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Activation of Toll-Like Receptor 5 on Breast Cancer Cells by Flagellin Suppresses Cell Proliferation and Tumor Growth

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Abstract

Increasing evidence showed that Toll-like receptors (TLRs), key receptors in innate immunity, play a role in cancer progression and development but activation of different TLRs might exhibit the exact opposite outcome, anti-tumor or pro-tumor effects. TLR function has been extensively studied in innate immune cells, so we investigate the role of TLR signaling in breast cancer epithelial cells. We found that TLR5 was highly expressed in breast carcinomas and that TLR5 signaling pathway is overly responsive in breast cancer cells. Interestingly, flagellin/TLR5 signaling in breast cancer cells inhibits cell proliferation and an anchorage-independent growth, a hallmark of tumorigenic transformation. In addition, the secretion of soluble factors induced by flagellin contributed to the growth-inhibitive activity in an autocrine fashion. The inhibitive activity was further confirmed in mouse xenografts of human breast cancer cells. These findings indicate that TLR5 activation by flagellin mediates innate immune response to elicit potent antitumor activity in breast cancer cells themselves, which may serve as a novel therapeutic target for human breast cancer therapy.

Introduction

Toll-like receptors (TLRs) are membrane-bound receptors that play key roles in both the innate and adaptive immune systems, particularly in inflammatory responses against pathogen infection (1-3). These receptors are primarily expressed on innate immune cells and recognize conserved pathogen-associated molecular patterns (PAMPs) (4-7). TLRs can also recognize some endogenous ligands (8,9). Activation of TLRs might play a role in cancer progression and development (10,11); however, activation of different TLRs might display completely different results. Several studies have shown that activation of TLR4 signaling by LPS protects tumor cells from immune attack and thus promotes tumor growth (12-14). Triggering of TLR9 on cancer cells has been shown to protect cancer cells against TRAIL-induced apoptosis and promote tumor cell proliferation (15). In contrast, activation of TLR3 induced an antiproliferative signaling in human breast cancer and melanoma (16,17). Thus, the function and biological importance of TLRs expressed on various tumor cells appears complex.

Unlike other TLR family members, TLR5 is not expressed on mouse macrophages and conventional dendritic cells. Instead, TLR5 expression is high in intestinal epithelial cells and lamina propria dendritic cells (18,19). It has been shown that TLR5 is highly expressed on gastric carcinoma cells (20,21). Recent evidence demonstrated that activation of TLR5 by its ligand flagellin elicits potent antitumor activity and thus inhibits colon tumor growth

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in vivo (21). In contrast, Sfondrini *et al.* showed that early administration of flagellin at the time of implanting mouse mammary cells resulted in increased tumor growth (22). Thus, the different results of these studies reveal the unique responses and susceptibilities to flagellin stimulation in different cancer cells. To date, the specific function and detailed mechanism of TLR5 signaling pathways in breast cancer cells is still poorly understood.

Here we report a comprehensive characterization of TLR5 expression and TLR5 signaling in human breast cancer cells themselvs. We found that TLR5 was highly expressed in human breast carcinomas and TLR5 signaling was overly functional in human breast cancer cells. Remarkably, triggering of TLR5 signaling pathway by flagellin in breast cancer cells inhibits cell proliferation and elicits the potential antitumor activity in mouse xenografts of human breast cancer.

Materials and Methods

Mice and Reagents

Six-week-old female athymic Nude mice (*Foxn1*^{nu}/*Foxn1*$^{+}$) were purchased from Harlan (Indianapolis, IN). *S. typhimurium* flagellin was purchased from InvivoGen (San Diego, CA) and used at the indicated concentrations. Antibodies against IκBα, phospho-IκBα, phospho-c-Jun NH2-terminal kinase (phospho-JNK), phospho^{Tyr701}-STAT1, phospho^{Tyr705}-STAT3, STAT1, STAT3, cyclin B1, cyclin D1, cyclin E2 and p21$^{Waf1/Cip1}$ were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho-ERK1/2 , ERK1 , PCNA (F-2), p27^{Kip1} (F-8), CDK2 (D-12) and TLR5 (H-127) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody to Gr-1 was purchased from BD Pharmingen (San Diego, CA). An antibody to F4/80 was purchased from AbD Serotec (Raleigh, NC). Anti-human TLR5 (anti-hTLR5) IgA, which is a blocking MAb to human TLR5, and matched human IgA isotype control were purchased from InvivoGen (San Diego, CA). Plasmid construct expressing NF-κB-luciferase reporter was described previously (23). Human breast cancer tissue arrays (BRC711 and T082) including 75 individual breast cancer specimens were purchased from US Biomax. Total RNA was isolated with TRIzol reagent (Invitrogen), and cDNA was prepared as previously described (24).

Generation of TLR5 knocked down MCF-7 cells

The silencing vector expressing siRNA targeting the human TLR5 was obtained from InvivoGen (San Diego, CA) (21).

Western blotting and Luciferase Assay

Tumor cells were starved with 1% FBS medium for 2 hours to decrease the signaling background and then treated with flagellin or other ligands for the indicated periods of time at 37°C. Immunoblotting with the appropriate antibodies was performed as described previously (12). The luciferase assay was conducted as described previously (24).

Immunofluorescence staining and Immunohistochemistry

Cells grown on a glass slide were fixed in 4% paraformaldehyde and permeabilized using 0.5% Triton X-100. Slides were washed with PBS and blocked with normal goat serum (1%) plus 1% bovine serum albumin in PBS for 20 min at room temperature. Slides were then incubated overnight with primary antibodies (1:100) against TLR5 in 1 × PBS with 1% bovine serum albumin. Samples were washed with PBS and incubated with Biotin-anti-rabbit secondary antibody (1:200) for 1 hour. Next, samples were washed with PBS and incubated with streptavidin-horseradish peroxidase conjugate solution for 20 min. After washing with PBS, the specifically bound antibodies were detected using TSA Plus Fluorescence Systems from Perkin Elmer (Boston, MA). The slides were then rinsed and mounted with DAPI mounting solution. Images were analyzed with a Zeiss Axioskop-2 microscope. Immunohistochemical analyses were performed with the ABC Elite kit (Vectorlabs, CA).

