# Colon cancer-derived factors activate NF-κB in myeloid cells via TLR2 to link inflammation and tumorigenesis

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Abstract. The exact mechanism for the contribution of NF-KB activation during colon carcinogenesis is unclear. The present study aimed to determine the manner in which colon cancer cells induce inflammatory responses in order to link tumor growth. Macrophages were stimulated with cultured medium from the supernatants of several colon cancer cell lines. Macrophage accumulation and NF-KB activation were observed in samples that were stimulated with supernatant from tumor cells that showed constitutive NF-KB activity. NF-kB activation in the stimulated macrophages was dependent on TLR2 and IKKB, but not TLR4. Various cytokines, such as IL-6, were induced in a TLR2-dependent manner. Tumor cells that were cultured with the supernatant of macrophages originally stimulated with the tumor cell cultured media grew more rapidly than those stimulated with the supernatant of unstimulated macrophages. Taken together, colon cancer-derived factors induce the accumulation of macrophages and activate NF-KB through a TLR2-dependent mechanism, suggesting an important link between inflammation and tumor growth.

## Introduction

Colorectal cancer is the second leading cause of cancerrelated death in industrialized nations (1). The development of colorectal cancer results from the sequential accumulation of activating mutations in oncogenes, such as *k*-ras, and mutations or deletions in the coding sequences of several tumor suppressor genes, including *p53*, adenomatous polyposis coli (APC) and  $\beta$ -catenin (2). In addition, the tumor microenviron-

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ment such as the infiltration of inflammatory cells play a role in the development of colon cancer (3).

NF-κB transcription factors are key regulators of innate and adaptive immune responses, inflammation and cell survival. Five members, p65 (RelA), c-Rel, RelB, p50/NF-κB1 and p52/NF-κB2, belong to the mammalian NF-κB family and are assembled by dimerization (4). In the classical pathway, a number of pro-inflammatory stimuli such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 $\alpha/\beta$  (IL-1 $\alpha/\beta$ ), lipopolysaccharide (LPS), and various drugs are capable of activating NF-κB. The activating signal from the receptors transmits mainly through IκB kinase (IKK)-dependent phosphorylation and degradation of the IκB inhibitory proteins and eventually activates the transcription of target genes, including cytokines, chemokines, and anti-apoptotic genes (5).

Findings of previous studies indicated the constitutive activation of NF-kB in various malignancies including colorectal cancer (6-11). A number of these reports suggested that constitutively active NF-kB is associated with harmful features of tumor cells, such as anti-apoptotic effects, cell growth, and metastasis (8,12,13). It is also reported that NF-KB activation in the tumor microenvironment, such as inflammatory cells, is associated with tumor promotion (4). However, the exact mechanism of the contribution of NF-kB activation in the tumor microenvironment that correlates to colorectal cancer development remains unclear. We conducted this study to investigate the manner in which colon cancer cells induced inflammatory response to correlate to tumor growth and found that colon cancer-derived certain NF-kB-dependent factor induced accumulation of macrophages and activated NF-KB in macrophages through TLR2.

# Materials and methods

*Cells*. The human colorectal carcinoma cell lines were maintained as instructed by the American Type Culture Collection (ATCC, Manassas, VA, USA). Bone marrow-derived macrophages (BMDMs) from C57BL/6 wild-type (WT), *Tnfr1*<sup>-/-</sup>, *Il-1r*<sup>-/-</sup>, *Tlr2*<sup>-/-</sup> and *Tlr4*<sup>-/-</sup> mice were cultured as previously described (14).  $I\kappa\kappa\beta^{-/-}$  macrophages were cultured from  $I\kappa\kappa\beta^{F/F}$ :

