate isotype controls (eBioscience, San Diego, CA) for 30 min at 4°C. For intracellular cytokine detection, cells were stimulated with 1× cell stimulation cocktail (eBioscience) for 10–12 h and were fixed and permeabilized using Cytofix/Perm Buffer™ Fixation/Permeabilization Solution Kit (eBioscience). The harvested cells were stained with FITC-labeled anti-CD4 and then with PE-labeled anti-EB13, or isotype-matched control antibody (eBioscience).
Finally, the stained cells were analyzed by flow cytometry and data analysis was done using FCS Express, V3 (De Novo).

Cytokine measurement

For IL-35 level measurement, SN from BCCs were harvested and determined using human IL-35 enzyme-linked immunosorbent assay (ELISA) kits (sensitivity: 15.6 pg/ml; Cusabio, China). For cytokine analysis of T<sub>conv</sub> culture system, T<sub>conv</sub> cells were cultured for 5 d after stimulation with rhIL-35 or BCCs’ SN. Cytokine production of IL-1β, IL-4, IL-6, IL-10, IL-12p70, IL-17a, TNF-α and IFN-γ was simultaneously determined by the commercially available Human High Sensitivity Panel (eBioscience). The assay was performed according to the manufacturer’s instructions and each sample was run in duplicate.

Western blotting

The harvested cells were washed with phosphate-buffered saline and suspended in radioimmunoprecipitation lysis buffer. The protein...
concentration was evaluated using the BCA Protein Assay (Beyotime). An aliquot of total protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was further electrotransferred onto polyvinylidene difluoride membranes. After being blocked using 5% non-fat dried milk for 1 h at room temperature, the membranes were probed with anti-EBI3 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p35 (1 μg/ml; R&D Systems), anti-phospho-STAT1(Y701) (1:1000), anti-STAT1 (1:1000), anti-phosphoSTAT3(1:1000), anti-STAT3 (1:1000), anti-phospho-STAT4(Y709) (1:1000), anti-STAT4 (1:1000) and anti-GAPDH (1:1000) (Cell Signal Technology, Beverly, MA) antibodies overnight at 4°C, followed by treatment with horseradish-peroxidase-conjugated secondary antibodies. The signals of labeled proteins were detected by enhanced chemiluminescence (Life Technologies, Carlsbad, CA).

CRISPR/Cas9 targeting and generation of lentiviral constructs

Single guide RNAs (sgRNA) targeted to ebi3 and p35, respectively, were designed and then cloned into pLenti CRISPR V2 (Hu6-sgRNA-EF1a-CAS9-puro) vectors (LncBio Shanghai, China). All plasmids were sequenced to confirm successful ligation (Supplementary Figure 2, available at Carcinogenesis Online). The following sgRNA sequences were utilized: (i) sg ebi3#1: 5'-GGGCGTGGACATGGAACCTGG-3', (ii) sg ebi3#2: 5'-GAACCCTTGACATCGTGATAG-3', (iii) sg ebi3#3: 5'-TGACCGTTGCCCTGGTCCAGG-3', (iv) sg p35#1: 5'-GGATTACATCTGAAACCTG-3', (v) sg p35#2: 5'-TAAACCAGTGCTCAGTTGG-3', (vi) sg p35#3: 5'-GATAAAA TCTAGATTTTGTGGA-3'.

The HEK293T cells were maintained as recommended by the manufacturer in 100 mm dishes. For each transfection, 4 μg CRISPR/Cas9 vector carrying the construct of interest, 4 μg psPAX2 envelope plasmid and 2 μg PMD2.G packaging plasmids were used. The transfection was carried out using jetPRIME (Polyplus, New York) following the manufacturer’s recommendations. Virus was harvested at 48 h post-transfection.