Cytokine microarray analysis

RayBio® Human Cytokine Antibody Array V was purchased from Ray Biotech (Norcross, GA). MCF-7 cells were treated with flagellin (0.1 μg/ml) or PBS (control) for 24 hours. Then, supernatants were collected and equal amounts of the supernatants were subjected to a human cytokine array, as previously described (21,25,26). The cytokine expression was determined by measuring the density of each spot using TotalLab TL100 software.

Cell cycle analysis

FACS was performed for cell cycle analysis. Flagellin-treated or non-treated cells in different days were detached with trypsin and stained with FITC BrdU Flow kit (BD Pharmingen™). Flow cytometry was performed by using a FACS Calibur, and data were analyzed with FlowJo software (Tree Star, OR).

Soft agar colony formation

Breast cancer cells (5×10^3) were plated in 0.35% low melting point agarose/growth medium onto six-well plates with a 0.6% agarose underlay. About 1.5 ml growth medium with or without flagellin at indifferent concentrations was added on the top of agarose and the medium was changed every 3–4 d, and incubated at 37°C in a humidified incubator for 14 days. At the end of the treatment, the cells were washed with PBS and incubated in a solution of 0.005% crystal violet and 10% formalin for 10 min and then rinsed with water. The integrated density of the colonies on each plate was determined using TotalLab TL100 software.

Xenograft model of human breast cancer

To establish breast cancer xenografts in nude mice, MDA-MB-468 cells (5×10^6) were mixed with Matrigel and injected under aseptic conditions into the mammary fat pads of nude mice (n=6 for each group). For MCF-7 xenografts, ovariectomized mice were first implanted *s.c.* with 60-day estradiol-releasing pellets (1.7 mg/pellet from Innovative Research) before 24 hours of cell injection. Then, MCF-7 cells (2×10^7) were mixed with Matrigel and injected into both fourth mammary fat pads of ovariectomized athymic nude mice. For peritumoral flagellin treatment, 10 days after injecting cells into the mammary fat pads of nude mice, we administered flagellin solution (1.0 μg in 50 μL) around the tumor site (1 injection/every 2 days for 3–4weeks). For i.v. flagellin treatment, one month after MDA-MB-468 xenografts were fully established, each mouse was injected with flagellin solution (2 μg in 100 μL) through the tail vein (1 injection/every 7 days for 6 weeks). The control group was injected with saline solution with the same method. The tumor was monitored and evaluated every 2 and 7 days for peritumoral and i.v. flagellin treatment, respectively. Tumors were measured in two dimensions, and volume was calculated according to the formula: $V=0.5 \times (\text{Length} \times \text{Width}^2)$.

Results

Expression of TLR5 in breast cancer

Although TLRs are mainly expressed and activated on innate immune cells such as macrophages and dendritic cells (DCs), TLRs may be functional in other cell types. To investigate the expression and potential function of TLR5 in breast cancer epithelial cells, we performed immunohistochemical staining on tissue arrays containing normal breast tissues and breast carcinomas (n=75) with different subtypes, stages and grades. We found that TLR5 was expressed in normal breast duct epithelium but not in the fibro fatty tissue of normal breast samples (Fig. 1A). Of all the breast cancer tissues tested, 60 tumor samples (80%) were positive for TLR5 and the majority of TLR5-positive cases were high-grade ductal carcinomas (Fig. 1A and supplementary Table 1). Specifically, TLR5 was highly expressed in most of invasive ductal carcinomas (IDC), and moderately expressed in medullary carcinomas and invasive lobular carcinoma (Fig. 1A). The staining was localized in the cytoplasm as well as on the cell surface, which was similar to the localization of TLR4 in ovarian cancer cells (14). However, no staining of TLR5 was observed in breast mucinous adenocarcinomas (Supplementary Table. 1).

We then analyzed the expression of TLR5 in breast cancer cell lines including MCF-7, MDA-MB-468, SKBR3, T47D, MDA-MB-231 and MDA-MB-435 cells. Immunoflorescence confirmed the expression of TLR5 in these six cell lines (Fig. 1B). Similarly to our observation on the tissue sections, TLR5 was distributed both inside the cell and on the cell surface in MCF-7, MDA-MB-468, SKBR3 and T47D cells. However, TLR5 was only localized in the cytoplasm in MDA-MB-231 and MDA-MB-435 cells (Fig. 1C). The expression of MyD88, which is a critical adaptor protein in TLR5 signaling pathway, was also detected in these breast cancer cell lines by western blotting (supplemental Fig. 1).

Activation of TLR5 signaling pathway by flagellin in breast cancer cells

We next asked whether TLR5 was also functional to activate TLR signaling pathway in breast cancer cells. To address this question, the six breast cancer cell lines were transfected with a construct containing a luciferase reporter under the control of NF-κB response element and stimulated with the TLR5 natural ligand flagellin in different concentrations. As shown in Fig. 2A, the NF-κB luciferase reporter activities in MCF-7, MDA-MB-468, SKBR3 and T47D cells were increased in a dose-dependent manner after challenging with flagellin. However, the other two cell lines, MDA-MB-231 and MDA-MB-435, appeared to be unresponsive to flagellin stimulation.