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Figure 1. NF- $\kappa$ B activation induces the accumulation of macrophages. (A) LOVO-WT/-KD was transplanted subcutaneously into BALB/c nude mice, and WT and KD tumors were stained immunohistochemically with an anti-F4/80 (red) antibody (magnification, x200). Tumors were also analyzed by DAPI (blue) staining (magnification, x200). The number of F4/80 positive macrophages were counted per field (magnification, x200) in LOVO-WT (n=5) or LOVO-KD (n=5) tumors. Values are the mean  $\pm$  standard error (SEM). \*P<0.05 as determined by the Student's t-test. (B) Macrophage migration was assayed quantitatively with a Boyden chamber. The indicated supernatants were added to the bottom wells of a 48-well Boyden chamber, and a membrane was placed on the wells. Macrophages were added to the upper wells at 5,000 cells/well. After 2.5-4 h incubation, the migrated cells were stained and then extracted. Stained cells were counted by a microscope. Values are the mean numbers per field (magnification, x200)  $\pm$  SEM. \*P<0.05 compared with the medium only as determined by the Student's t-test.

Mx1-Cre mice after poly (IC) injection (15). IKK $\gamma$  knock-down cells were previously described (6).

*Reagents*. The polyclonal anti-phospho-IκBα(Ser32), antiphospho-ERK1/2, anti-phospho-Jun N-terminal protein kinase (JNK) (Thr183/Tyr185) and anti-phospho-p38 (Thr180/Tyr182) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-F4/80 antibody was obtained from Invitrogen (Carlsbad, CA, USA). The other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

*Biochemical and cell proliferation assays.* Protein lysates were prepared from tissues and cultured macrophages, separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto Immobilon membranes (Millipore, Billeca, MA, USA), and analyzed by immunoblotting. Cell numbers were determined using a cell counting kit, according to the manufacturer's protocol (Dojindo Laboratories, Kumamoto, Japan). Culture supernatants were assayed using a mouse chemokine antibody array (RayBiotech, Inc. Redwood City, CA, USA). Levels of IL-6 were measured using enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN, USA). Migration assay (Boyden chamber assay). Macrophage migration was assayed quantitatively with a Boyden chamber. The supernatants of cancer cells were added to the bottom wells of a 48-well Boyden chamber (Neuro Probe, Inc.), and a 10  $\mu$ m pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe, Inc., Gaithersburg, MD, USA) was placed on the wells. Cells were trypsinized, resuspended and macrophage suspension (5,000 cells/well) was added to the upper wells, and the chamber was incubated 2.5-4 h at 37°C, 5% CO<sub>2</sub>. The cells that adhered to the upper surface of the filter membrane were removed by scraping with a rubber blade, and those that moved through the filter and stayed on the lower surface of the membrane were considered to be migrated cells. The migrated cells were stained, and then extracted using a kit component (QCM<sup>™</sup> colorimetric cell migration assay, Chemicon International, Billerica, MA, USA) after cutting each stained area in evenly sized pieces.

Immunofluorescence and immunohistochemistry. The sections were deparaffinized and incubated overnight at 4°C with antiphospho-I $\kappa$ B $\alpha$  (Cell Signaling Technology) antibody or control Abs. Binding of the primary antibody was detected with antirabbit IgG Abs (Vector Laboratories, Burlingame, CA, USA), followed by visualization with 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA). For immunofluorescence, the sections were incubated with anti-F4/80 (Serotec, Raleigh, NC, USA), followed by secondary Alexa555 IgG antibody (Molecular probes, Carlsbad, CA, USA).

*Transfection*. HEK293 cells were seeded into 6-well plates and cotransfected with NF- $\kappa$ B-Luc reporter plasmid and the expression vectors of TLR2 or TLR4 using an Effectene transfection reagent (Quiagen, Hilden, Germany). Each sample contained 100 ng of Renilla luciferase vector (Promega, Madison, WI, USA) for the standardization of transfection efficacy. Total DNA amount was kept constant by supplementation with the empty vector.

Statistical analysis. Data are expressed as the mean  $\pm$  standard error of the mean (SEM). Significant differences were detected using the Student's t-test. P $\leq$ 0.05 was considered to be statistically significant.

