The Effect of Lipopolysaccharide on Noxa Expression Is Mediated through IRF1, 3, and 7

Sujan Piya* and Tae-Hyoung Kim*

Department of Biochemistry, Chosun University School of Medicine, Gwangju 61452, Republic of Korea

Introduction

The lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, elicits the secretion of cytokines, such as interferons, that stimulate the host defense system. Previously, we demonstrated that interferons induce interferon regulatory factors (IRFs) 1, 3, and 7, which regulate the transcription of Noxa and alter the expression profiles of Bcl-2 family proteins in tumors. However, the immediate consequences of LPS stimulation on Noxa and BH3 expression in tumor cells remain uncharacterized. In this study, we determined that LPS induced Noxa expression in CT26 cells. Furthermore, studies in HCT116 parental and HCT116 p53-deficient cells revealed that LPS-mediated Noxa was independent of p53. Meanwhile, IRF1, 3, and 7 in CT26, HCT116 parental, and HT116 p53-deficient cells were upregulated by LPS stimulation, suggesting that LPS induces the expression of these IRFs in a p53-independent manner. The responsiveness of IRF1, 3, 4, and 7 binding to the Noxa promoter region to LPS indicated that IRF1, 3, and 7 activated Noxa expression, whereas IRF4 repressed Noxa expression. Together, these results suggest that LPS directly affects Noxa expression in tumor cells through IRFs, implicating that it may contribute to LPS-induced tumor regression.

Keywords: LPS, BH3 profiles, Noxa, tumor surveillance, interferon regulatory factors (IRFs)
the subsequent formation of pores in the mitochondrial outer membrane [18], leading to the permeabilization of the mitochondrial outer membrane and the release of pro-apoptotic mitochondrial proteins, such as cytochrome c, Apaf-1, and Smac [19]. The anti-apoptotic Bcl-2 family members bind to and inhibit Bax and Bak to protect the mitochondria from damage caused by pro-apoptotic BH3-only proteins. Noxa is a BH3-only pro-apoptotic protein that can bind to and inhibit anti-apoptotic protein Mcl-1 by releasing Bax and Bak from Mcl-1 [20]. Although Noxa was originally considered to be a sensitizer that neutralized Bax and Bak, recent reports have demonstrated that Noxa directly activates Bax and Bak to cause mitochondrial membrane permeabilization [21].

We have previously shown that IFNs, which are key mediators of LPS in vivo, alter the expression profiles of Bcl-2 family proteins, based on BH3 profiles in vitro, where expression of pro-apoptotic Bcl-2 family proteins becomes elevated and anti-apoptotic Bcl-2 family proteins become downregulated. In addition, treatment of CT26 cell-generated tumor-bearing mice with LPS resulted in alterations in the expression profiles of Bcl-2 family proteins in tumor tissues similar to those seen in vitro in IFN-treated CT26 cells [22]. In this study, we further examined whether LPS itself affects the expression profiles of Bcl-2 family proteins in tumor cells. We found that LPS alters the expression levels of Bcl-2 family proteins, especially those of Noxa, which is transcriptionally regulated by IRF1, 3, 7, and 4 in tumor cells. Collectively, these results suggest that LPS-induced tumor regression results, in part, from activation of immune cells to produce cytokines, such as IFNs, and alterations in the expression profiles of Bcl-2 family proteins in tumor cells.

Materials and Methods

Cell Culture and Reagents

HCT116 parental cells and CT26 cells were cultured in McCoy’s 5A medium or DMEM supplemented with 10% FBS, 2 mM l-glutamate, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO₂ in a humidified incubator. LPS derived from Escherichia coli was purchased from Sigma-Aldrich (USA). Anti-actin antibody was purchased from Chemicon International (USA). Anti-Bad, anti-Bcl-2, anti-Bcl-xL, anti-Bim, anti-IRF1, anti-IRF3, and anti-FLAG antibodies were purchased from Cell Signaling Technology (USA). Anti-Bak antibody was purchased from Upstate Biotechnology (USA). Anti-Bax, anti-IRF4, and anti-GFP antibodies were purchased from Santa Cruz Biotechnology Inc. (USA). Anti-Bid and anti-IRF7 antibodies were from Prosci Inc. (USA). Anti-Mcl-1 antibody was from BD Transduction Laboratories (USA). Anti-Noxa and anti-p53 antibodies were from Oncogene Research Products (USA), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit/-mouse IgG were purchased from Jackson ImmunoResearch Lab (USA).