To verify that the activation of NF-κB following flagellin incubation was due to TLR5 activation, we first pre-incubated MCF-7 cells with an anti-TLR5 blocking antibody in different concentrations and then challenged the cells with flagellin. We found that the flagellin induced NF-κB luciferase reporter activity can be suppressed by anti-TLR5 blocking antibody in a dose-dependent manner (Fig. 2B). Then, we further established an MCF-7 tumor cell line stably expressing TLR5 shRNA (Supplemental Fig. 2). The phosphorylation levels of IκB, ERK, and JNK after flagellin stimulation were increased in MCF-7 wild-type cells but not in the knocked down cells (Fig. 2C). We next asked if flagellin/TLR5 signaling pathway in MCF-7 cells can activate the expression of cytokines and/or chemokines in canonical TLR downstream signaling. Interestingly, we found that upon flagellin stimulation, there was a striking induction of TNF-α, IL-1β, IL-6 and IL-8 mRNA in MCF-7 cells but not in TLR5 knocked down cells (Fig. 2D). Thus, these data collectively suggested that flagellin activated TLR5-dependent signaling pathway in breast cancer cells. In order to further analyze the profile of cytokines and chemokines induced by flagellin in breast cancer cells, the flagellin-stimulated tumor cell supernatant was used for cytokine microarray. Flagellin substantially increased production of several chemokines including epithelial cell-derived neutrophil-activating peptide-78 (ENA-78), MIP3α, monocyte chemotactic protein-1(MCP-1), macrophage-derived chemokine (MDC), IL-6, Gro-α and Osteoprotegerin (Fig. 2E). Interestingly, the induced chemokines upon flagellin stimulation are mostly involved in leukocyte recruitment (27). Thus, activation of TLR5 by flagellin initiated TLR signaling pathway transduction and induced the secretion of several cytokines and chemokines in breast cancer cells.

Flagellin inhibits tumor cell proliferation and colony formation *in vitro*

To investigate the role of Flagellin/TLR5 signaling on tumor cell proliferation, a BrdUrd incorporation assay was performed. MCF-7 cells were treated with flagellin (0.1 μg/ml) for periods up to 3 days and the number of BrdUrd incorporated cells was determined daily by flow cytometry. As shown in Fig. 3A, the percentage of BrdUrd incorporated cells was decreased from 46% to 32% after 1 day of flagellin treatment, suggesting that flagellin inhibited cell proliferation. Moreover, after 2 and 3 days of treatment, the percentage of BrdUrd positive cells was further decreased and maintained to 13% of the cells. However, we could not detect the population of cells in sub-G_0/G_1 phase (data not shown), indicating that flagellin did not induce apoptosis.

To further characterize the effect of flagellin on tumor cell proliferation, a colony formation assay was performed in MCF-7 cells after challenging with flagellin with increased concentrations. This experiment was designed to assay the ability of cells to grow unattached to a surface and therefore suspended in agar. Thus, it could be used to measure the tumorigenicity of tumor cells. As shown in Fig. 3B, the colony formation ability of MCF-7 was significantly decreased by flagellin in a dose-dependent manner. This phenomenon was TLR5 dependent since knocking down TLR5 in the cells could block the suppressive activity of flagellin (Fig. 3C). Flagellin also inhibited colony formation of MDA-MB-468, SKBR3 and T47D cells in higher concentration (10 μg/ml) (Fig. 3D). However, for the other two flagellin non-responsive cells, MDA-MB-231 and MDA-MB-435, no significant decrease of colony number was observed even in the presence of flagellin with the highest concentration. Thus, the activation of TLR5 by flagellin inhibited proliferation and colony formation of breast cancer cells *in vitro*.

Soluble factors mediate the anti-proliferation effect of flagellin

To gain more insights into the molecular mechanism in the anti-proliferation effect of flagellin on breast cancer cells, we analyzed the expression level of various proteins involved in cell cycle regulation upon flagellin treatment. We found that the levels of cyclin B1, cyclin D1 and cyclin E2 were significantly decreased after flagellin treatment. A marginal increase of CDK inhibitor p27 was also observed at early stage of flagellin treatment (Fig. 4A). To analyze whether treatment of MCF-7 cells with flagellin results in secretion of soluble factors that might contribute in an autocrine fashion to the growth-inhibitive activity, cells were treated for 4 hours with flagellin, washed, and replenished with fresh medium for another 6 hours. Then the medium was collected as conditioned medium. We found that conditioned medium from MCF-7 cells treated with flagellin decreased the colony size in comparison to the untreated control or the conditioned medium from flagellin-treated TLR5 knocked down cells (Fig. 4B). Thus, at least in part, flagellin-induced soluble factors mediate the anti-proliferation effect on breast cancer cells. To further investigate the molecular mechanisms in activation of cytokine signaling by flagellin-induced soluble factors in breast cancer cells, the phosphorylation levels of STATs were analyzed by western blotting after flagellin treatment. As expected, the phosphorylation of STAT1 and STAT3 was induced by flagellin in later stage, suggesting that cytokine-induced signaling pathways were activated in breast cancer cells (Fig. 4C). Thus, these data indicate that the soluble factors induced by flagellin in an autocrine fashion are involved in an anti-growth effect of flagellin on breast cancer cells.

Activation of TLR5 by flagellin inhibits tumor growth *in vivo*

To determine the effect of flagellin on tumor growth *in vivo*, we first used MCF-7 breast cancer cell xenograft mouse model. After the palpable tumors were established, the mice were injected with or without flagellin solution (1.0 μg in 50 μL) around the tumor site and administrated every 2 days. As shown in Fig. 5A, tumors in the control group increased from 52 ± 13 to 428 ± 178 mm^3, whereas tumors in the flagellin treated group increased only from 48 ± 12 to 162 ± 35 mm^3. The average weight of tumors from the control group was 425 ± 11 mg, whereas the average weight in flagellin treated group only 168 ± 5 mg (Fig. 5A), suggesting that flagellin retarded tumor growth in the xenograft mouse breast tumor model. Flagellin had no significant effect on the body weight of mice, indicating low toxicity of flagellin at the test dosage and conditions (data not shown). However, in the mouse inoculated with TLR5 shRNA-expressing MCF-7 cells, although the cells maintained similar growth rate *in vivo* compared to wild-type cells, flagellin failed to inhibit tumor growth *in vivo* (Supplemental Fig. 3), indicating that TLR5-dependent anti-tumor activity is involved in this flagellin response.