## **Results and Discussion**

NF-*kB* activation in colorectal carcinoma induces accumulation of macrophages. Previously, we showed that tumor expansion was strongly suppressed in colon cancer cells (LOVO) by NF-kB inhibition established by stable knockdown of IKKy (LOVO-KD) (6). It is reported that macrophage is one of the major inflammatory cells in cancer tissues (16). To determine whether NF-kB activation is associated with the accumulation of macrophages, tissues from a xenograft model were stained with anti-F4/80 antibody, which is a marker for macrophages. As shown in Fig. 1, macrophages were accumulated in the tumor tissues of the control LOVO (LOVO-WT), whereas relatively few macrophages were found in the tissue of LOVO-KD (Fig. 1A). We also stained the tissues from other cells, such as DLD-1, and found similar results (data not shown). These results suggested that NF-κB activation in cancer cells is associated with macrophage accumulation.

We then investigated the ability of macrophage accumulation of tumor cells by cell migration assay. The supernatants of cells with constitutive NF- $\kappa$ B activity (LOVO-WT and SW620) and cells without NF- $\kappa$ B activity (LOVO-KD and SW480) (6) were used. It was found that only cells with constitutive NF- $\kappa$ B activity showed the ability of macrophage accumulation, suggesting that macrophage accumulation is NF- $\kappa$ B-dependent (Fig. 1B).

Colorectal carcinoma with constitutive active NF- $\kappa$ B activation induces NF- $\kappa$ B activation in macrophages. Human colon cancer tissues were immunohistochemically stained with anti-phospho-I $\kappa$ B $\alpha$ , which is a marker of NF- $\kappa$ B activation, and found that NF- $\kappa$ B is strongly activated especially in myeloid cells (Fig. 2A). Therefore we investigated whether colon cancer cells are capable of directly activating NF- $\kappa$ B in macrophages (murine bone marrow-derived macrophages; BMDMs). The supernatant of cells with constitutive NF- $\kappa$ B activity (LOVO-WT and SW620) and cells without NF- $\kappa$ B activity (LOVO-KD and SW480) were used. As shown in Fig. 2B, supernatants of LOVO-WT and SW620 cells activated NF- $\kappa$ B determined by I $\kappa$ B $\alpha$  degradation and phosphorylated



Figure 2. Colorectal carcinoma with constitutive active NF-κB activation induces NF-κB activation in macrophages. (A) Human colon cancer tissues were stained with anti-phospho-IκBα (magnificantion, x200). (B) BMDMs were cultured with the supernatant of LOVO-WT, LOVO-KD, SW480 and SW620. At the indicated times, cells were lysed and immunoblotted with anti-IκBα or anti-phospho-IκBα. (C) BMDMs from WT, *Tlr2<sup>-/-</sup>*, *Tlr4<sup>-/-</sup>* or *Iκκβ<sup>-/-</sup>* mice were incubated with the supernatant of LOVO-WT. At the indicated times, cells were lysed and immunoblotted with anti-IκBα or anti-phospho-IκBα. (D) 293T cells were cotransfected with the NF-κB-Luc reporter plasmid and the expression vectors encoding TLR2 or TLR4. After 24 h, cells were cocultured with the supernatant of the indicated cells for 8 h. Luciferase activity is presented as a fold induction relative to the basal level of vector-transfected cells. Values are the mean ± SD.

I $\kappa$ B $\alpha$ . In contrast, as in the case of macrophage accumulation, supernatants of LOVO-KD and SW480 cells were not capable of activating NF- $\kappa$ B in macrophages (Fig. 2B). We also used THP-1 human macrophages and found consistent results

with those of mouse BMDMs (data not shown). These results suggest that colon cancer cells with NF- $\kappa$ B activity activate NF- $\kappa$ B in macrophages.