Western Blotting

Cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) in the presence of 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, 2 mM sodium fluoride, and 1 mM sodium orthovanadate. The resulting soluble lysates were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane purchased from Bio-Rad (USA). This membrane was first probed with specific primary antibodies and then with HRP-conjugated anti-IgG antibody. Proteins were then visualized using Immobilon Western Chemiluminescent HRP Substrate purchased from Millipore (USA).

Chromatin Immunoprecipitation (ChIP) Analysis

Wild-type CT26 (2 × 10⁸) cells were seeded into 100 mm dishes and treated with LPS for the indicated durations. ChIP analysis was performed as described in our previous report [22]. The Noxa promoter was amplified by PCR using specific primers published in our previous report [22].

Results

Expression Profiles of Bcl-2 Family Proteins in Response to LPS

LPS itself has been shown to induce apoptosis in cultured tumor cells, implying that it may affect the expression of regulators of apoptosis in tumor cells [10, 11]. We previously reported that IFNs, key mediators of LPS-induced tumor regression, can alter the expression levels of members of the Bcl-2 family in tumor cells in vitro and in vivo [22]. Therefore, we hypothesized that LPS itself may directly alter the expression levels of Bcl-2 family proteins instead of through mediator cytokines, such as IFNs. To address this hypothesis in tumor cells in vitro, mouse colorectal carcinoma CT26 cells were treated with LPS in culture and the expression levels of Bcl-2 family proteins were measured by immunoblotting. LPS stimulation resulted in the elevation of expression levels of the pro-apoptotic Bcl-2 proteins Noxa, Bim, Bad, Bak, and Bax. In addition, LPS stimulation reduced the expression of Bid and Bcl-xL. Interestingly, Mcl-1 and Bcl-2 expression levels did not change in response to LPS (Fig. 1).

Noxa was first identified as a transcriptional target of p53 and a major player in p53-induced apoptosis in response to DNA-damaging agents [23]. Noxa targets and causes damage to the mitochondria, leading to apoptotic cell death in a BH3-dependent manner [23, 24]. Therefore, we examined
the LPS-mediated alteration of Bcl-2 family protein expression in HCT116 parental and HCT116 p53-deficient cells. As expected, expression profiles of Bcl-2 family proteins in HCT116 parental cells, with the exception of decreased expression of Bcl-2, were similar to those seen in LPS-stimulated CT26 cells (Fig. 2A). The expression levels of Bcl-2 family proteins in HCT116 p53-deficient cells were similar to those in HCT116 parental cells. This lack of difference in Bcl-2 family protein expression profiles in HCT116 parental and HCT116 p53-deficient cells indicates that LPS alters the expression profiles of Bcl-2 family proteins in a p53-independent manner (Fig. 2B). These results suggest that transcription factors, such as IRF3, in the downstream portion of the LPS-induced signaling pathway may be responsible for changes in Bcl-2 family protein levels [25].

Increased Expression of IRFs in Response to LPS

Noxa is a selective inhibitor of Mcl-1, and Bad is a selective inhibitor of Bcl-2 and Bcl-xL [26, 27]. In addition, the expression profiles of Bcl-2 family proteins in response to LPS in HCT116 cells (Fig. 2A) indicate that the expression of Bcl-2 and Bcl-xL had decreased, suggesting that increased Bad may not be critical for the inhibition of Bcl-2 and Bcl-xL. However, the expression level of Mcl-1 was not changed in response to LPS stimulation, indicating that elevated Noxa expression may be critical for the responsiveness of tumor cells to LPS. As shown in Fig. 2, p53 is dispensable for Noxa induction in response to LPS. Moreover, we previously found that IFN-γ is a key mediator of LPS-induced tumor regression and influences BH3 expression profiles in vivo and in vitro, and IRFs are
regulators of Noxa expression [22]. Therefore, we examined the expression levels of IRF1, 3, and 7 in CT26, HCT116 parental, and HCT116 p53-deficient cells. In these cells, IRF1, 3, and 7 were significantly elevated in response to LPS in a p53-independent manner (Fig. 3), suggesting that these IRFs may play key roles in Noxa induction in response to LPS.