To gain more evidence with other breast cancer cells, we established MDA-MB-468 xenograft tumor model, in which the tumors are estrogen-independent (28). Flagellin also significantly retarded tumor growth and decreased tumor weight when administrated with flagellin around the tumor sites (Fig. 5B). To access the antitumor activity of flagellin in mice with established larger tumor burden, we further allowed MDA-MB-468 tumor to grow to 80-100 mm^3. Then, we administrated flagellin by i.v. injection at 2 μg per-mouse and injected every week. We found that the systemic administration of flagellin also inhibited tumor growth and decreased tumor weight (Fig. 5C). Since systemic administration of flagellin might also elicit apoptotic signaling and systemic inflammatory responses in mice (29), we further analyzed the potential toxicity of flagellin by i.v. treatment. Similar to previous report, systemic administration with flagellin induced serum production of IL-6 in early time point. However, the level of IL-6 was return to baseline concentration after 24 hours later (Supplementary Fig. 4A). Moreover, histological examination of the livers and spleens revealed no obvious inflammation, necrosis, or fibrosis between control and treatment groups (Supplementary Fig. 4). Body weights as well as spleen, lung and heart weights were unaffected in flagellin-treated groups (Supplementary Fig. 4 and data not shown). Thus, these data collectively indicate that flagellin exhibits minimal toxic effects in normal animals when systemic administrated by i.v. injection at the test dosage.

TLR5 activation by flagellin leads to increased tumor necrosis and neutrophil infiltration *in vivo*

To further analyze the mechanisms for TLR5/flagellin signaling pathway mediated tumor suppression *in vivo*, the histopathology of xerograft tumor was then investigated by haematoxylin and eosin stain. As shown in Fig. 6A, examination of tumor sections revealed that tumor necrosis and leukocytes infiltration were increased in the flagellin treated group. Moreover, immunohistochemical staining for cell proliferation marker PCNA in tumor sections indicated that while PCNA was noticeably higher in MCF-7 cell-injected mice, the expression of this protein was inhibited by treatment of flagellin (Fig. 6B). To determine the infiltrated cell type in flagellin-treated tumors, we performed immunohistochemistry with tumor xenografts using antibodies against neutrophil and macrophage specific marks (Gr-1 and F4/80, respecitively). There were no obvious F4/80 positive cells in tumor sections either from control or flagellin-treated groups (Supplementary Fig. 5). However, Gr-1 positive cells were significantly increased around necrotic areas of tumor sections from flagellin-treated groups (Fig. 6B), indicating TLR5 activation by flagellin leads to increased neutrophil infiltration *in vivo*. As neutrophils have been shown to exhibit potent antitumor activity in tumor microenvironment (21,30,31), these data indicate that the increase of neutrophil infiltration could be an important mechanism for TLR5/flagellin induced antitumor activity *in vivo*.

Discussion

Recent evidence suggests that TLRs, key receptors in innate immunity, play a role in tumorigenesis but different TLRs exhibit either anti-tumor or pro-tumor activities (11). In this study, we investigated the expression and function of TLR5 in breast cancer epithelial cells. We found that TLR5 was specifically expressed in the ductal epithelium of normal breast tissues, implicating a role of TLRs in inflammatory responses of the mammary tissue against pathogen infection (32). Interestingly, we also found that most of human breast cancer samples expressed TLR5 and there was an enhanced expression of TLR5 in some subtypes of breast carcinomas. Because the endogenous ligands for TLR5 in tumors are not known, we used flagellin, the natural ligand of TLR5, to examine the activity of TLR5 signaling in breast cancer cells. Similar to other reports in immune sentinel cells and fibroblasts (33,34), activation of TLR5 by flagellin in breast cancer cells induced the secretion of pro-inflammatory cytokines and chemokines. It is worthwhile to notice that not every breast cancer cell line we tested was responsive for flagellin stimulation and there was no simple correlation between TLR5 expression and flagellin sensitivity in these breast cancer cell lines. Since we also observed the differences in subcellular localization of TLR5 between flagellin sensitive and non-sensitive cell lines, the surface localization of TLR5 in flagellin sensitive cells might explain why these cells are highly responsive for flagellin stimulation, which is similar to other TLRs (35).

In fibroblasts and dendritic cells (DCs), TLR5/flagellin signaling pathway has been shown to promote cell proliferation and prevent apoptosis by inducing p27 degradation, which can be antagonized by type I IFNs (33,34). In contrast, our results show that activation of TLR5 by flagellin in breast cancer cells inhibits cell proliferation and anchorage independent growth. As cell specific TLR5 signaling might account for our different results, we also observed significant decrease of cell cyclin proteins with marginal increase of p27 levels in breast cancer cells upon flagellin treatment. We found that flagellin-induced soluble factors exhibited suppressive activity on tumor cell proliferation, suggesting that the effect of flagellin on cancer cell proliferation is not primarily mediated by the direct intracellular mechanism of TLR5 as previously reported in fibroblasts and DCs (33,34). In these flagellin-induced soluble factors, several factors like MIP3α and IL-6 have been shown to inhibit proliferation of various types of cells including breast cancer cells (36,37). However, in other scenarios these factors have also been reported to promote cell proliferation and tumor growth (38,39). The different results are dependent upon different cell types and different combinations with other factors in tumor microenvironment (40). Moreover, we observed flagellin induced phosphorylation of STAT1 and STAT3 in breast cancer cells at late stages, further suggesting cytokine-induced signaling pathways were activated in breast cancer cells after flagellin treatment. As a key mediator of cytokine-induced gene expression, STAT1 is activated by many cytokines including type I and type II IFNs (41). While we failed to detect the secretion of type I and type II IFNs upon flagellin treatment for 24 hours (data not shown), the phosphorylation of STAT1 suggests that other factors may be involved in cytokine-induced signaling pathway upon flagellin treatment and initiate a unique TLR5 signaling pathway in breast cancer cells to inhibit cell growth.

As a receptor highly expressed in intestinal epithelial cells, including colon cancer cells, TLR5 has been shown to mediate anti-tumor activity in a mouse xenograft model of human colon cancer (21). Consistent with this study, our data show that administration of flagellin *in vivo* retards tumor growth in the xenograft mouse model of human breast cancer. As we observed *in vitro*, flagellin induced secretion of endogenous cytokines and chemokines including MIP-3α, ENA-78, GRO-α and MDC. These factors have been shown to attract monocytes, neutrophils, and lymphocytes to the site of infection (23-27). In agreement with the report by Rhee SH *et al.* demonstrating that infiltration of neutrophils induced by flagellin exert potent anti-tumor responses in a mouse xenograft model of human colon cancer, we observed increased tumor necrosis and leukocyte infiltration upon flagellin treatment either by peritumoral or i.v. injection. Interestingly, a recent report also observed that flagellin induced expression of several chemokines to recruit neutrophils and monocytes *in vitro* in prostate cancer cells (42). Thus, these data suggest that the flagellin-induced pro-inflammatory factors further recruit neutrophils at the tumor site to modulate the growth of breast tumors.

