Original Article

Lipopolysaccharide Activates Immunologic Defense against Colorectal Carcinoma in a Tumor Xenograft Model

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Key Words
Colorectal cancer;
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Background. Lipopolysaccharides (LPS) are the major antigens expressed on the outer membrane of gram-negative bacteria. LPS induces a strong response in a normal immune system. Since tumor cells are suppressed by the immune system, immunotherapy is considered an efficient strategy against cancers. In this study, we assumed that LPS was able to activate the immune system to prevent tumor progression.
Aims. We aim to investigate the potential anti-tumor effects of LPS on colorectal cancer (CRC) in a HCT-15 cells-derived tumor xenograft model.
Methods. LPS was intravenously injected in the HCT15-derived tumor xenografts at day 7 and day 14, respectively, after tumor implantation for observing whether LPS was able to suppress tumor initiation and progression. Meanwhile, intravenous immunoglobulin (IVIG) was used to reduce immune response. The tumor volumes and body weight were recorded.
Results. We found that LPS significantly reduced initial tumor growth (injected at day 7 with 100 mm^3 tumor size) in the HCT-15 bearing mice. Meanwhile, the anti-tumor effect was neutralized by co-injection of immunosuppressor IVIG. In addition, the body weight was recovered at day 14 in LPS group compared to PBS control group due to reduction of tumor volume in LPS group. We also found that LPS significantly eradicated tumor volume at later stage (injected at day 14 with 400 mm^3 tumor size) but led to strong weight reduction.
Conclusions. We demonstrated that LPS ameliorated small tumor initiation and big tumor progression in a CRC tumor xenograft model. The results suggested the immune system may be activated to suppress tumors.
Limitations. The mouse model used in this study has normal immune function, but the immune activity in cancer patients is often inhibited by the tumor microenvironment. Therefore, whether LPS can induce the immune activity in cancer patients needs further investigation.
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Colorectal carcinoma (CRC) is one of the most common and malignant cancer types. Accumulated evidence supports the development of CRC is caused by a complex and multistep interaction between genetic mutations and environmental stresses.[1,2] Mutations on gatekeeper gene *adenomatous polyposis coli* (APC) are found in ~80% of all human CRC.[3] APC acts as a canonical tumor suppressor interacting with Axin and GSK-3β in that promotes phosphorylation of β-catenin and results in subsequent protea-

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some degradation of β-catenin.[4] When APC mutation occurs, β-catenin is dephosphorylated, leading to translocate to nucleus and promote oncogene transcription.

Recently, immunotherapies, such as targeting PD-1 to reactivate $CD8^+$ T cells, are a potential strategy against CRC.[5] It reveals that intact immune system is necessary to against tumors. Besides reactivation of $CD8^+$ T cells, our body also contains innate immunity that is also responsible for eradicating tumor cells, including macrophages and natural killer cells.[6,7] To our knowledge, macrophages are known and divided to M1 and M2 type, whereas M1 is known to suppress tumors and M2 is to enhance tumor progression.[8] Since the immune system is the first line of defense against infected cells and tumors, the reactivation of immune cells evolved by well-known antigen may inhibit tumor progression.

Lipopolysaccharides (LPS), also known as lipoglycans and endotoxins, are the major components expressed on the outer membrane of all gram-negative bacterial species. LPS serves as a major molecule for the mammalian innate immune system to evolve defense responses against bacterial infection.[9] Since LPS is able to stimulate immune activity such as M1 differentiation,[10,11] we assumed that the activation of the immune system derived by LPS may suppress tumor growth. Classically, LPS from the infecting pathogens is released by the LPS binding proteins (LBP) in the serum, then LBP transferred LPS to CD14 on monocytes, dendritic cells, or macrophages.[12] CD14 splits LPS into monomers and presents them to the Toll-like receptor 4 (TLR4) complex. Therefore, LPS/TLR4 complex induces multiple signaling components such as NF-κB and IRF3 to produce cytokines.[13-15] We intended to investigate the evocation of immunological activity to suppress tumors in this study.

TLR4 expresses in immune cells, however, TLR4 is also highly expressed in intestinal stroma is correlated to CRC progression.[16] Literature has indicated that upregulation of TLRs (TLR1, TLR2, TLR4, and TLR8) in tissues of colorectal cancer are related to high expression of inflammatory cytokines (interleukin-6, interleukin-8, and interferon-α), resulting in higher possibility of CRC recurrence.[17] The LPS/TLR4 in CRC cells actually activates PI3K/AKT pathway to promote downstream β1 integrin function and increase tumor metastasis.[18] Moreover, LPS blockade promotes immunotherapy against CRCs and attenuates distant metastasis to the liver.[19] For the controversial observations, we intended to investigate whether LPS promotes CRC progression directly on tumor cells or reduces tumor progression through activating immune system for eradicating tumors in a tumor xenograft animal model.