Colorectal carcinoma induces NF- $\kappa$ B activation via TLR2 in macrophages. We then analyzed how the supernatant of colon cancer cells activated NF- $\kappa$ B in macrophages. TNFR, IL-1R and TLRs are considered to be important receptors for the activation of NF- $\kappa$ B. Therefore, BMDMs of WT,  $Tlr2^{-/-}$ ,  $Tlr4^{-/-}$ ,  $Tnfr1^{-/-}$ ,  $Il-1r^{-/-}$  and  $I\kappa\kappa\beta^{-/-}$  mice were stimulated with the supernatant of LOVO-WT. In WT,  $Tlr4^{-/-}$ ,  $Tnfr1^{-/-}$  and  $Il-1r^{-/-}$  macrophages, NF- $\kappa$ B was strongly activated by the supernatant of LOVO-WT as determined by p-I $\kappa$ B $\alpha$  and I $\kappa$ B $\alpha$ degradation (Fig. 2C, data not shown). In contrast, NF- $\kappa$ B was not activated in  $Tlr2^{-/-}$  and  $I\kappa\kappa\beta^{-/-}$  macrophages, suggesting that the supernatant of colon cancer cells-induced activation of NF- $\kappa$ B is mediated via TLR2 and IKK $\beta$  (Fig. 2C).

To analyze the involvement of TLR2 in greater detail, NF- $\kappa$ B activity was measured by NF- $\kappa$ B reporter. Cells were transiently transfected with NF- $\kappa$ B-Luc together with plasmidexpressing TLR2, and stimulated with the supernatants of cancer cells at 24 h after the transfection. NF- $\kappa$ B was activated by the supernatants of LOVO-WT and SW 620, whereas those of LOVO-KD and SW480 were not able to activate NF- $\kappa$ B. We also analyzed the effect of TLR4 expression and found no effect from the cancer supernatants. These results suggest that colorectal carcinoma induces NF- $\kappa$ B activation via TLR2 signaling (Fig. 2D).

*Colorectal carcinoma induces MAPK activation via TLR2 in macrophages.* We analyzed whether the supernatant of LOVO-WT activates p38, ERK, or JNK, which is also activated via TLR2 ligands such as peptidoglycan (17). BMDMs from *Tlr2<sup>-/-</sup>, Tlr4<sup>-/-</sup>* mice were similarly stimulated with the supernatant of LOVO-WT as above. As expected, p38, ERK and JNK were activated by the supernatant of LOVO-WT via TLR2, but not via TLR4 (Fig. 3A).

NF-κB and MAPK pathways transcriptionlly regulate the expression of inflammatory cytokines in macrophages (18). Thus, we analyzed the expression of inflammatory cytokines in wild-type BMDMs with the supernatant of LOVO-WT using a cytokine protein array and found that several cytokines, including IL-6, were increased (Fig. 3B). In wild-type BMDMs, IL-6 expression was increased by LOVO-WT and SW620, but not by LOVO-KD as determined by ELISA. In *Tlr2<sup>-/-</sup>* BMDMs, IL-6 was not increased even by the supernatant of LOVO-WT and SW620 (Fig. 3C).

NF- $\kappa B$  activation in colorectal cells is critical for tumor proliferation. We have previously reported that LOVO-WT showed more proliferative cells relative to LOVO-KD cells determined by PCNA staining in a xenograft model, whereas no significant difference was found *in vitro* (6). We investigated an effect of proliferation of LOVO-WT cells cultured with macrophage supernatant stimulated with LOVO-WT cells. LOVO-WT cells cultured with the supernatant of BMDMs stimulated with LOVO-WT grew more rapidly than with the supernatant of unstimulated BMDMs (Fig. 3D).

NF- $\kappa B$  is considered to be one of the most important signaling pathways for the development of overt inflammation-

16 OVO-WT cells / well (x 103) 14 12 10 LOVO-WT-treated BMDM Sur LOVO-KD-treated BMDM 12 24 hrs Cancer cell NF-KB Cell growth TLR2 NF-KB Inflammation Macrophage Figure 3. NF-KB activation in colorectal cells is critical for tumor proliferation. (A) BMDMs from WT, Tlr2-1- or Tlr4-1- mice were incubated with the supernatant of LOVO-WT. At the indicated times, cells were lysed and immunoblotted with the antibodies against the indicated proteins. (B) Cytokine secretion from BMDMs by the supernatant of LOVO-WT was analyzed using a protein array. (C) WT or Tlr2-1- BMDMs were treated with or without the supernatant of LOVO-WT, LOVO-KD or SW620 for 24 h, and IL-6 levels were determined by ELISA. (D) LOVO-WT cells were cultured with the supernatant of macrophages stimulated with or without LOVO-WT cells. Cell proliferation was measured by cell counting under a microscope. The values are the mean  $\pm$  SD. (E) Models of this study.



