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Original Article

Overexpressed indoleamine 2, 3-dioxygenase induced T cell apoptosis via Fas/FasL and Bcl-2/Bax-related apoptotic pathways in metastatic breast cancer

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Abstract: IDO is reported to be related to regulatory T cells (Tregs) infiltration and promoted tumor progression, however, the direct influence of breast cancer-derived IDO on the apoptosis of T cells is remains unclearly. In this study, pIRES2-EGFP-IDO and pIRES2-EGFP empty vectors were cloned and transfected with breast cancer 4T1 cell lines by method of lipofection. Meanwhile, T cells isolated from the mice spleen were co-cultured with untreated 4T1 cells, pIRES2-IDO-4T1 cells and pIRES2-EGFP-4T1 cells for 48 h to explore the effects of IDO on T cells. The expression of IDO was detected by RT-PCR and Western Blot, and then the apoptosis of co-cultured T cell lines were analyzed by flow cytometry, using simple-T cells as control. Moreover, the expressions of apoptosis-related proteins including Fas/FasL, Bcl-2/Bax were assessed by Western blot, followed by analysis of BcL-2/Bax ratio. The results revealed that IDO was expressed in pIRES2-IDO-4T1 cells, and T cells in pIRES2-IDO-4T1/T cells co-culture system showed significantly increased apoptosis ($P<0.01$) compared with simple-T cells, 4T1/T cells and pIRES2-EGFP-4T1/T cells. Furthermore, Fas, FasL and Bax were significantly up-regulated in pIRES2-IDO-4T1/T cells co-culture system in comparison with simple-T cells, while Bcl-2 was markedly decreased ($P<0.01$). BcL-2/Bax ratio in pIRES2-IDO-4T1/T cells co-culture system was significantly reduced ($P<0.01$). To conclude, our results implied that IDO may trigger the apoptosis of T cells by regulating Fas/FasL and Bcl-2/Bax-related apoptotic pathways.

Keywords: Indoleamine 2, 3-dioxygenase, breast cancer, T cell, apoptosis

Introduction

As a malignancy occurred in the glandular epithelial tissue of the breast, breast cancer has become the most commonly diagnosed cancer and the major leading cause of cancer-related death among females, comprising 23% of the total cancer cases and 14% of the cancer deaths [1]. Currently, breast cancer affects approximately 12% of women worldwide [2]. In the past two decades, mortality rates of breast cancer have been decreasing in many developed countries, however, increasing incidence and mortality have been seen in most developing countries [3]. Breast cancer is characterized by distant metastasis which is closely correlated with the main cause of death. The pathogenesis of breast cancer reflects not only the consequence of somatic mutations aberrantly regulating oncogenes and tumor suppressor genes, but also the effect of the abnormal function of tumor micro-environment.

The tissue microenvironment where a tumor arises prevents tumor development and progression. Indoleamine 2, 3-dioxygenase (IDO) has been found to act at multiple levels to create a more hospitable environment for tumor development and metastasis. IDO is an enzyme that catalyzes the first and rate-limiting step in tryptophan catabolism to N-formylkynurenine, leading to T cell proliferation suppression [4]. Moreover, degradation of tryptophan may also contribute to the inhibition of T cell proliferation and lead to cancer escaping from immune surveillance in tissue microenvironments [5-8]. Human IDO gene has been approved to be overexpressed in multiple kinds of cancers, including colon cancer [9], gastric cancer [10], endometrial cancer [11], hepatic cancer [12], lung cancer [13], ovarian cancer [14], and renal cancer [15]. Further Reports demonstrated that IDO expression was positive related with focal T cell infiltration, tumor size, distal metastasis, lymph nodes metastasis and

vascular invasion [9, 11, 12, 14]. The mechanism of IDO-related cancer metastasis has been proved to induce the decreasing of IDO-induced T cell activation, proliferation, IFN-γ secretion [16], and $CD8^+$ cytotoxic T cells in *in vitro* co-culture assay [17]. Meanwhile, previous studies have suggested that up-regulated expression of IDO in primary breast cancer promotes breast cancer progression and correlates with increase of infiltrated Tregs and lymph node metastasis [18, 19].