Material and Methods

Animal husbandry

Male nude mice were purchased from BioLASCO Taiwan Co., Ltd. All the mice were housed under a 12 hour-light cycle at 22 °C. All the animal experimental procedures were approved by the Chang Gung University, Taoyuan, Taiwan.

HCT-15 culture and tumor xenograft model

HCT-15 cells were cultured in F12K medium (Gibco) supplied with 10% of fetal bovine serum (Gibco) and incubated at 37 °C with 5% CO_2. The HCT-15 xenograft model was described as our previous study.[20] In brief, 2×10^6 HCT-15 cells were suspended in 100 ul of phosphate buffered saline (PBS) and subcutaneously injected into the right legs of 5-weeks-old nude mice. The tumor volume was recorded every 2 to 3 days after inoculation. After 7- or 14-days post-transplantation, mice bearing HCT-15 cells (n = 3 for each treatment) were divided three groups by intravenously injected (i.v.) with 100 ul of PBS as control group, 100 ng of LPS, or 1 mg of intravenous immunoglobulin (IVIG) combined with LPS. LPS and IVIG were resuspended in 100 ul of PBS for *i.v.* treatment.

Statistical analysis

Statistical analysis was performed using GraphPad Prism V5.01 software (GraphPad Software, Inc., California, USA). Student's t-test was used to com-

pare two groups and ANOVA was used to compare data with more than two groups. Data are expressed as mean ± SD and the significant difference was acceptable as $p < 0.05$.

Results

LPS reduced CRC progression in the xenograft model

To determine the potential effects of LPS on enhancing or inhibiting tumor progression, a HCT-15 cells-derived tumor xenograft model was used. After 7-days tumor inoculation, the tumor volume was 100 mm^3. Then, the HCT-15 bearing mice were intravenously injected with LPS, IVIG, or LPS + IVIG. We found LPS significantly decreased implanted tumor growth (Fig. 1A and 1C), but the anti-tumor effect was attenuated by IVIG combination (Fig. 1A and 1C). However, IVIG had no effect against tumor progression (Fig. 1B). The results revealed that LPS inhibited tumor growth. Meanwhile, tumor implantation led to reduction of body weight (Fig. 1D), but LPS diminished tumors with increase of body weight compared to PBS group at day 14 (Fig. 1D).

LPS decreased tumor burden in the CRC xenograft model

To further evaluate the potential anti-tumor effects of LPS against CRC in late stage, LPS was intravenously injected into HCT-15 bearing mice at 14 days-post tumor implantation, whereas tumor volume was 400 mm^3. We found that LPS promoted tumor necrosis and significantly reduced HCT-15 tumor volume compared to PBS group (Fig. 2A and 2B). However, LPS treatment caused bodyweight reduction dramatically after LPS injection at day 17 (Fig. 2C). The result was consistent with Fig. 1D. We observed the weight reduction was recovered in 3 days (Fig. 2C). The results implied that LPS caused high levels of inflammation derived from stimulation of immune system, that significantly eradicate existing tumor burden.