Generation of recombinant stable cell lines

To knock out gene expression of ebi3 or p35, MDA-MB-231 cells were transduced with CRISPR/Cas9 virus for 24 h using 8 μg/ml polybrene (LncBio). Two days after transduction, selection of recombinant cell lines was performed in the presence of 2.0 μg/ml puromycin (Solarbio, Beijing, China).

Statistical analysis

IHC scores of different subgroups were compared by chi-square test. Kaplan-Meier curves were analyzed for relevant variables. The log-rank test was used to analyze the differences in survival time among the patient subgroups. The Cox’s proportional hazard regression model was used to evaluate risk factors associated with the prognoses. Pearson’s correlation coefficient (r value) was calculated assuming linear relationship between variables. The data represent the mean values ± the standard error of mean. Comparison between groups was performed using unpaired Student’s t-test with GraphPad Prism5 software. A P ≤ 0.05 (two-sided) was considered statistically significant.

Results

IL-35 was expressed in breast cancer tissues and was positively associated with poor prognosis

We first examined the expression of IL-35 in breast cancer tissues. Since IL-35 is a heterodimeric protein, which is composed of EBI3 and p35 subunits, we detected the expression of EBI3 and p35 using serial sections by IHC. As shown in Figure 1A, EBI3 and p35 were co-expressed in the cytoplasm of most cancer cells and part of tumor-infiltrating lymphocytes. Furthermore, the expression of EBI3 and p35 was correlated strongly (r = 0.77, P < 0.0001) (Figure 1B and C). These results demonstrated that BCCs expressed IL-35.

Table 1. Association between IL-35 expression levels and clinico-pathological features of breast cancer patients

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<th>χ²</th>
<th>P-value</th>
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<tr>
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<td>17</td>
<td>7.878</td>
<td>0.005**</td>
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<tr>
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<td>25</td>
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<td>15</td>
<td>25</td>
<td>7.878</td>
<td>0.005**</td>
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<td>29</td>
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<td>27</td>
<td>16</td>
<td>7.878</td>
<td>0.005**</td>
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<tr>
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<td>4.654</td>
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<tr>
<td>Positive</td>
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<td>23</td>
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According to 7th edition of UICC TNM classification of malignant tumors. Bold values in italics are statistically significant. *P ≤ 0.05, **P ≤ 0.01.
similar function, we tested the inhibitory effect of BCCs’ SN on T
conv cell proliferation using neutralizing IL-35 mAb and CCK-8 assay. As shown in Figure 2A, both SN from 231 and MCF-7 cells markedly suppressed the proliferation of T
conv cells and IL-35-blocking could partially reverse these effects. Therefore, BCCs’ SN inhibited T
conv cell proliferation in an IL-35-dependent manner.

BCC-derived IL-35 induced iTr35 cells

Given our finding that IL-35 secreted by BCCs suppressed the proliferation of T
conv cells strongly, we next sought to determine whether BCC-derived IL-35 could convert T
conv cells to iTr35 cells. We monitored that, compared with control, T
conv cells activated with anti-CD3/CD28 in the presence of BCCs’ SN simultaneously upregulated the expression of IL-35 at both mRNA and protein levels (Figure 2B–D). Single-cell analysis by intracellular cytokine staining also suggested that treatment with BCCs’ SN or rhIL-35 induced the expression of IL-35 in T
conv cells (Figure 2E). These data confirmed that BCC-derived IL-35 converted T
conv cells into suppressive iTr35 cells.

Cytokine secretion profiles of T
conv cells treated by BCCs’ SN

Next, we analyzed the cytokine secretion profiles of T
conv cells treated with BCCs’ SN, including IL-1β, IL-4, IL-6, IL-10, IL-12p70, IL-17a, TNF-α and IFN-γ (Figure 3A–H). Secretion of IL-10 and IL-12p70 was obviously upregulated, whereas the secretion of Th1-type cytokine IFN-γ and Th17-type cytokine IL-17 was markedly depressed in both IL-35 groups and BCCs’ SN-treated groups (Figure 3A–D). Furthermore, compared with BCCs’ SN-treated T
conv cells, the addition of IL-35 functional mAb reversed these effects. In addition, we also found that the levels of IL-1β and IL-4 were significantly increased in BCCs’ SN-treated groups compared with those in the control group, but these changes were uncorrelated with IL-35 treatment (Figure 3E and F).

