RESEARCH ARTICLE

IL-12 Regulates B7-H1 Expression in Ovarian Cancer-associated Macrophages by Effects on NF-ϰB Signalling

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Abstract

    <u>Background and Aim</u>: B7-H1, a co-inhibitory molecule of the B7 family, is found aberrantly expressed in ovarian cancer cells and infiltrating macrophage/dendritic-like cells, and plays a critical role in immune evasion by ovarian cancer. IL-12, an inducer of Th1 cell development, exerts immunomodulatory effects on ovarian cancer. However, whether IL-12 regulates B7-H1 expression in human ovarian cancer associated-macrophages has not been clarified. Therefore, we investigated the effects of IL-12 on the expression of B7-H1 in ovarian cancer-associated macrophages and possible mechanisms. <u>Methods</u>: PMA induced THP-1-derived macrophages or human monocyte-derived macrophages were treated with recombinant IL-12 (rIL-12) or infected with adenovirus carrying human IL-12 gene (Ad-IL-12-GFP) for 24 h, then cocultured with the SKOV3 ovarian cancer cell line for another 24 h. Macrophages were collected for real-time PCR and Western blot to detect the expression of B7-H1, and activation of the NF-ϰB signaling pathway. Moreover, supernatants were collected to assay for IL-12, IFN-γ and IL-10 by ELISA. In addition, monocyte-derived macrophages treated with IFN-γ were cocultured with SKOV3 and determined for the expression of B7-H1. Furthermore, the expression of B7-H1 in monocyte-derived macrophages was also evaluated after blocking NF-ϰB signaling. <u>Results</u>: The expression of B7-H1 was significantly upregulated in monocyte-derived macrophages treated with rIL-12 or Ad-IL-12-GFP compared with the control groups (*p*<0.05), accompanied by a remarkable upregulation of IFN-γ (*p*<0.05), a marked downregulation of IL-10 (*p*<0.05) and activation of NF-ϰB signaling. However, the upregulation of B7-H1 was inhibited by blocking the NF-ϰB signaling pathway (*p*<0.05). Expression of B7-H1 was also increased (*p*<0.05) in monocyte-derived macrophages treated with IFN-γ and cocultured with SKOV3. By contrast, the expression of B7-H1 in THP-1-derived macrophages was significantly decreased when treated in the same way as monocyte-derived macrophages (*p*<0.05), and IL-10 was also significantly decreased but IFN-γ was almost absent. <u>Conclusions</u>: IL-12 upregulates the expression of B7-H1 in monocyte-derived macrophages, which is possible though inducing the secretion of IFN-γ and further activating the NF-ϰB signal pathway. However, IL-12 downregulates the expression of B7-H1 in THP-1-derived macrophages, associated with a lack of IFN-γ and inhibition of expression of IL-10.

Keywords: Ovarian cancer - B7-H1 - IL-12 - IFN-γ - NF-ϰB signaling pathway

Introduction

    Ovarian cancer, the most common and aggressive gynecologic cancer, is the foremost cause of death from gynecologic malignancies in the developed world (Torres et al., 2009; Wang et al., 2014). Recently, immunotherapy has shown great promise for cancer treatment (Jain et al., 2012), however, resistance of cancer cells to immunotherapy remains a definite challenge. Studies have demonstrated that B7-H1 play a critical role in ovarian cancer immune evasion (Dong et al., 2002; Dong et al., 2003).

    B7-H1, also known as CD274 or programmed death-1-ligand 1 (PD-L1), is a cell surface protein of B7 family, it

is thought to be involved in the regulation of cellular and humoral immune responses through the PD-1 receptor on activated T and B cells. Interaction of PD-L1 expressed on cancer cells and PD-1 receptor expressed on T cells has been known to inhibit immune responses by inducing T-cell apoptosis, impairing cytokine production and diminishing the cytotoxicity of activated T cells, and may endow tumors with a mechanism to escape host immune destruction (Wang et al., 2014). B7-H1 mRNA is widespread in human tissues, although cell surface protein expression is restricted to a fraction of macrophage-lineage cells (Dong et al., 1999; Dong et al., 2002). B7-H1 can be found on activated T lymphocytes and aberrantly expressed by numerous human tumors (Dong

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et al., 2002; Wintterle et al., 2003; Curiel et al., 2003; Jun Konishi et al., 2004; Ohigashi et al., 2005., Elhag et al., 2012). Experimental evidences suggested that human ovary tumor and its tumor-infiltrating macrophages also highly expressed B7-H1 (Dong et al., 2002; Dong et al., 2003), but the underlying mechanism for the upregulation remains unclear.