Dynamic interaction between tumor and tumor microenvironment is essential for tumor growth, angiogenesis and metastasis (43). Activation of TLRs on tumor cells has been shown to modulate tumor microenvironment and elicit pro- or anti-tumor activities. Manipulating TLR signaling to enhance its anti-tumor effects in tumor microenvironment has been investigated and reported by several studies (44,45). In the present study, we identified that TLR5 was highly expressed and activated in breast carcinomas and cancer cells. Furthermore, we showed that activation of TLR5 by flagellin in breast cancer cells modulate the production of pro-inflammatory cytokines to elicit a potent anti-tumor activity in breast cancer, which may serve as a novel therapeutic target for human breast cancer therapy.

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Reference

1. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010; 140:805–20. [PubMed: 20303872]
2. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. Science. 2010; 327:291–5. [PubMed: 20075244]
3. Beutler B. Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. Immunol Rev. 2009; 227:248–63. [PubMed: 19120489]
4. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998; 282:2085–8. [PubMed: 9851930]
5. Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature. 1998; 395:284–8. [PubMed: 9751057]
6. Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, Barrett SL, et al. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol. 2003; 4:1247–53. [PubMed: 14625549]
7. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000; 408:740–5. [PubMed: 11130078]
8. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007; 13:1042–9. [PubMed: 17767165]
9. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007; 13:1050–9. [PubMed: 17704786]
10. Kluwe J, Mencin A, Schwabe RF. Toll-like receptors, wound healing, and carcinogenesis. J Mol Med. 2009; 87:125–38. [PubMed: 19089397]
11. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. Nat Rev Cancer. 2009; 9:57–63. [PubMed: 19052556]

12. Huang B, Zhao J, Li H, He KL, Chen Y, Chen SH, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. Cancer Res. 2005; 65:5009–14. [PubMed: 15958541]

13. Szczepanski MJ, Czystowska M, Szajnik M, Harasymczuk M, Boyiadzis M, Kruk-Zagajewska A, et al. Triggering of Toll-like receptor 4 expressed on human head and neck squamous cell carcinoma promotes tumor development and protects the tumor from immune attack. Cancer Res. 2009; 69:3105–13. [PubMed: 19318560]

14. Kelly MG, Alvero AB, Chen R, Silasi DA, Abrahams VM, Chan S, et al. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res. 2006; 66:3859–68. [PubMed: 16585214]

15. Chiron D, Pellat-Deceunynck C, Maillasson M, Bataille R, Jego G. Phosphorothioate-modified TLR9 ligands protect cancer cells against TRAIL-induced apoptosis. J Immunol. 2009; 183:4371–7. [PubMed: 19734228]

16. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol. 2006; 176:4894–901. [PubMed: 16585585]

17. Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P. Toll-like receptor 3 expressed by melanoma cells as a target for therapy? Clin Cancer Res. 2007; 13:4565–74. [PubMed: 17671143]

18. Uematsu S, Akira S. Immune responses of TLR5(+) lamina propria dendritic cells in enterobacterial infection. J Gastroenterol. 2009; 44:803–11. [PubMed: 19547909]

19. Miao EA, Andersen-Nissen E, Warren SE, Aderem A. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. Semin Immunopathol. 2007; 29:275–88. [PubMed: 17690885]

20. Schmausser B, Andrulis M, Endrich S, Muller-Hermelink HK, Eck M. Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with Helicobacter pylori. Int J Med Microbiol. 2005; 295:179–85. [PubMed: 16044857]

21. Rhee SH, Im E, Pothoulakis C. Toll-like receptor 5 engagement modulates tumor development and growth in a mouse xenograft model of human colon cancer. Gastroenterology. 2008; 135:518–28. [PubMed: 18538140]

22. Sfondrini L, Rossini A, Besusso D, Merlo A, Tagliabue E, Menard S, et al. Antitumor activity of the TLR-5 ligand flagellin in mouse models of cancer. J Immunol. 2006; 176:6624–30. [PubMed: 16709820]

23. Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, et al. A toll-like receptor that prevents infection by uropathogenic bacteria. Science. 2004; 303:1522–6. [PubMed: 15001781]

24. Cai Z, Shi Z, Sanchez A, Zhang T, Liu M, Yang J, et al. Transcriptional regulation of Tlr11 gene expression in epithelial cells. J Biol Chem. 2009; 284:33088–96. [PubMed: 19801549]

25. Rhee SH, Keates AC, Moyer MP, Pothoulakis C. MEK is a key modulator for TLR5-induced interleukin-8 and MIP3alpha gene expression in non-transformed human colonic epithelial cells. J Biol Chem. 2004; 279:25179–88. [PubMed: 15069060]

26. Rhee SH, Kim H, Moyer MP, Pothoulakis C. Role of MyD88 in phosphatidylinositol 3-kinase activation by flagellin/toll-like receptor 5 engagement in colonic epithelial cells. J Biol Chem. 2006; 281:18560–8. [PubMed: 16644730]

27. Kobayashi Y. Neutrophil infiltration and chemokines. Crit Rev Immunol. 2006; 26:307–16. [PubMed: 17073556]

28. Bartholomeusz C, Gonzalez-Angulo AM, Kazansky A, Krishnamurthy S, Liu P, Yuan LX, et al. PEA-15 inhibits tumorigenesis in an MDA-MB-468 triple-negative breast cancer xenograft model through increased cytoplasmic localization of activated extracellular signal-regulated kinase. Clin Cancer Res. 2010; 16:1802–11. [PubMed: 20215547]

29. Rolli J, Loukili N, Levrand S, Rosenblatt-Velin N, Rignault-Clerc S, Waeber B, et al. Bacterial flagellin elicits widespread innate immune defense mechanisms, apoptotic signaling, and a sepsis-like systemic inflammatory response in mice. Crit Care. 2010; 14:R160. [PubMed: 20731882]