**Table 2. Multivariate analysis of prognostic factors associated with overall survival of breast cancer patients**

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<tr>
<th>Variables</th>
<th>Unfavorable versus favorable</th>
<th>Overall survival</th>
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<td></td>
<td>Hazards ratio (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>IL-35</td>
<td>Negative/low versus high</td>
<td>3.779(1.046–13.653)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>≥ 60 versus &lt; 60</td>
<td>2.843(1.035–7.811)</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Well/moderate versus poor</td>
<td>5.055(1.361–18.774)</td>
</tr>
<tr>
<td>lymph node involvement</td>
<td>Negative versus positive</td>
<td>4.893(1.344–17.811)</td>
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Bold values in italics are statistically significant . *P ≤ 0.05.

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Figure 2. BCC-derived IL-35 inhibited proliferation of T
conv cells and induced iTr35. Purified CD4+CD25−CD45RO+T
conv cells were cultured with IL-35 or BCCs’ SN, and anti-CD3/CD28 for 5 days. (A) CCK-8 assays showed the effects of BCCs’ SN on proliferation of T
conv cells. (B–D) All cells were collected for analysis of IL-35 level by real-time RT-PCR (B, C) and western blot (D), respectively. (E) Flow cytometry quantification of IL-35 in cells after activation for 10–12 h with the cell-stimulation cocktail, then cells were stained with anti-EBI3 or isotype control antibody. Data are representative of at least three independent experiments and presented as mean ± SEM of three replicates (A–C). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.
Inhibitory receptor expression on T<sub>conv</sub> cells treated by BCCs' SN

It has been reported that nTregs express surface inhibitory receptors (IRs), such as CD73, LAG3 and CTLA-4, which contribute to the suppressive function of Tregs themselves (15–17). In this study, we also detected the surface expression of IRs on T<sub>conv</sub> cells treated by BCCs' SN. As shown in Figure 3I, the percentage of CD73<sup>+</sup> T<sub>conv</sub> cells is upregulated in BCCs' SN-treated groups. However, BCCs' SN treatment had no effect in the induction of LAG3 and CTLA-4, which had been described as mediators of nTregs suppression.

BCC-derived IL-35 signaled through STAT1 and STAT3

On the basis of the earlier results, we further explored the molecular mechanisms involved in T<sub>conv</sub> cell proliferation inhibition and iTT35 cell induction by BCC-derived IL-35. The STAT protein family played a pivotal role during cellular differentiation and immunoregulation (18). Published reports showed that both IL-27 and IL-12, which shared subunits with IL-35, induced the phosphorylation of STAT1, STAT3 and STAT4 (19). The treatment of mouse T<sub>conv</sub> cells with IL-35 resulted in intracellular phosphorylation of STAT1 and STAT4 (20). In this study, we examined...
the phosphorylation status of STAT1, STAT3 and STAT4 in $T_{\text{conv}}$ cells treated by BCC-derived IL-35 by western blot. As shown in Figure 4A, increased p-STAT1 and p-STAT3 expressions were observed in IL-35-treated groups and BCCs’ SN-treated groups compared with control $T_{\text{conv}}$ cells, and the addition of IL-35-blocking mAb in BCCs’ SN-treated groups could partially reverse these effects. Moreover, the expression of p-STAT4 was not detectable in all groups (Figure 4A). These indicated that BCC-derived IL-35 inhibited $T_{\text{conv}}$ cell proliferation and converted $T_{\text{conv}}$ cells into iT$\lambda$35 cells through activation of STAT1/STAT3 pathway.