**IRF1, 3, and 7 Are Recruited to the Noxa Promoter in Response to LPS**

As mentioned above, we previously reported that IRFs 1, 3, 4, and 7 bind to IRF elements in the mouse Noxa promoter region in response to IFNs [22]. In addition, we showed that IRFs 1, 3, and 7 are activators of Noxa expression, whereas IRF4 is a negative regulator or repressor of Noxa expression. Therefore, we investigated whether these IRF-binding elements participate in the regulation of Noxa induction in response to LPS. We performed ChIP on LPS-treated and -untreated CT26 cells. One IRF1-binding element (-3454 to -3223) (Fig. 4A), four IRF3-binding elements (-6259 to -5957 and -5113 to -4750) (Fig. 4B), and six IRF7-binding elements (-3812 to -3633, -2208 to -2057, and -641 to -417) (Fig. 4D) were occupied by IRF1, IRF3, and IRF7 proteins, respectively, in LPS-treated CT26 cells, but not in LPS-untreated CT26 cells. These results indicate that these elements are responsible for Noxa induction in response to LPS. By contrast, two IRF4-binding elements (-4755 to -4589 and -2208 to -2057) were bound by IRF4 in LPS-untreated CT26 cells, and IRF4 was no longer bound to these elements in LPS-treated CT26 cells (Fig. 4C), suggesting that IRF4 may function as a repressor of Noxa expression in response to LPS. The plausible binding elements of IRF1, 3, 4, and 7 are depicted in Fig. 4E.

**Discussion**

It is theorized that the component effective against human cancer in a mixed bacterial vaccine, known as Coley’s toxin, contains LPS [28–30]. The mechanisms surrounding LPS-mediated tumor regression have been studied and LPS is recognized as a potent stimulator of immune cells and inducer of various cytokines, including IFNs and TNF-α [5, 6, 31–33].

We previously reported that tumor cells are sensitized by altering the expression profiles of Bcl-2 family proteins in response to LPS, possibly through IFNs, which was highlighted by the finding that knockdown of Noxa expression in tumor cells does not result in regression of tumor tissues in response to LPS [22]. These findings emphasize that LPS-mediated tumor regression may be processed by not only immunological stimulation, but also sensitization of tumor cells to cell death. The sensitization of tumor cells by LPS is thought to be mediated by IFNs or other cytokines produced by activated immune cells [6, 22, 33].

In this study, we demonstrated that LPS directly alters the expression profiles of Bcl-2 family proteins in tumor cells in vitro (Figs. 1 and 2) in a manner similar to IFN induction in vitro and LPS induction in tumors in vivo [22]. On the basis of previous reports on selective interactions between pro-apoptotic and anti-apoptotic proteins of Bcl-2 family members, [34, 35], we can speculate that elevated Bad and Noxa levels by LPS in tumor cells can selectively inhibit Bcl-2/Bcl-xL and Mcl-1, respectively. The increased level of Bad in response to LPS may not influence the mitochondrial status because the expression levels of Bcl-2

![Fig. 3](image-url)
and Bcl-xL, two selective target proteins of Bad, did not change and decreased, respectively. However, the increased level of Noxa, a selective inhibitor of Mcl-1, may significantly influence the mitochondrial status since the expression level of Mcl-1 did not change. It is also possible that the increased Noxa protein may directly activate Bax and Bak.

Fig. 4. ChIP analysis of the mouse Noxa promoter region.
CT26 cells were treated with LPS (10 µg/ml) for 24 h and analyzed by ChIP using IRF1 (A), IRF3 (B), IRF4 (C), and IRF7 (D). Input DNA from cell lysates before immunoprecipitation was used as a positive control. Elutes from beads left untreated with antibodies were used as the negative controls. Data are representative of three independent experiments. Data are presented in a separate panel for each IRF. (E) Schematic of functional IRF-binding elements in the mouse Noxa promoter.
in addition to inhibiting Mcl-1 to trigger the oligomerization of Bax and Bak, leading to mitochondrial membrane permeabilization.

Cumulative evidence from previous studies demonstrates that Noxa expression is mediated by various transcription factors, such as p53 [23], IRFs [22, 36], HIF [37], CREB [38], c-myc [39, 40], p73 [41], FKHR1L1 [42], PRDM1 [43], FoxO1 [44], and PAX3-FoxO1 fusion protein [45]. Among these factors, we assumed that IRFs may be involved in Noxa induction in response to LPS, because LPS stimulates the secretion of IFNs, which activate the expression of Noxa, as we previously reported [22]. Indeed, we showed that IRF 1, 3, and 7 bind to several sites in the Noxa promoter (Fig. 4), indicating that LPS may activate IRFs to increase the expression level of Noxa in tumor cells. Nevertheless, it is possible that in addition to IRFs, other transcription factors can be recruited to the Noxa promoter in response to LPS.

Together, our results indicate that LPS orchestrates the complicated process of LPS-induced tumor regression by directly and indirectly affecting tumor cells and immune cells. We believe that this study increases our understanding of the role of LPS in LPS-mediated tumor regression at the molecular level.

Acknowledgments

This study was supported by funding from Chosun University, 2017.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References


21. Dai H, Smith A, Meng XW, Schneider PA, Pang YP,