30. Challacombe JM, Suhrbier A, Parsons PG, Jones B, Hampson P, Kavanagh D, et al. Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with ingenol-3-angelate. J Immunol. 2006; 177:8123–32. [PubMed: 17114487]

31. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci U S A. 2006; 103:12493–8. [PubMed: 16891410]
32. Gonen E, Vallon-Eberhard A, Elazar S, Harmelin A, Brenner O, Rosenshine I, et al. Toll-like receptor 4 is needed to restrict the invasion of Escherichia coli P4 into mammary gland epithelial cells in a murine model of acute mastitis. Cell Microbiol. 2007; 9:2826–38. [PubMed: 17608742]
33. Hasan UA, Caux C, Perrot I, Doffin AC, Menetrier-Caux C, Trinchieri G, et al. Cell proliferation and survival induced by Toll-like receptors is antagonized by type I IFNs. Proc Natl Acad Sci U S A. 2007; 104:8047–52. [PubMed: 17463087]
34. Hasan UA, Trinchieri G, Vlach J. Toll-like receptor signaling stimulates cell cycle entry and progression in fibroblasts. J Biol Chem. 2005; 280:20620–7. [PubMed: 15788393]
35. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, et al. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol. 2006; 8:1327–36. [PubMed: 17128265]
36. Broxmeyer HE, Sherry B, Cooper S, Ruscetti FW, Williams DE, Arosio P, et al. Macrophage inflammatory protein (MIP)-1 beta abrogates the capacity of MIP-1 alpha to suppress myeloid progenitor cell growth. J Immunol. 1991; 147:2586–94. [PubMed: 1918979]
37. Shen WH, Zhou JH, Broussard SR, Freund GG, Dantzer R, Kelley KW. Proinflammatory cytokines block growth of breast cancer cells by impairing signals from a growth factor receptor. Cancer Res. 2002; 62:4746–56. [PubMed: 12183434]
38. Beider K, Abraham M, Begin M, Wald H, Weiss ID, Wald O, et al. Interaction between CXCR4 and CCL20 pathways regulates tumor growth. PLoS One. 2009; 4:e5125. [PubMed: 19340288]
39. Sasser AK, Sullivan NJ, Studebaker AW, Hendey LF, Axel AE, Hall BM. Interleukin-6 is a potent growth factor for ER-alpha-positive human breast cancer. FASEB J. 2007; 21:3763–70. [PubMed: 17586727]
40. Knupfer H, Preiss R. Significance of interleukin-6 (IL-6) in breast cancer (review). Breast Cancer Res Treat. 2007; 102:129–35. [PubMed: 16927176]
41. Watson CJ. Stat transcription factors in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia. 2001; 6:115–27. [PubMed: 11467447]
42. Galli R, Starace D, Busa R, Angelini DF, Paone A, De Cesaris P, et al. TLR stimulation of prostate tumor cells induces chemokine-mediated recruitment of specific immune cell types. J Immunol. 2010; 184:6658–69. [PubMed: 20483744]
43. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008; 454:436–44. [PubMed: 18650914]
44. Lu H, Wagner WM, Gad E, Yang Y, Duan H, Amon LM, et al. Treatment failure of a TLR-7 agonist occurs due to self-regulation of acute inflammation and can be overcome by IL-10 blockade. J Immunol. 2010; 184:5360–7. [PubMed: 20308630]
45. Conforti R, Ma Y, Morel Y, Paturel C, Terme M, Viaud S, et al. Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. Cancer Res. 2010; 70:490–500. [PubMed: 20068181]

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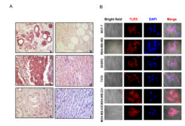

Figure 1. TLR5 expression in breast cancer

A, human breast tissue arrays were subjected to immunohistochemical analyses with anti–TLR5 primary antibody and biotinylated second antibody. Red indicates positive staining. Representative images from tissues with different histological types are shown. (a) normal breast tissue, (b) No TLR5 expression in fibrofatty tissue of normal breast, (c) Strong TLR5 expression in invasive ductal carcinoma, (d) Moderate TLR5 expression in medullary carcinoma, (e) Moderate TLR5 expression in invasive lobular carcinoma, (f) No positive signal was detected in rabbit IgG isotype control. Magnification, 40X. B, Expression of TLR5 in breast cancer cells was detected by immunoflorescence. DAPI was used for nucleic staining. Magnification, 100X.

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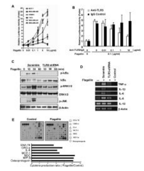

Figure 2. Flagellin activates TLR5-dependent signaling pathway in breast cancer cells

A, Breast cancer cells were transfected with NF-κB reporter plasmid and analyzed for luciferase activity induced by indicated flagellin doses. B, MCF-7 cells were transfected with NF-κB reporter plasmid and analyzed for luciferase activity induced by indicated flagellin doses in the presence of anti-hTLR5 MAb or human IgA control. C, MCF-7 and knocked down (TLR5-shRNA) cells were treated with flagellin (0.1 μg/ml) by indicated time points and phosphorylation of IκB, ERK, and JNK were analyzed by immunoblotting with phosphorylation-specific and control antibodies. D, MCF-7 cells were induced by flagellin (0.1 μg/ml) for 6 hours for the expression of cytokine mRNAs analyzed by RT-PCR. E, equal amounts of supernatant from control cells and flagellin-stimulated MCF-7 cells were used for human cytokine microarray analysis *(upper panel)*. Cytokines with significant increase were measured by spots intensity and presented as a ratio to mock control *(lower panel)*. All data were representative results of three independent experiments.

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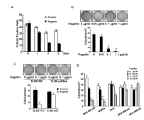

Figure 3. Flagellin inhibits proliferation and colony formation of tumor cells *in vitro*

A, MCF-7 cells were stimulated for different days with 0.1 μg/ml flagellin and assessed by BrdUrd incorporation and DNA content flow cytometry. The percentage of BrdUrd positive cells for flagellin-treated and untreated control group was shown. B, Top, four representative 35-mm plates of MCF-7 cells or (C) knocked down cells cultured for 14 days in the presence of the indicated concentrations of flagellin. At the end of the treatment the cells were fixed and stained with crystal violet. *Bottom*, quantitative data of colony formation assays in the top (B and C). colonies>1 mm in diameter were counted. **, p<0.001; relative to untreated cells. D, Quantitative data of colony formation assays in T47D, MDA-MB-231 and MDA-MB-435 cells treated with the indicated concentrations of flagellin.