Although overexpression of IDO have been proposed to be correlated with Tregs infiltration and promoted tumor progression, the direct influence of breast cancer-derived IDO on T cells apoptosis has not been clearly elucidated in breast cancer yet. Thus, in this study, IDO-overexpressed breast cancer 4T1 cell lines were co-cultured with T cells to investigate the relationship between IDO and T cell survival and explored the mechanism of IDO function of T cell apoptosis in tumor microenvironment, providing a new sight into the development of novel and efficient IDO-targeted therapies of breast cancer treatment.

Materials and methods

Cell lines

Balb/c mice (6-8 weeks) were kept in the central animal care facility in Tianjin Medical University (Tianjin, China) with food and water. Mice were sacrificed and spleen T lymphocytes were isolated from the spleens. Metastatic breast cancer 4T1 cell lines were provided by Department of Immunology from Tianjin Medical University. The whole procedure was performed in sterile condition. Single cell suspension was prepared from the spleens excised from the Balb/c mice. Red blood cells were lysed and the cells were suspended in 0.9% saline with bovine serum albumin (BSA). The peripheral blood mononuclear cells were separated by ficoll. Further, $CD3^+$ T cells were separated by CD3 Micro Beads (Miltenyi-Biotec, Bergisch Gladbach, Germany) and incubated with RPMI-1640(Gibco, Grand Island, NY) containing 10% FBS (HyClone, Logan, UT) and 100 U/ml IL-2 at 37°C. All mice in this study were used in accordance with the guidelines issued by the Chinese Council on Animal Care and animal experimental protocol approved by the ethical committee of Tianjin Medical University.

Vectors and transfection

PIRES2-EGFP-IDO and pIRES2-EGFP control vectors were designed and cloned by Takara Biotechnology (Dalian) Co., LTD. Plasmids were transfected into 4T1 cells by Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Briefly, 4T1 cells were suspended at 2×10^6/ml in culture medium and seeded into culture bottle. PIRES2-EGFP-IDO and pIRES2-EGFP vectors were stably transfected into 4T1 cells by Lipofectamine 2000 until the cells reached 80-90% confluence. After further culturing, stably expressed plasmids of each group were selected and analyzed.

Cell flow cytometry

T cells were co-cultured with untreated 4T1 cells, pIRES2-IDO-4T1 cells and pIRES2-EGFP-4T1 cells for 48 h. After centrifuging, the detection of apoptosis was performed by flow cytometry FACScan (BD Company, Franklin Lakes, NJ, USA) using the Annexin V-FITC apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) and Anti-CD3/PE antibody (Santa Cruz Biotechnology, Santa Cruz, CA), following the manufacturer's instructions.

RT-PCR

Total RNA was extracted from 4T1 cells using Trizol reagent (Invitrogen, Carlsbad, USA), The primers (Takara Biotechnology Co., LTD., Dalian, China) for amplification of IDO gene were 5'-CACCATGGCGTATGTGTGGAA-3' (forward) and 5'-TGCCAGGACACAGTCTGCATAAG-3' (reverse), 138 bps, while the primers for amplification of β-actin were 5'-CCTGGGCATGGAGTCCTGTG-3' (forward) and 5'-AGGGGCCGGACTCGTCATAC-3 (reverse), 131 bps. Cycle parameters were 90°C for 10 min, followed by 35 cycles of 90°C for 30 s, 58°C for 30 s and 75°C for 45 s, with a final extension at 72°C for 10 min. Amplified products were further analyzed by agarose gel electrophoresis.