Discussion

In this study, we uncovered the potential anti-tu-

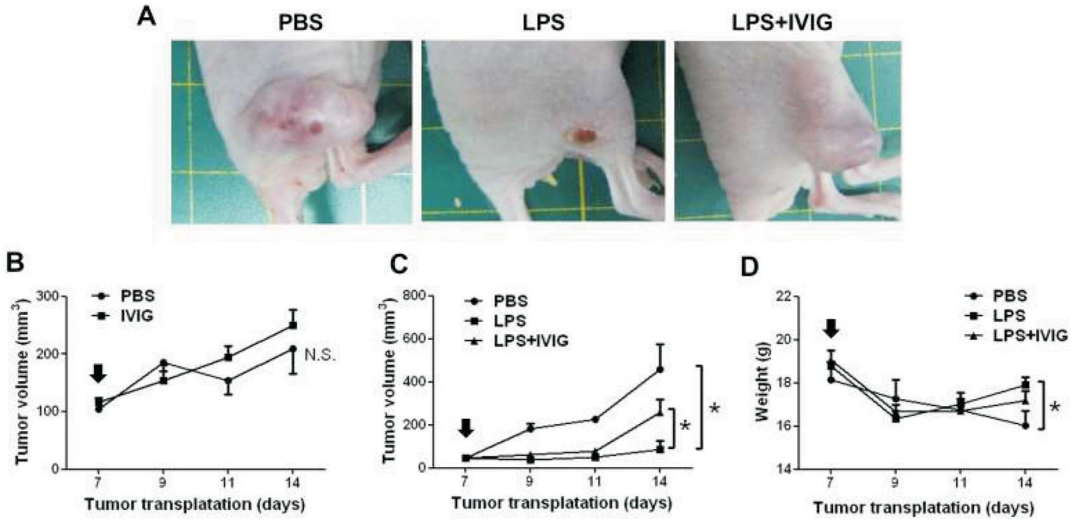

Fig. 1. LPS inhibited HCT-15 tumor initial progression. (A) Representative images of HCT-15 bearing nude mice that received various indicated treatments at 7-days-post transplantation, including PBS, LPS, and IVIG + LPS. (B and C) Growth curves of HCT-15 tumors were recorded in mice treated with IVIG (n = 3), LPS (n = 3), and LPS + IVIG (n = 3) compared to PBS control (n = 3). (D) Bodyweight changes in HCT-15 bearing mice treated with LPS, LPS + IVIG compared to PBS control. Agent injection is indicated by the black arrow. * $p < 0.05$.

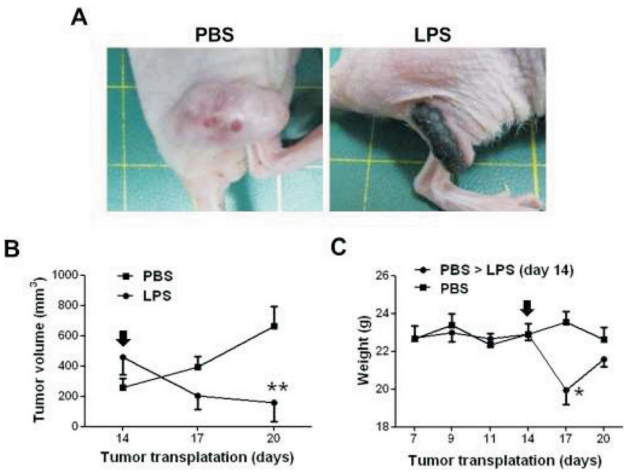

Fig. 2. LPS enhanced tumor necrosis and reduced HCT-15 tumor volume. (A) Representative images of HCT-15 bearing nude mice that received various indicated treatments at 14-days-post transplantation. PBS and LPS were intravenously injected. (B) Growth curves of HCT-15 tumors were recorded in mice treated with LPS (n = 3) compared to PBS control (n = 3). (C) Bodyweight change were also recorded in HCT-15 bearing mice treated with LPS compared to PBS control. Agent injection is indicated by the black arrow. * $p < 0.05$, ** $p < 0.01$.

mor effects of LPS against CRC progression in a colorectal HCT15 cancer cells-derived xenograft model. In this model, HCT-15 cells were subcutaneously implanted into nude mice and the mice were intravenously injected with LPS at day 7 and day 14 to evaluate the anti-tumor effects at an early or late stage, respectively. In addition, to confirm LPS effects on the innate immune system, intravenous immunoglobulin (IVIG), an immunosuppressant for the monocyte-macrophage system,[21] was co-injected with LPS at day 7 after tumor inoculation. We found that LPS significantly delayed the tumor progression and reduced tumor burden, but the anti-tumor effects were inhibited by IVIG. These findings suggested that LPS treatment may ameliorate tumors by activating an innate immune defense.

In general, tumor-bearing mice showed gradual decrease in body weight, tumor size from 0 to 400 mm^3, day 0 to day 7 (Fig. 1D by PBS injection) but steady when tumor size from 300 to 600 mm^3, day 7 to day 14 (Fig. 2C by PBS injection). On the other hand, LPS treatment dramatically caused loss of body weight but rapidly recovered in small tumor (Fig. 1D) and large tumor (Fig. 2C). Moreover, body weight increased after LPS injection because tumor growth was inhibited (Fig. 1D at day 14). We speculated that body weight is an indicator representing adverse effect of injected agent. The effect that LPS dramatically decreased body weight after i.v. injection was speculated due to inflammatory cytokine storm derived by activation of immunity.

Since LPS binds to TLR4 which is expressed in macrophages, we speculate that LPS activates macrophages to prevent tumors in this study. To our knowledge, macrophages execute various functions, including defense against invading pathogens. According to literature, macrophages are usually classified M1 (classical-activated macrophages) and M2 (tumor-associated macrophages, TAMs) phenotype.[22] Generally, M1 macrophages enhance inflammation response against invading pathogens and initial tumor cells, but M2 macrophages play an immune suppressive role to trigger tumor progression. M1 macrophages express CD14, CD68, CD80, and CD86 and secrete pro-inflammatory cytokines such as IL-12, tumor necrosis factor (TNF)-α, CXCL-10, interferon (IFN)-γ and nitric oxide synthase (NOS). Otherwise M2 macrophages express CD163, MGL1, and MGL and secrete anti-inflammatory cytokines such as IL4, IL-10, and IL-13 and express abundant arginase-1, mannose receptor (MR, CD206), and scavenger receptors.[23-25] LPS is transferred to CD14 and consequently bind to TLR4 on macrophages which belongs to M1 phenotype. A previous study has indicated that LPS induces activation of M1 macrophages.[26] Although the animal model used in this study is a T cell deficient mice, we observed LPS injection diminished tumor growth. We speculated that LPS specifically activated on M1 macrophage to suppress tumor progression.