IL-12 is produced by macrophages and dendritic cells, and promotes their proinflammatory and proimmunogenic activities, it is a disulfide linked heterodimer with a molecular mass of 70 kDa that is composed of two subunits with masses of 40 and 35 kDa (Kobayashi et al., 1989; Stern et al., 1990). IL-12 has been shown to stimulate antitumor responses in several models of solid tissue tumors. Injection of IL-12 encapsulated in polymeric microspheres directly into subcutaneous tumors results in a vigorous NK and cytotoxic T cell response against the tumor and its metastasis (Whitworth et al., 2011). Numerous evidences suggest that IL-12 is a promising candidate for ovarian cancer immunotherapy. Research has confirmed that IL-12 upregulated B7-H1 in experimental autoimmune encephalomyelitis (EAE) mice (Cheng et al., 2007), but the effects of IL-12 to ovarian cancer-associated macrophages on the expression of B7-H1 and its possible mechanisms have not been expounded.

Materials and Methods

Cell preparations

Human monocytic cell line THP-1 and human ovarian cancer cell line SKOV3 were kept by our laboratory, and were cultured in RPMI 1640 (Invitrogen, USA) supplemented with 10% FBS (Gibco, USA), 100 mg/ml penicillin, and 100 mg/ml streptomycin at $37^{\circ C}$ with 5% CO_2. To generate THP-1-derived macrophages, 1×10^6 cells/well were seeded into six-well plates and treated with 320 nM phorbol-12-myristate-13-acetate (PMA) (Biyuntian, Shanghai, China) for 24 h. Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Paque density centrifugation from peripheral blood donated by healthy volunteers. The monocytes were acquired by a plastic adsorptive process as described (Bever et al., 1989; Wu et al., 2009) and seeded into six-well plates at a density of 5×10^5 cells/well, then were induced into macrophages by 320 nM PMA in RPMI 1640 supplemented with 10% FBS, 100 mg/ml penicillin, and 100 mg/ml streptomycin for 24 h at $37^{\circ C}$ with 5% CO_2.

Cells treatments and coculture

After a thorough wash to remove all PMA, THP-1-derived macrophages or human monocyte-derived macrophages were treated with 20 ng/ml rIL-12 (PeproTech, USA) or Ad-IL-12-GFP (constructed by our laboratory) (Cheng et al., 2012) for 24 h, then cocultured with SKOV3 cells (in the upper insert of six-well transwell apparatus with 0.4 μm pore size, 2×10^5 cells per well) with equivalent volume medium. After 24 h coculture, macrophages were collected for real-time PCR and western blot, the conditioned mediums were centrifuged to remove cellular debris, and then tested for IL-10, IL-12 and IFN-γ by ELISA. In addition, human monocyte-

Table 1. Primers sequences for real-time PCR

CD274	Forward: 5'- GGT GGT GCC GAC TAC AAG -3'
	Reverse: 5'- ATT GGT GGT GGT GGT CTT AC -3'
IFN-γ	Forward: 5'- CAT TCA GAT GTA GCG GAT-3'
	Reverse: 5'-GTA TTG CTT TGC GTT GGA-3'
p35	Forward: 5'- CTG GAC CAC CTC AGT TTG-3'
	Reverse: 5'-TCA GAA GTG CAA GGG TAA AA-3'
p40	Forward: 5'-GTG GAG TGC CAG GAG GAC A -3'
	Reverse: 5'-TCT TGG GTG GGT CAG GTT T-3'
β-actin	Forward: 5'- CTG GGA CGA CAT GGA GAA AA -3'
	Reverse: 5'- AAG GAA GGC TGG AAG AGT GC -3'

derived macrophages were treated with 25 ng/ml IFN-γ (PeproTech, USA), and then cocultured with SKOV3; the expression of B7-H1 was determined by real-time PCR and western blot as mentioned above.

Inhibition of NF-κB signaling pathway

Monocyte-derived macrophages were pre-treated with 10 μM Bay11-7082 (Biyuntian, Shanghai, China), a specific inhibitor of NF-κB signaling pathway, for 1 h, then treated with rIL-12 or Ad-IL-12-GFP, cocultured with SKOV3 in the same way.