Western blot

Total cell lysate was extracted from cells transfected with pIRES2-EGFP-IDO plasmid or

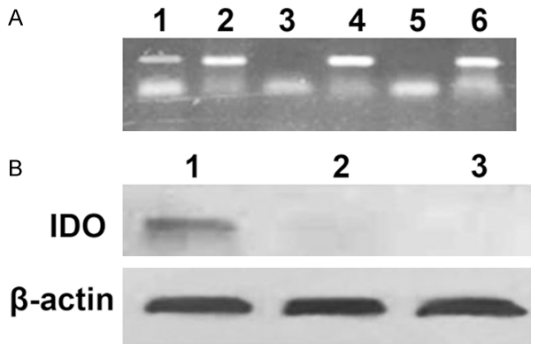

Figure 1. The expression of IDO in 4T1 cells. A. The expression of IDO mRNA in 4T1 cells was detected by RT-PCR (Lane 1, 3, 5 were PCR products amplified by primers for IDO, lane 2, 4, 6 were PCR products amplified by primers for β-actin. 1, IDO could be detected in 4T1 cells transfected with pIRES2-IDO; 3, IDO could not be detected in pIRES2-EGFP-4T1 cells; 5, IDO could not be detected in untreated cells). B. The expression of IDO protein in 4T1 cells was detected by western blot (1, IDO expressed in pIRES2-IDO-4T1 cells, 2, IDO unexpressed in pIRES2-EGFP-4T1 cells, 3, IDO unexpressed in untreated 4T1 cells). β-actin was also detected in those cell lysates as loading control.

pIRES2-EGFP vector using RIPA buffer (PBS containing 1% nonidet P-40, 0.5% sodium deoxycholate acid, 0.1% SDS, PMSF (100 ng/ml), and aprotinin (66 ng/ml)). Proteins were electrophoresed by 12% SDS-PAGE and transferred onto the nitrocellulose membranes which were blocked by 5% milk. Subsequently, IDO protein were detected using rabbit anti-mouse antibody and goat anti-rabbit-HRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Fas/FasL, Bcl-2/Bax proteins were incubated with corresponding rabbit anti-mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit IgG-HRP antibodies. Actin protein was detected using a mouse anti-actin mAb (Chemicon International, Temecula, CA) and anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) with the standard blotting and detection techniques.

Statistical analysis

SPSS15.0 software was conducted to statistical analysis. The experimental data were shown as mean ± SD. One-way ANOVA was used with Tukey's multiple comparison tests for multiple groups. The significance level was set at α=0.05.

Results

IDO expression could be detected in 4T1 cells transfected with pIRES2-IDO

As shown in **Figure 1**, the expression of IDO could be detected in mRNA level by RT-PCR, and IDO protein could be observed in pIRES2-IDO-4T1 cell lines rather than in pIRES2-EGFP-4T1 cell lines or untreated 4T1 cells detected by Western Blot assays.

IDO induced T cell apoptosis

In order to investigate the effect of IDO on T cell survival, T cells were co-cultured with pIRES2-IDO-4T1 cells, pIRES2-EGFP-4T1 cells and 4T1 cells. After 48 h-co-culturing, T cell apoptosis was analyzed by flow cytometry (**Figure 2A-D**). 45.7% apoptotic cells were found in pIRES2-IDO-4T1/T cells co-culture system, while only 34.4%, 33.6% and 11.2% in pIRES2-EGFP-4T1/T cells, 4T1/T cells and simple-T cells (untreated cells). The apoptotic rate in pIRES2-IDO-4T1/T cells group significantly increased 0.33, 0.36 and 3.08 folds compared with pIRES2-EGFP-4T1/T cells, 4T1/T cells and simple-T cells, respectively (P<0.05). As shown in **Figure 2E**, apoptotic cells were significantly increased in pIRES2-IDO-4T1/T cells when comparing with other groups.

IDO triggered T cell apoptosis through both intrinsic and extrinsic apoptosis pathways

Fas antigen is a death domain-containing member belonging to the tumor necrosis factor receptor (TNFR) family. Fas and Fas ligand (FasL) have been proved to play a central role in mediating apoptosis and to be implicated in the pathogenesis of various malignancies and diseases of the immune system. In addition, Bcl-2-family proteins and its multidomain protein Bax are central regulators of cell life and death.