However, the major limitation is that mouse model used in this study has normal immune function, but actually the immune activity is often suppressed in the tumor microenvironment of cancer patients. Therefore, whether LPS can induce the immune activity in cancer patients needs further investigation. Recent literature has revealed that immunotherapies are promising to fight against tumors. Since TAMs contrib-

ute to tumor progression as a therapeutic target,[25] the TAM-targeted therapeutics are mainly focus on the strategies to eliminate M2 or educate M2 to transfer to M1 phenotype.[27] For example, zoledronic acid (ZA) is a potent agent to modulate macrophages phenotypes against tumors. ZA is able to reverse the polarity of TAMs from M2-like to M1-like by attenuating IL-10, VEGF, and MMP-9 expression with recovery of iNOS expression.[28,29] Another agent capable of repolarizing TAMs to M1 phenotype is CP-870,893, which is a CD40 antibody. According to a previous study, administration of CD40 antibody is able to induce macrophage-dependent tumor regression *in vivo*.[30] There is exhausted immunity in patients with tumors. Although we observed that LPS was able to diminish tumor growth, the priority was to converse M2 to M1 in tumor patients. Subsequently, stimulation of M1-like macrophages by adequate antigens may help eradicate tumors.

However, LPS also enhance tumor proliferation and progression through binding to TLR4 in tumors. It indicates that LPS is not a potent agent to stimulate M1 macrophages in tumor patients. Compared to LPS, an intrinsic cytokine IFNγ also binds to and activates macrophages, leading to induction of M1 differentiation.[31,32] IFNγ is a pro-inflammatory marker secreted by T helper type 1 (Th1)-type T cells, natural killer cells, and activated antigen-presenting cells. Besides activating M1 differentiation, IFNγ, however, induces PD-L1 expression in macrophages and dendritic cells, resulting in suppression of $CD8^+$ T cells. Meanwhile, IFNγ also induces PD-L1 expression in tumors. STAT1 is reported involved in transcription of PD-L1. Therefore, targeting PD1-PD-L1 interaction is potent to reactivate $CD8^+$ T cells, leading to evoke $CD8^+$ T cells to against tumors. Although the findings about LPS to suppress tumors in this study is not potent enough to perform in tumor therapeutics, IFNγ combined with anti-PD-1 antibody may be an alternative selection.[33,34]

Conclusion

We investigated and validated the anti-tumor effect of LPS in a HCT15-derived xenograft CRC model. We speculated that LPS activated the immune system to inhibit tumor growth since IVIG suppressed the anti-tumor effect. This study suggested activation of the immune system is capable of eradicating colorectal tumors and the immunotherapeutic strategies have potential against CRC in clinical practice.

Declare

The authors have declared that no competing interests exist.

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原　著

於大腸癌細胞異植鼠中試驗脂多醣激活免疫系統抑制大腸癌的生長

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背景　脂多醣 (LPS) 是革蘭氏陰性細菌外膜表達的主要抗原。LPS 在正常免疫系統中誘導強烈反應。在這項研究中，我們假設 LPS 能夠激活免疫系統以防止腫瘤進展。

方法　在腫瘤植入小鼠後第 7 天和第 14 天分別將 LPS 靜脈注射到 HCT15 衍生的異植腫瘤模型中，以觀察 LPS 是否能夠抑制腫瘤的發生和發展。

結果　我們發現 LPS 顯著降低小鼠的初始腫瘤生長。同時，共同注射免疫抑製劑 IVIG 可中和抗腫瘤作用。由於注射 LPS 組有效抑制初始腫瘤生長，因此與 PBS 控制組相比，LPS 組在第 14 天體重得以恢復。此外 LPS 可以根除較大的腫瘤，但體重卻顯著降低。

結論　我們證明在 CRC 腫瘤異種移植模型中 LPS 改善了小腫瘤的發生和大腫瘤的進展。

研究限制　此研究所用老鼠模型為正常免疫功能，但癌病人的免疫活性往往受到癌細胞微環境的抑制，因此，LPS 是否可誘發癌病人的免疫活性需進一步探討。

關鍵詞　大腸癌、脂多醣。