Real-time PCR analysis

Total RNA was extracted from macrophages with Trizol reagent (TaKaRa, Japan). After confirming RNA purity and assaying concentration, 1000 ng aliquots of total RNA from each sample were reverse-transcribed into cDNAs using PrimeScript RT Reagent Kit (TaKaRa, Japan) following the manufacturer's protocol. Equal amounts of cDNA for each sample were used as template for real-time PCR. Real-time PCR was performed using SYBR Premix ExTaq (TaKaRa, Japan). Relative gene expression was calculated using the $2^{-\Delta\Delta ct}$ method with β-actin as calibrator. The primers used were described in Table 1.

Western blot analysis

Macrophages were lysed in RIPA buffer (Biyuntian, Shanghai, China) containing 1 mM PMSF, a protease inhibitor, for 30 minutes on ice, followed by centrifuging for 30 min at 12,000 rpm, $4^{\circ C}$. The protein concentrations were determined by BCA protein assay kit (Biyuntian, Shanghai, China). Equal total proteins were electrophoresed with 12% SDS-PAGE gel, followed by transferring to PVDF membranes using a wet transblot system (Bio-Rad, USA). The membranes were blocked for 2 h at room temperature with 5% nonfat milk and incubated overnight at $4^{\circ C}$ with specific primary antibodies (rabbit anti-human B7-H1, 1:1000; mouse anti-human β-actin, 1:1000). After washing for 3 times, the membranes were incubated for 1h with HRP-conjugated secondary antibody (1:1000). After further washing, the immunoreactive bands were visualized with the enhanced chemiluminescence (ECL) reagent (Millpore, USA). The bands were quantified with quanitity one software (Bio-Rad, USA).

To assay NF-κB signaling pathway, the nuclear and cytoplasmic protein of monocyte-derived macrophages were extracted with a nuclear extract kit (Biyuntian, Shanghai, China) according to the manufacturer's recommendation.
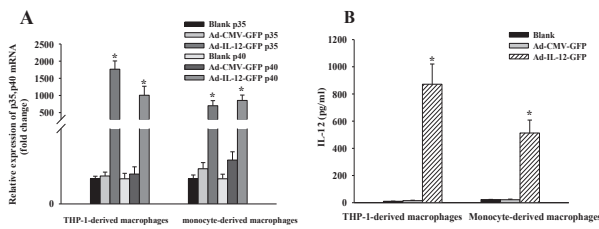
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Figure 1. Adenovirus-mediated IL-12 Had a Markedly Expression. Total RNA and supernatants was prepared after THP-1-derived macrophages and monocyte-derived macrophages were infected with Ad-IL-12-GFP, Ad-CMV-GFP or without treatment for 48 h. (A) Real-time PCR was performed to detect p35, p40. β-actin was amplified in each sample as endogenous control. (B) ELISA for IL-12 in the supernatants. All results were run in triplicate at least. *stands for *p*<0.05 versus control groups.
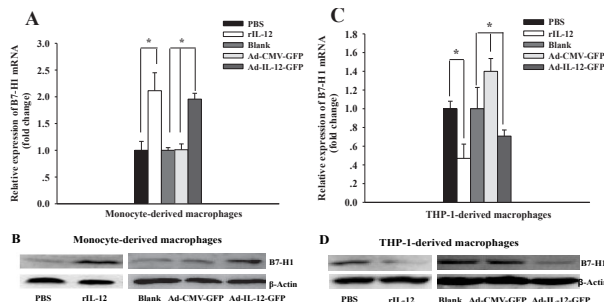

Figure 2. IL-12 Increased B7-H1 Expression in Monocyte-derived Macrophages and Decreased the Expression of B7-H1 in THP-1-derived Macrophages. (A, C) Real-time PCR for B7-H1 mRNA expression, *stands for *p*<0.05. (B, D) Western blot for B7-H1 expression.

ELISA analysis

The supernatants from above experiments were assessed for IL-12, IL-10 and IFN-γ using a commercially available ELISA kit (Biolegend, USA) following the manufacturer's instructions.

Statistical analysis

Data were analyzed with paired t test or One-way ANOVA using SPSS version 19.0 software. *p*<0.05 was considered statistically significant.