**Discussion**

Tumor progression is a multistep process depending on both tumor behavior and the immune function of the host. Immune function is generally compromised in most patients with cancer (21–23). It is reported that there are increased numbers of functionally suppressive Tregs in the peripheral blood and tumor microenvironment of patients with cancer, including breast cancer, colorectal cancer and lung cancer (24–26). Moreover, elevated Tregs number in tumor microenvironment is coincident with an increase of intratumoral IL-35$^+$Tregs level and these IL-35$^+$Tregs are significantly associated with cancer development and progression (4,5). In this study, we found that BCCs also expressed and secreted IL-35 in addition to nTregs and iTr35 cells. Furthermore, elevated IL-35 expression in BCCs was significantly correlated with more aggressive tumor phenotypes, including poor differentiation, PR-negative expression and estrogen receptor-negative expression. We also assessed the prognostic value of IL-35 by a long-term follow-up investigation and found that BCC-derived IL-35 was an independent unfavorable prognostic factor for breast cancer.

As an inhibitory cytokine, IL-35 has three known biological functions: suppression of T-cell proliferation, the conversion of $T_{\text{conv}}$ cells into iT$\lambda$35 cells and downregulation of Th17 cell development and differentiation (14,27). To determine whether
BCC-derived IL-35 also have these effects, we first examined the proliferation inhibition of T\textsubscript{core} cells. The results showed that BCC-derived IL-35 obviously suppressed the proliferation of T\textsubscript{core} cells. Meanwhile, BCC-derived IL-35 also induced the conversion of these suppressed T\textsubscript{core} cells into iT\textsubscript{Tr}35 cells in an IL-35-dependent manner. The induction of iT\textsubscript{Tr}35 cells further raised the possibility of more IL-35 accumulation and infectious tolerance in tumor environment, which orchestrated a positive feedback loop contributing to maximal immunosuppressive effect. Moreover, the inhibitory effect of BCC-derived IL-35 on T\textsubscript{core} cells could be blocked by IL-35 mAb treatment, which further underlined the role of BCC-derived IL-35 in the inhibition of antitumor immunity and provided possibility for using of IL-35 mAb in breast cancer therapy. Our results suggest that BCC-derived IL-35 may promote tumor progression by exhausting tumor infiltratory T\textsubscript{core} cells and enhance iT\textsubscript{Tr}35 cells population in breast cancer microenvironment.

Another important finding of this study was that BCC-derived IL-35 treatment led to the change of cytokine production in T\textsubscript{core} cells. A previous study showed that both T\textsubscript{core} cells and IL-35-treated T\textsubscript{core} cells had similar production of cytokines in a mouse model (14). However, in contrast to the previous report, our study demonstrated that BCC-derived IL-35 induced higher levels of IL-10 and IL-12 (IL-12p70) secretion and lower levels of IFN-\gamma and IL-17a secretion in T\textsubscript{core} cells, compared with control group in humans. It was known that IL-12 shared p35 subunit with IL-35; therefore, we speculated that evaluated IL-12p70 level might be attributed to high level of IL-35 that was secreted by BCCs and iT\textsubscript{Tr}35 cells induced by BCC-derived IL-35. On the other hand, increased secretion of IL-10 could promote T\textsubscript{core} cells to differentiate into inhibitory iT\textsubscript{Tr}IL-10 cells and suppress the function of Th1 and Th17 cells (28). And it was further confirmed by our results that Th1-type cytokine IFN-\gamma and Th17 cytokine IL-17a were dramatically decreased in both rHL-35 treatment group and BCC-derived IL-35 treatment group. IFN-\gamma and IL-17a played important antitumor roles by inducing the differentiation of Th1 and Th17 cells, respectively, and inhibiting Th2 formation (29,30). The earlier results proved that BCC-derived IL-35 not only directly suppressed the proliferation of Th1 and Th17 cells, but also indirectly inhibited their functions by promoting the secretion of IL-10 in T cells, which further clearly pointed out the role of BCC-derived IL-35 in maintaining suppressive tumor microenvironment. However, IL-35 mAb treatment rebounded IFN-\gamma and IL-17a production of T\textsubscript{core} cells and brought about the recovery of BCC-derived IL-35-induced proliferation inhibition of T\textsubscript{core} cells.