Therefore, the expression of Fas/FasL, and Bcl-2/Bax were evaluated after 48 h-co-culturing. As shown in **Figure 3**, the expression of Fas, FasL and Bax were all up-regulated in T cells in pIRES2-IDO-4T1/T cells co-culture system, while the expression of Bcl-2 was down-regulated. The differences (using pIRES2-EGFP-4T1/T cells co-culture system as control) were all statistically significant (P<0.05).

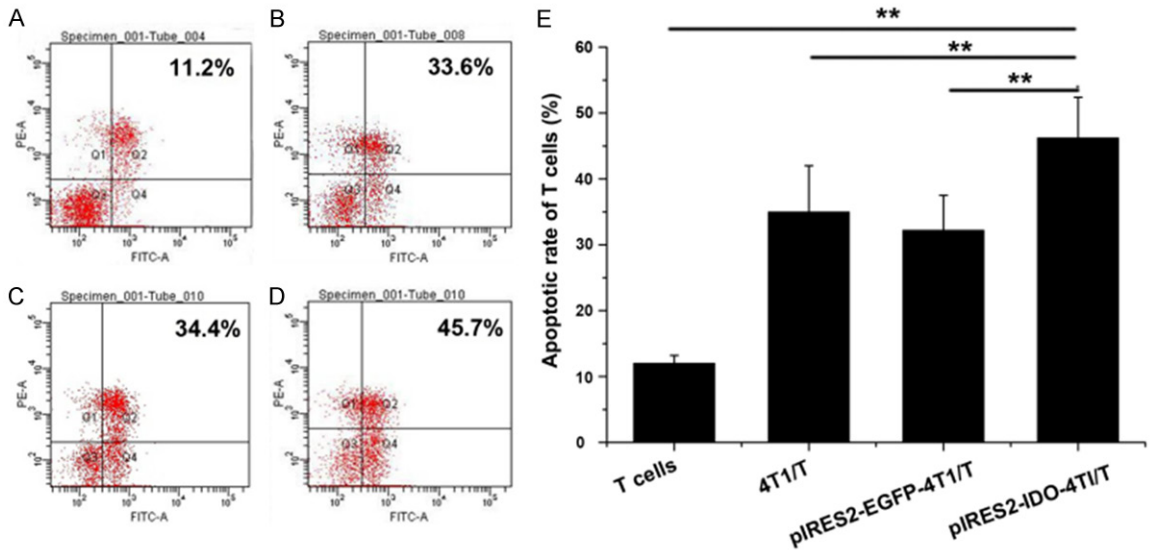

Figure 2. Apoptosis analysis of T cells. A. 11.2% apoptotic rate was detected in single T cells (without co-culture); B. 33.6% apoptotic rate was detected in 4T1/T cells co-culture system; C. 33.4% apoptotic rate was detected in pIRES2-EGFP-4T1/T cells co-culture system; D. 45.7% apoptotic rate was detected in pIRES2-IDO-4T1/T cells co-culture system. E. Semi-quantitatively for T cell apoptosis of 4 groups. $^{**}P<0.01$, compared with pIRES2-IDO-4T1/T cells co-culture system.

IDO induced disturbed balance of BcL-2/Bax ratio

Previous studied have demonstrated that the Bax/Bcl-2 ratio determines cell apoptosis. Thus, we analyzed the ratio of Bax/Bcl-2 in single T cells, 4T1/T cells co-culture system, pIRES2-EGFP-4T1/T cells co-culture system and pIRES2-IDO-4T1/T cells co-culture system. As shown in **Figure 4**, the ratio of BcL-2/Bax was obviously decreased in pIRES2-IDO-4T1/T cells co-culture system compared with that in T cells. Additionally, there were significant differences between 4T1/T cells co-culture system, pIRES2-EGFP-4T1/T cells co-culture system and pIRES2-IDO-4T1/T cells co-culture system.