Results

IL-12 was over-expressed in Ad-IL-12-GFP-transfected macrophages and the supernatants

Experiments were divided into blank (without adenovirus), Ad-CMV-GFP and Ad-IL-12-GFP three groups. Adenovirus transduction was evidenced by GFP expression. Compared to the control groups, Ad-IL-12-GFP group had significantly higher p35, p40 gene expression (Figure 1 A) and higher IL-12 protein expression and secretion (Fig 1 B).

IL-12 treatment increased B7-H1 expression in ovarian cancer-associated monocyte-derived macrophages while decreased B7-H1 expression in ovarian cancer-associated THP-1-derived macrophages

Researches have demonstrated that PMA-induced macrophages and M2-polarized macrophages shared
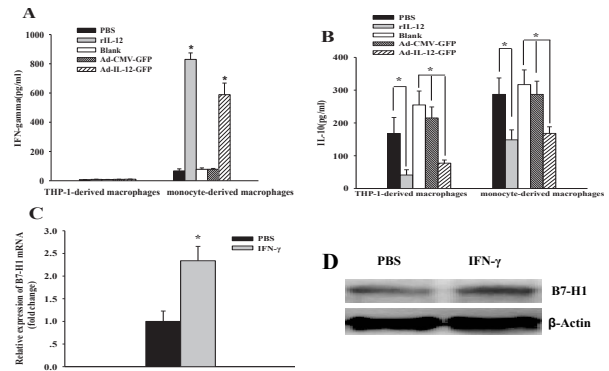

Figure 3. Effects of IL-12 on IFN-γ and IL-10. The supernatants was collected after coculture for 24 h and assessed by ELISA for IFN-γ (A) and IL-10 (B). IFN-γ increased the expression of B7-H1 at both mRNA (C) and protein (D) levels in monocyte-derived macrophages. *stands for *p*<0.05.

the same profile (Tjiu et al., 2009). The phenotype and function of M2-polarized macrophages were very similar to that of TAM (tumor-associated macrophages), but M2-polarized macrophages were still not equaled to TAM (Grivennikov et al., 2010, 2010; Mantovani et al.). So, to imitate tumor environment *in vivo*, further investigate the regulatory effect of IL-12 on B7-H1 in ovarian cancer-associated macrophages, coculture system was used. Monocytes cells were induced by PMA, after thoroughly removing PMA, treated with 20 ng/ml rIL-12, and then cocultured with SKOV3 cells without direct contact in a transwell apparatus. The results showed that the expression of B7-H1 in monocyte-derived macrophages was significantly increased in rIL-12 treated group compared with PBS control group (Figure 2 A and B). In view of the advantages of adenovirus-mediated gene therapy, we also used adenovirus carrying human IL-12 gene to infected monocyte-derived macrophages. In accordance with rIL-12 treatment, B7-H1 expression in monocyte-derived macrophages was also remarkably elevated in Ad-IL-12-GFP group compared with blank group and Ad-CMV-GFP group (Figure 2 A and B).

While THP-1-derived macrophages were treated as the same as monocyte-derived macrophages with IL-12, surprisingly, we obtained the opposite results, the expression of B7-H1 in THP-1-derived macrophages treated with rIL-12 or Ad-IL-12-GFP exhibited markedly reduction compared with the control groups (Figure 2 C and D).

IL-12 increased the level of IFN-γ and decreased the level of IL-10

To further explore the possible mechanism of IL-12 exerts function in regulating B7-H1 expression; we investigated the expression of IFN-γ firstly, because IFN-γ has been well known to induce the expression of B7-H1 (Lee et al., 2005; Lee et al., 2006; Kondo et al., 2010). It was found that rIL-12 and Ad-IL-12-GFP treated groups produced a higher level of IFN-γ in the supernatants of monocyte-derived macrophages cocultured with SKOV3 system. However, IFN-γ was almost not founded in the supernatants of THP-1-derived macrophages cocultured with SKOV3 system (Figure 3 A). This might be account for the above opposite results of B7-H1 expression. To
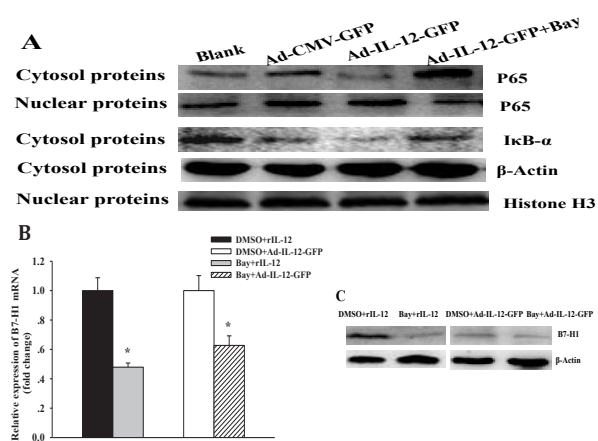
A