associated carcinogenesis. Our results demonstrate that NF- $\kappa$ B is a candidate for a critical regulator of the inflammatory response in the tumor microenvironment of the sporadic cancers without overt inflammation (Fig. 3E). However, this study examined an *in vitro* analysis only; therefore, an *in vivo* analysis should be performed to confirm the results.

In the tumor tissues, we showed that NF- $\kappa$ B activation is a critical regulator in tumor cells and the surrounding cells. It has been reported that constitutive NF- $\kappa$ B activation in tumor cells accumulated myeloid cells such as neutrophils and macrophages via the expression of IL-8, Gro- $\alpha$ , RANTES and MCP-1 (19-21). These chemokines or cytokines are regulated by NF- $\kappa$ B activity, as we have shown in a previous study (6).

The term 'classically activated macrophages' has been used to designate the effector macrophages that are produced during cell-mediated immune responses (22). Whether inflammation promotes or inhibits carcinogenesis remains controversial. However, previous investigations into tumor-associated macrophages (TAMs) and the inflammation-associated carcinogenesis model (23), suggest that inflammation promotes the development of cancer. Although we did not expect colon cancer development to depend upon inflammation, our results showed that NF- $\kappa$ B, a major activator of the inflammatory response, plays a crucial role in cancer progression.

In the present study, factors from colon cancer cells with constitutive active NF- $\kappa$ B were found to activate macrophages via TLR2. Factors such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) were found to activate TLR2. However, the reason for this activation has yet to be determined. Mouse lung carcinoma is regarded as one of the most potent macrophage activators leading to the production of IL-6 through the activation of TLR2 and TLR6 (24). Biochemical purification led to the identification of the extracellular matrix proteoglycan versican (24). We therefore examined the versican expression in human colon cancer cells and found a weak expression in mRNA and protein levels. Additionally, versican expression was not associated with NF-KB activity and thus we concluded that as yet unknown factors may be responsible for the activation in colon cancer cells.

Previous studies have indicated that the induction of a suppressive macrophage population in tumors may occur through the MyD88-dependent activation of NF- $\kappa$ B (25). Irrespective of the stimulus, these TAMs produce high levels of IL-10, and are capable of inhibiting immune responses to neo-antigens that are expressed by tumor cells, as well as de-activate neighboring macrophages. Studies also suggest that regulatory macrophages contribute to angiogenesis, thereby promoting tumor growth (26). Naugler *et al* have reported that MyD88-dependent IL-6 secretion from Kupffer cells, resident macrophage in liver, is critical for the development of chemical-induced liver cancer (27). These observations suggest that TLR/MyD88 systems in myeloid cells play a significant role in cancer progression and may be a potent treatment target for cancer.

Previously, we reported that NF- $\kappa$ B activation was observed in more than 50% of cancer cells in the carcinoma tissues and was involved in angiogenesis (6). In addition to angiogenesis, we have shown that constitutive NF- $\kappa$ B activation is associated with macrophage activation, which induced enhanced inflammation and cell growth. Regarding cell growth, we have shown that various growth factors and cytokines such as GCSF, GM-CSF and IL-6 were produced from activated macrophages and these factors may prove to be ideal candidates as a therapeutic target.

In conclusion, our results indicate that NF- $\kappa$ B activation in colon cancer cells may induce inflammation via TLR2, thus raising the prospect of anti-inflammatory intervention targeting macrophages in the chemoprevention of colon cancer. Our results also show that the IKK $\beta$ /NF- $\kappa$ B signaling pathway is an attractive target for the development of anticancer drugs.

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