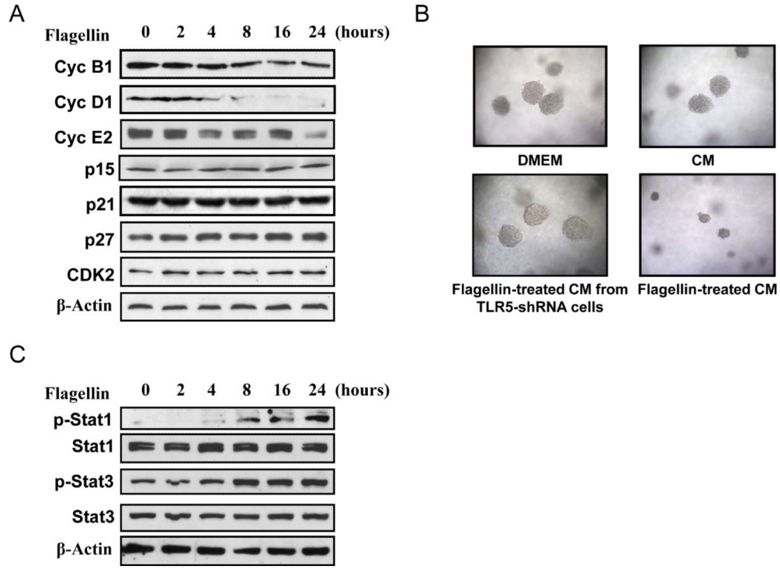

Figure 4. The anti-proliferation effect of flagellin is mediated by soluble factors

A, MCF-7 cells were treated with flagellin (0.1 μg/ml) at different time points and the expression of cell cycle proteins were examined by western blots. B, MCF-7 cells were treated with different conditioned medium indicated below the image: DMEM, Dulbecco's Modified Eagle Medium; conditioned medium from MCF-7; conditioned medium form TLR5-shRNA MCF-7 cells treated with flagellin (0.1 μg/ml); conditioned medium form MCF-7 treated with flagellin (0.1 μg/ml). Four representative images were taken from 35-mm plates of MCF-7 cells cultured for 14 days. Magnification: X40 C, MCF-7 cells were treated with flagellin (0.1 μg/ml) at different time points and the phosphorylation levels of STAT1 and STAT3 proteins were examined by western blots.

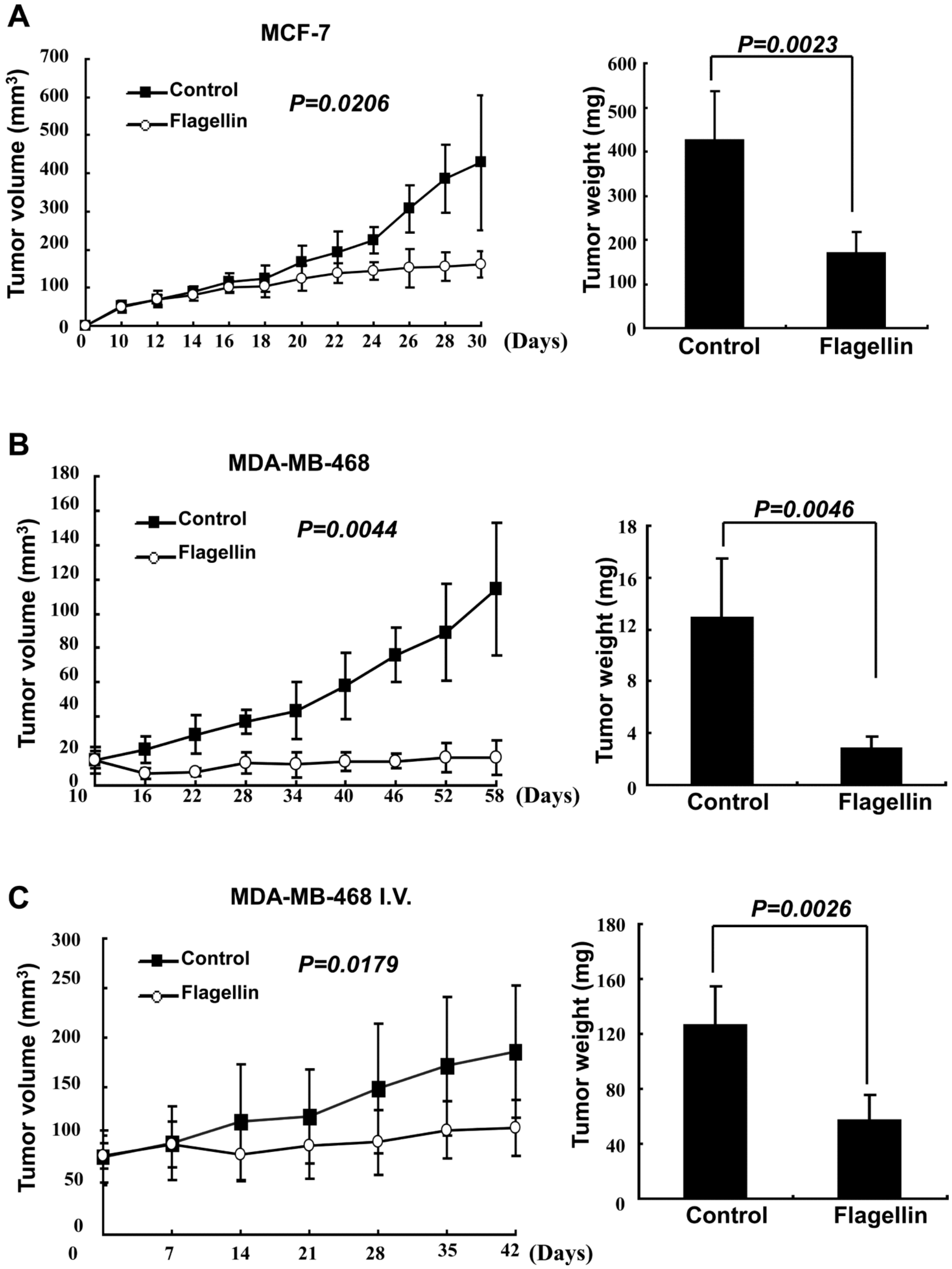

Figure 5. Flagellin inhibits solid tumor growth in breast cancer cell xenograft mouse model