B

C

Figure 4. NF-κB Signaling Pathway was Involved in the Upregulation of B7-H1 in IL-12 Treatment Monocyte-derived Macrophages. (A) IL-12 activated NF-κB signaling pathway. Nuclear and cytoplasmic proteins were extracted after coculture for 24 h, western blot to assay NF-κB signaling pathway. β-Actin and Histone H3 as cytosol protein and nuclear protein control, respectively. Bay11-7082 inhibited the upregulation of B7-H1 inducing by IL-12 in monocyte derived-macrophages. (B) Real-time PCR for B7-H1 expression, *stand for *p*<0.05 versus the control groups. (C) Western blot for B7-H1 protein expression

further explore the function of IFN-γ on regulation B7-H1, 25 ng/ml IFN-γ was used to treat monocyte-derived macrophages for 24 h before coculture with SKOV3. The results showed that the expression of B7-H1 in monocyte-derived macrophages was dramatically increased in IFN-γ treated group (*p*<0.05) (Figure 3 C and D). These results have suggested that IFN-γ may be involved in the IL-12 upregulating expression of B7-H1 in monocyte-derived macrophages.

In other side, we measured IL-10 in the coculture supernatants by ELISA. The date showed that IL-10 was significantly reduced in both rIL-12 and Ad-IL-12-GFP treated groups of the two kinds of macrophages (Figure 3 B). Previous researches have suggested that IL-10 is an effectively cytokine that upregulated B7-H1 (Strome et al., 2003; Wu et al., 2009), so it has been presumed that IL-12 might decreased the expression of B7-H1 by inhibiting IL-10.

Involvement of NF-κB activation in B7-H1 induction in monocyte-derived macrophages

We demonstrated that IL-12 upregulated B7-H1 might be though inducing the expression of IFN-γ. Previous studies have illustrated that IFN-γ could activate NF-κB in human cells (Cheshire et al., 1997; Deb et al., 2001; Luo et al., 2005) and the promoter region of the human B7-H1 gene has an NF-κB motif (Chen et al., 2009). Therefore, whether B7-H1 induction by IL-12 is mediated by NF-κB activation was investigated. Monocyte-derived macrophages were treated with Ad-IL-12-GFP, then cocultured with SKOV3, the nuclear translocation of NF-κB p65, indicating NF-κB activation, was elevated, and was nearly completely blocked by Bay11-7082 (Figure 4 A). Corresponded with the change of nuclear p65 concentration, western blot also showed that the cytosol

concentrations of p65 and IkB-α were declined. When pre-treated monocyte-derived macrophages with Bay11-7082 for 1 h, then treated with rIL-12 or Ad-IL-12-GFP for 24 h, cocultured with SKOV3 for another 24 h, the expression of B7-H1 in monocyte-derived macrophages was also been inhibited compared with DMSO group (Figure 4 B and C).

Discussion

Although our understanding of ovarian cancer immune evasion has greatly improved in recent years, the molecular mechanisms are still largely unknown. Recent studies reveal that B7-H1 possesses dual functions of co-stimulation of naive T cells and inhibition of activated effector T cells to sustain immune homeostasis. Aberrant expression and dysregulation of B7-H1 have been reported in ovarian cancer cells and tumor-associated macrophages (Dong et al., 2003). Therefore, development of strategies targeting B7-H1 signals provides a novel and promising approach to improve the outcome of ovarian cancer therapy.

