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

Hai-Yu Xiong, Ting-Ting Ma, Bi-Tao Wu, Yan Lin, Zhi-Guang Tu*

Abstract

**Background and Aim:** 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. **Methods:** 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. **Results:** 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. **Conclusions:** 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
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 proinmunogenic activities, it is a disulphide 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°C with 5% CO₂. To generate THP-1-derived macrophages, 1x10⁶ cells/well were seeded into six-well plates and treated with 320 nM phorbol-12-myristate-13-acetate (PMA) (Biyuntian, Shanghai, China) according to the manufacturer’s instructions. Equal total proteins were electrophoresed in 12% SDS-PAGE gel, followed by transferring to PVDF membranes using a wet transblot system (Bio-Rad, 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°C with specific primary antibodies (rabbit anti-human B7-H1, 1:1000; mouse anti-human β-actin, 1:1000). 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°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 (Millipore, USA). The bands were quantified with quantity one software (Bio-Rad, USA).

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; the expression of B7-H1 was determined by real-time PCR and western blot as mentioned above.

### Table 1. Primers sequences for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD274</td>
<td>Forward: 5′-GTT GGT GCC GAC TAC AAC 3′&lt;br&gt;Reverse: 5′-ATT CTT GGT GGT GGT CTT CTT A-3′</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward: 5′-CAT TCA GAT GTA GCG GAT 3′&lt;br&gt;Reverse: 5′-GTA TTT CTT CTC GTT GTA GGA 3′</td>
</tr>
<tr>
<td>p35</td>
<td>Forward: 5′-CTG GAC GAC CTC AGT ATG TTG 3′&lt;br&gt;Reverse: 5′-CTC TGG GTG GAT CTC TTT T 3′</td>
</tr>
<tr>
<td>p40</td>
<td>Forward: 5′-CTG GAT TGC CAG GAC GAC A-3′&lt;br&gt;Reverse: 5′-TTC GAC GAT GAT CAG GAC A-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5′-CTG GGA CGA CAT GGA GAA A-3′&lt;br&gt;Reverse: 5′-AAG GAA GGC TGG AAG AGT GC 3′</td>
</tr>
</tbody>
</table>

Table 1. Primers sequences for real-time PCR

Monocyte-derived macrophages were pretreated 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 Ex Taq (TaKaRa, Japan). Relative gene expression was calculated using the 2^ΔΔct 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; then treated with 10 μM Bay11-7082 for 30 minutes on ice, followed by centrifuging for 30 min at 12,000 rpm, 4°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°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 bands were visualized with enhanced chemiluminescence reagent (ECL, Millipore, USA). The bands were quantified with Quantity One software (Bio-Rad, USA).

To assay NF-κB signaling pathway, the nuclear and cytoplasmic protein of monocye-derived macrophages were extracted with a nuclear extract kit (Biyuntian, Shanghai, China) according to the manufacturer’s recommendation.
**IL-12 Regulates B7-H1 Expression in Ovarian Cancer-associated Macrophages**

**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 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 equal to TAM (Griivennikov 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 found 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
Hai-Yu Xiong et al


after IL-12-challenge, but IL-12 was not derived-macrophages. (B) Real-time PCR for B7-H1 expression, the upregulation of B7-H1 inducing by IL-12 in monocyte and nuclear protein control, respectively. Bay11-7082 inhabited signaling pathway. β-Actin and Histone H3 as cytosol protein κB signaling pathway. Nuclear and cytoplasmic proteins were Monocyte-derived Macrophages.

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

Figure 4 A). Corresponded with the change of nuclear p65, indicating NF-κB activation, was elevated, and cocultured with SKOV3, the nuclear translocation of NF-κB p65, indicating 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

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 cucture with SKOV3 system was almost not detected in the supernatants. These results were performed to THP-1-derived macrophages, we didn’t obtain the same results as monocyte-derived macrophages without IL-12 treatment cocultured with SKOV3 system where as the downregulation of B7-H1 by IL-10 whether was decreasing of IL-10 in the supernatants of monocyte-derived macrophages, 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

This work was supported by the National Natural Science Foundation of China (Grant No. 81172016).

References


and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *JEM*, **170**, 827-45.