MCF-7 (A) and MDA-MB-468 (B) xenograft tumors were treated with PBS or flagellin solution (1μg/50μl for every 2 days) around the tumor sites. Tumor growth curves of control or flagellin-treated groups are shown. Tumor weight of control or flagellin-treated groups are shown in the right. C, MDA-MB-468 xenograft tumors were allowed to reach between 80-100 mm^3 before treatment. Then, PBS or flagellin solution (2μg in 100 μL) were injected through tail vein (1 injection/ week). Tumor growth curves of control or flagellin-treated groups are shown. Tumor weight of control or flagellin-treated groups are shown in the right.

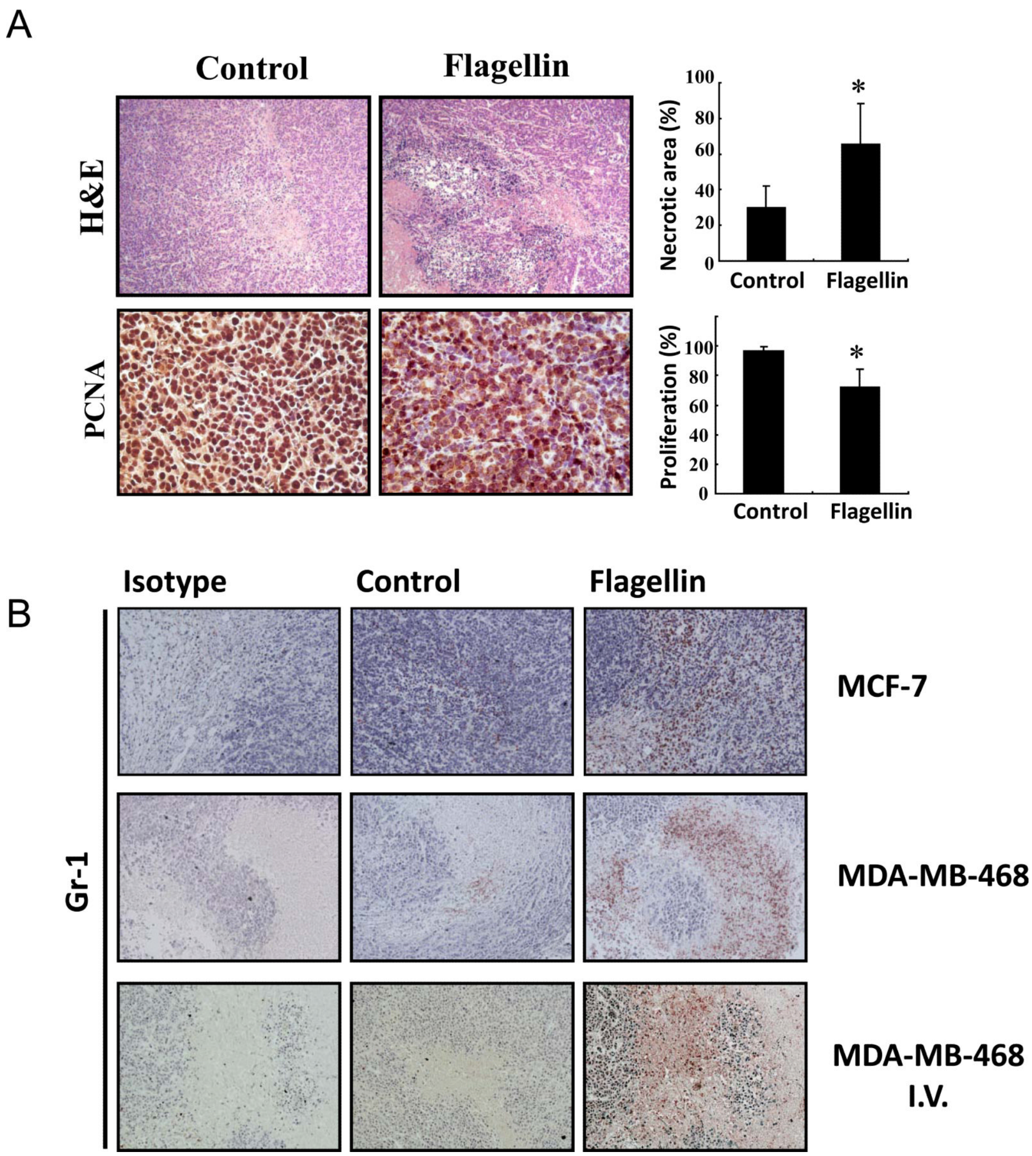

Figure 6. Increased tumor necrosis and neutrophil infiltration in flagellin-treated tumors

A, H&E staining of MCF-7 xenograft tumor sections showed more prevalent necrosis and leucocytes infiltration in flagellin-treated tumors (*Top panel*). The percentage necrosis area was calculated from five sections per tumor. Mean ± s.d. values of data from five tumors are shown (*Top right*). Cell proliferation marker PCNA staining of tumor sections showed decreased cell proliferation in flagellin-treated tumors (*Bottom panel*). The ratio of PCNA-positive to total tumor cells was calculated from five sections per tumor. Mean ± s.d. values of data from five tumors are shown (*Bottom right*). Magnification: H&E, 10X; PCNA, 40X. * $P < 0.05$. B, Tumor sections from MCF-7 and MDA-MB-468 xenografts were stained by neutrophil-specific marker Gr-1. Representative images from tumor sections with flagellin peritumoral or i.v. treatment are shown. Red indicates positive staining. Magnification: 10X.