Discussion

It has been observed that IDO was often over-expressed in malignant cancer patients [20]. However, poorly understing of breast cancer-derived IDO on T cells apoptosis remains. In our study, we therefore attempted to co-cultured overexpressed IDO 4T1 cell lines with T cells to detect T cell apoptosis, and expressions of Fas/FasL and Bcl-2/Bax. Our results demonstrated that higher level of IDO in breast cancer 4T1 cells significantly induced T cell apoptosis; meanwhile Fas, FasL and Bax were up-regulated, while Bcl-2 was decreased. These outcomes suggested that overexpressed IDO induced the apoptosis of T cells via Fas/FasL and BcL-2/Bax-associated cell apoptotic pathways.

Apoptosis and related forms of cell death have central importance in tumor development, homeostasis, tumor surveillance, and the function of immune system. Apoptosis may be triggered via cell-intrinsic traits such as immortalization, non-responsiveness to inhibitory growth signals, and apoptosis resistance, as well as cell-extrinsic traits including angiogenesis, invasive and metastatic capabilities, and immune escape. The intrinsic pathway emerges from mitochondria, whereas in the extrinsic pathway, apoptosis is activated by the interaction of ligands with receptors belonging to the tumor necrosis factor death-receptor (TNFR) superfamily. The most commonly investigated apoptotic induction pathway is the Fas/FasL pathway, in which Fas Ligand (FasL) induces apoptosis in Fas-expressing cells.

Fas (also called Apo-1 or CD95), is a death domain-containing member of the TNFR. It plays a critical role in physiologically regulating programmed cell death and has been involved