It has been reported that surface IRs LAG3, CD73 and CTLA-4 mediated the immunosuppression function of nTregs (13,31,32). In this study, we also tested whether these membrane IRs changed in rHL-35-treated and BCCs’ SN-treated T\textsubscript{core} cells. The results showed that BCCs’ SN facilitated CD73 expression of T\textsubscript{core} cells. In general, Tregs preferentially express CD73, which converts adenosine monophosphate to highly immunosuppressive adenosine and enhances suppressive function of Tregs (33). Therefore, elevated CD73 may promote inhibitory function of BCCs’ SN-induced T\textsubscript{core} cells. Nevertheless, no difference on the surface expression of LAG3 and CTLA-4 was observed between rHL-35-treated or BCCs’ SN-treated T\textsubscript{core} cells, and control group. This result was supported by the work of Collison LW that multi-inhibitory receptors were at same level in both T\textsubscript{core} cells and iT\textsubscript{Tr}35 cells (14).

Lastly, we explored the molecular mechanisms involved in the induction of iT\textsubscript{Tr}35 cells by BCC-derived IL-35. As IL-35 receptor uses IL-12R\beta2 and gp130, it is possible that signaling is mediated via the STAT family of transcription factors (20). In fact, previous studies from mouse models revealed that IL-35 induced the transcription of STAT1 and STAT4 in T\textsubscript{core} cells and STAT1 and STAT3 in B cells (8,20). In humans, only STAT1 and STAT3 were activated by IL-35 expressed by tumor-infiltrating Tregs in colorectal cancer during the induction of iT\textsubscript{Tr}35 cells (24). Our previous results confirmed that IL-35 treatment of human monocyte-derived dendritic cells resulted in phosphorylation of STAT1 and STAT3 (34). These data suggested the possibility that IL-35 might use different signaling components in different species or different types of cells, or different results derived from different sources of IL-35. In this study, we demonstrated that STAT1 and STAT3 but not STAT4 were involved in the induction of iT\textsubscript{Tr}35 cells by BCC-derived IL-35. STAT1 is a well-known transcription factor, which plays a critical role in the generation of immunosuppressive function of Tregs and regulatory B cells (8,35,36). On the other hand, activation of STAT3 is important for enhancing human iTreg phenotype and function (37). Therefore, the activation of both STAT1 and STAT3 led to the phenotypic differentiation and functional performance of iT\textsubscript{Tr}35 cells induced by BCC-derived IL-35.

In conclusion, BCCs expressed and secreted IL-35. BCC-derived IL-35 inhibited T\textsubscript{core} cell proliferation and further converted suppressed T\textsubscript{core} cells into iT\textsubscript{Tr}35 cells via activation of STAT1/STAT3 signaling pathway. This might constitute a positive feedback loop: BCC-derived IL-35 inhibited the proliferation of tumor-infiltrating lymphocytes and further induced them into iT\textsubscript{Tr}35 cells, which subsequently produced more IL-35 and iT\textsubscript{Tr}35 cells in tumor microenvironment (Figure 4B). By this means, BCCs promoted their own development and progression and led to the poor prognosis of breast cancer patients. Our study provided evidence for a novel tumor-evading mechanism in breast cancer and suggested that IL-35 might be a potential therapeutic target for the treatment of breast cancer.

Supplementary material
Supplementary data are available at Carcinogenesis online.

Funding
Natural Science Foundation of China (31570919, 31270970 and 81602476); Taishan Scholar Foundation; the Science and Technology Project of Shandong, China (2008GQ10002035 and 2012G0021821); Science and Technology Project of Jinan of China (201202197).

Conflict of Interest Statement None declared.

References