IL-12 has been well recognized as a proinflammatory mediator, and it was widely used for cancer treatment (Tahara et al., 1995; Wysocka et al., 1995; Herpen et al., 2008; Labbe et al., 2009; Whitworth et al., 2011). IL-12 can induce IFN-γ expression and secretion of T cells, NK cells and macrophages (Wysocka et al., 1995; Puddu et al., 1997), the promoter region of the human B7-H1 gene has an NF-κB motif (Chen et al., 2009). IFN-γ upregulated B7-H1 expression in some cells via NF-κB signal pathway activation (Kondo et al., 2010; Huang et al., 2013). However, the molecular mechanisms of action regulating the gene expression of B7-H1 vary in both different cell types and distinct stimuli. In this study, we demonstrated that IL-12 induced different B7-H1 expression in different type of macrophages cocultured with SKOV3 cells and further elucidated the possible molecular mechanisms.

It was founded in current study that B7-H1 and IFN-γ were higher expressed in monocyte-derived macrophages, and the IL-10 level in the supernatants was markedly reduced in IL-12 treated groups compared with control groups. Moreover, NF-κB signaling pathway was also activated in IL-12 treated groups, and the upregulation of B7-H1 in IL-12 treated group was inhibited by pre-treating with Bay11-7082. Together, these results indicate that NF-κB signaling pathway is one mechanism mediating upregulation of B7-H1 expression by IL-12 in monocyte-derived macrophages cocultured with SKOV3 cells.

Consistent with our findings, IL-12 has been shown to regulated B7-H1 expression via IFN-γ in other cell types (Eppihimer et al., 2002; Cheng et al., 2007). Studies have suggested that B7-H1 expression was significantly increased on microvascular endothelial cells *in vitro* and *in vivo* after IL-12-challenge, but IL-12 was not effective at inducing B7-H1 expression in tissues of IFN-γ-deficient mice. These data showed that elevation of B7-H1 expression was induced by IL-12 through IFN-γ (Eppihimer et al., 2002). Comparable and similar data were observed in EAE mice. IL-12 treatment increased the number of antigen presenting cells positive for B7-H1

expression in EAE mice but not in IFN-γ-deficient EAE mice (Cheng et al., 2007). Combined with our current studies, it therefore can be inferred that IFN-γ might play an important role in inducing B7-H1 in monocyte-derived macrophages by IL-12.

Interestingly, when the same treatments were performed to THP-1-derived macrophages, we didn't obtain the same results as monocyte-derived macrophages. Although the level of IL-10 in coculture supernatants was markedly reduced as same as in monocyte-derived macrophages, B7-H1 expressions were reduced in THP-1-derived macrophages treated with IL-12, and IFN-γ was almost not detected in the supernatants. These results were different from our previous study, in which THP-1-derived macrophages without IL-12 treatment cocultured with SKOV3 showed upregulation of B7-H1 expression as same as monocyte-derived macrophages (Xiong et al., 2014). However, other studies have provided data suggesting that IL-10 could induce B7-H1 expression, whereas neutralizing antibody against IL-10 significantly blocked B7-H1 expression (Kuang et al., 2009; Bloch et al., 2013). So we speculate that IL-12 down-regulated B7-H1 expression in THP-1-derived macrophages cocultured with SKOV3 main through inhibiting the expressions of IL-10, since IFN-γ was absent. But the evidences is still not insufficient due to we didn't used recombinant IL-10 to rescue B7-H1 expression. Furthermore, the decreasing of IL-10 in the supernatants of monocyte-derived macrophages coculture with SKOV3 system whether also downregulated B7-H1 expression as well as the downregulation of B7-H1 by IL-10 whether was offseted by the upregulation by IFN-γ remain need further investigation.

The expression of B7-H1 was regulated by different mechanisms in different cells or conditions. Studies have founded that TLR4 activation protects T24 cells from CTLs killing by upregulation B7-H1 expression. While blocking ERK pathway can restore sensitivity of T24 cells to CTL-mediated killing by downregulation B7-H1 expression (Wang et al., 2014). In DC subsets, ERK/p38 MAP-kinases and PI3K were involved in the differential regulation of B7-H1 expression (Karakhanova et al., 2010). Our research illustrated that IL-12 regulated B7-H1 expression via NF-κB signaling pathway in monocyte-derived macrophages.

In summary, we have demonstrated for the first time that B7-H1 is elevated in monocyte-derived macrophages while declined in THP-1-derived macrophages treated by IL-12 and cocultured with SKOV3. Analysis of the supernatants, we find the level of IFN-γ is significantly different, which might be one of the mechanisms mediating modulatory the effects of IL-12 on B7-H1 in the two kinds of macrophages.

Acknowledgements

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