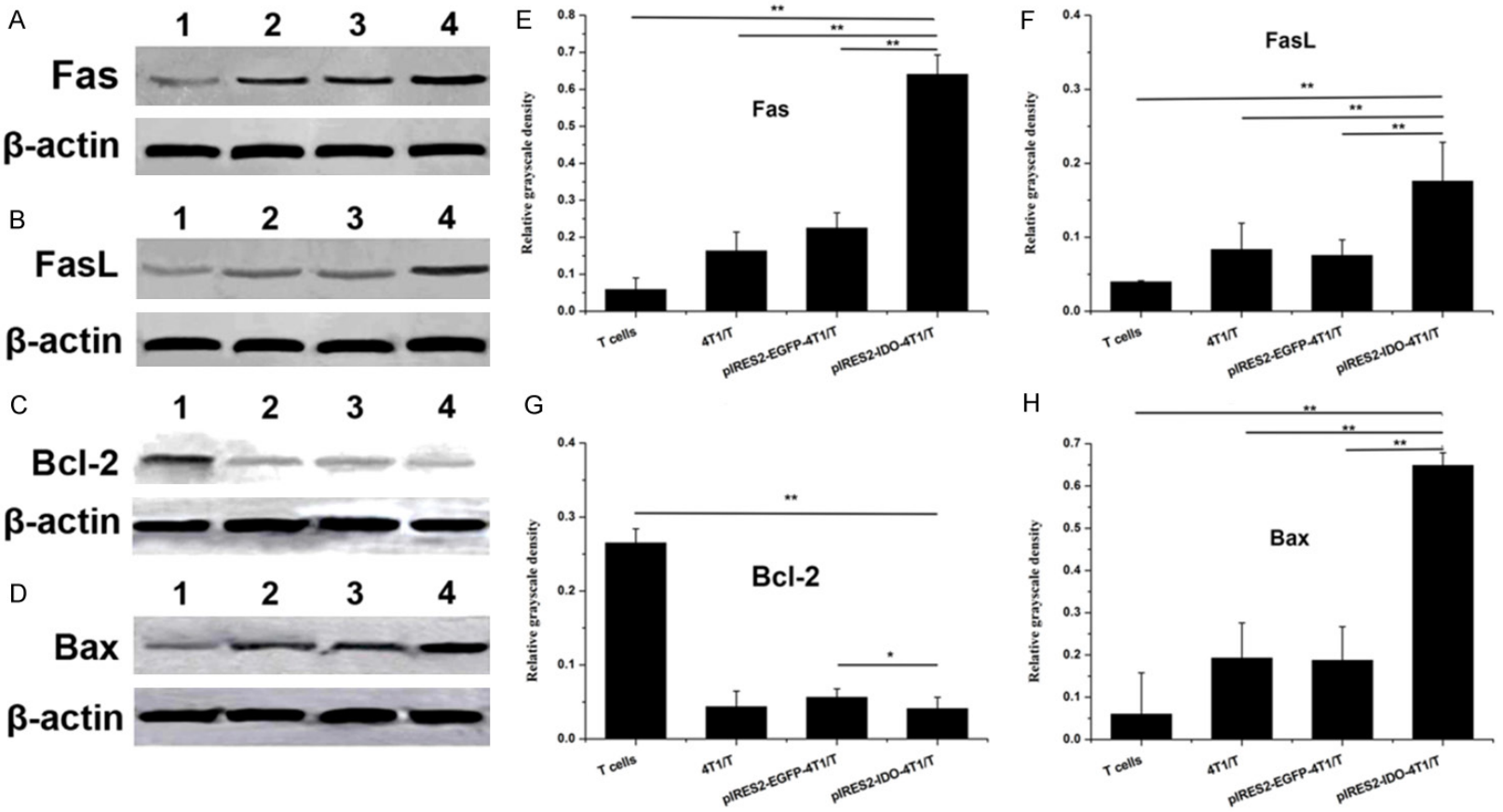

Figure 3. The expression of apoptosis related proteins in T cells. A. The expression of Fas protein; B. The expression of FasL protein; C. The expression of Bcl-2 protein; D. The expression of Bax protein. E. Semi-quantitatively for Fas; F. Semi-quantitatively for FasL; G. Semi-quantitatively for Bcl-2; H. Semi-quantitatively for Bax. (1). Single T cells; (2). 4T1/T cells co-culture system; (3). PIRES2-EGFP-4T1/T cells co-culture system; (4). PIRES2-IDO-4T1/T cells co-culture system. $^{*}P<0.05$, $^{**}P<0.01$, compared with pIRES2-IDO-4T1/T cells co-culture system.

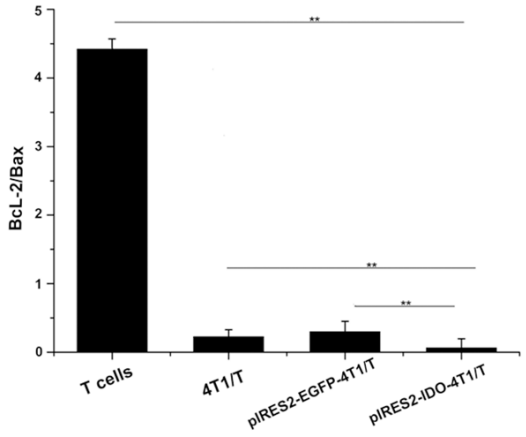

Figure 4. BcL-2/Bax ratio in single T cells, 4T1/T cells co-culture system, pIRES2-EGFP-4T1/T cells co-culture system and pIRES2-IDO-4T1/T cells co-culture system. $^{**}P<0.01$, compared with pIRES2-IDO-4T1/T cells co-culture system.

in the pathogenesis of various malignancies and diseases of the immune system [21]. The ligand of Fas, FasL regulates lymphocyte interactions, and constitutive FasL expression by certain human and mouse malignant cells may contribute to immune evasion. Furthermore, co-expression of Fas and FasL by activated T cells leads to the phenomenon of activation-induced cell death [22]. Therefore, we examined the expression of Fas and FasL in T cells co-cultured with IDO-overexpressed 4T1 cells. The results showed that up-regulated expressions of Fas and FasL were both found after 48 h-coculturing. Additionally, posttranslational modifications of FasL have profound influence on its activity because only membrane-bound or multimerized FasL induce cell death, whereas soluble FasL may inhibit this. Hohlbaum A M et al [23] also demonstrated that both the cytotoxic and the proinflammatory effects of FasL are mediated by the transmembrane form of FasL. Thus, up-regulated Fas and FasL in the IDO-4T1/T cells co-cultured system indicated that IDO-induced T cell apoptosis might be mediated by extrinsic apoptosis pathway.

Consistently, the intrinsic pathway is regulated by proteins of the B-cell lymphoma 2 proteins (BCL-2) family and proceeding via regulating apoptosis by releasing killing effectors from mitochondria to cytochrome c, leading to mitochondrial outer membrane permeabilization (MOMP). As sensors and gatekeepers, proteins of the Bcl-2 gene family play a pivotal role in controlling the execution machinery of apoptosis through the mitochondrial pathway. Kluck et al [24] revealed that Bcl-2 inhibited cytochrome c translocation, thereby blocking caspase activation and the apoptotic process. Bax, the pro-apoptotic protein, plays an important role in the cell death. Oltval et al [25] found that overexpressed Bax accelerated programed cell death induced by cytokine deprivation in an IL-3-dependent cell line, and also countered the death repressor activity of Bcl-2. Some researchers also yielded to a conclusion that the disturbed balance of Bcl-2/Bax ratio determines survival or death following an apoptotic stimulus [25, 26]. Accordingly, in this study, the expression of Bax was showed to be up-regulated, while Bcl-2 was down-regulated, indicating the intrinsic apoptosis pathway was also activated. In addition, BcL-2/Bax ratio in pIRES2-IDO-4T1/T cells co-culture system was significantly reduced. These outcomes revealed the mechanisms of T cell apoptosis induced by IDO might due to intrinsic pathway.

The above results coincided with the previous reports that local T-cell-based immune-escape enhanced IDO activation in the tumor microenvironment might represent predominant immunoregulatory mechanism by which tumor cells can escape the immune system. The characterization of IDO in cancer immune escape has contributed an important mechanism in the aspect of metabolic alterations. It has been discovered that T cells were sensitive to IDO activation, for the deprivation of tryptophan stopped T cell proliferation and resulted in the inability of T cell activation [5]. Recent reports deepened the understanding of the IDO mechanism to murine immunoregulatory $CD8^+$ dendritic cells (DCs) because their ability to suppress delayed-type hypersensitivity responses to tumor-associated peptides was blocked by exposure to 1-methyl-tryptophan [27]. For the mechanisms, IDO derived from cancer cells as well as nodal regulatory DCs were all found to contribute to cancer immune escape [28]. Moreover, the IDO mechanism is used by cultured human macrophages and dendritic cells to suppress in vitro T cell proliferation. Tryptophan restriction also led to the induction of Tregs and immune suppression [29]. Furthermore, it is has been indicated that the interaction between IDO and Tregs is a mutual effect, in which high level of IDO promotes the differ-

entiation, activation, and maturation of Tregs, further providing the immunosuppressive tumor microenvironment for cancer immune escape [30].

However, this study also has some limitations Primarily, the mechanism of IDO in the T cell apoptosis only focusing on Fas/FasL and BcL-2/Bax was studied in vitro without further confirmation, the results are further needed to be proved by more experiments or clinical researches. In addition, more studies are still needed to enrich the mechanism of this immune escape induced by IDO through intrinsic pathway and extrinsic pathway.

Conclusion

In conclusion, our study contributed to the detailed mechanism of IDO mediating the apoptosis of T cells in breast cancer cells, and shed a new light on the exploration of targets against metastatic breast cancer via Fas/FasL-induced apoptotic pathway and Bcl-2/Bax-associated pathway. Understanding the key molecular of IDO mediating T cells apoptosis of breast cancer can pave the way for the development of novel and efficient IDO-targeted therapies of breast cancer treatment.

Disclosure of conflict of interest

None.

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