行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

探討類鐸受體四號對於腫瘤生長之影響

計畫類別：■個別型計畫 □整合型計畫
計畫編號：NSC 99 －2320－B－039－001－MY2
執行期間： 2010 年 01 月 01 日至 2011 年 7 月 31 日

執行機構及系所：中國醫藥大學醫學系微生物科

計畫主持人：李哲欣
共同主持人：謝政蓉
計畫參與人員：無

成果報告類型(依經費核定清單規定繳交)：□精簡報告 ■完整報告

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Toll-like Receptor 4 Signaling Promotes Tumor Growth

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Summary: Chronic inflammation is a potential risk factor for tumor progression. The molecular mechanisms linking chronic inflammation and tumor growth have proven elusive. Herein, we describe a new role for Toll-like receptor 4 (TLR4) in tumor-associated macrophages (TAMs) in promoting tumor growth. TAMs can remodel tumor microenvironment and promote tumor growth. With the use of mice lacking TLR4 signaling, we show that TLR4 signaling influences tumor growth and that TLR4 signaling is a critical upstream activator of nuclear factor-kappa B (NF-κB) in TAMs. TLR4-deficient TAMs produce neither proinflammatory cytokines nor angiogenic factors, and activate no NF-κB activity in tumor cells. Furthermore, using macrophage/tumor cell coculture system and adoptive transfer of macrophages with functional TLR4 macrophages to TLR4-deficient mice bearing tumors, we demonstrate an essential role for TLR4 signaling in inducing NF-κB activity in tumor cells and enhancing tumor growth. Antibody neutralization experiments reveal that TAMs are stimulated by heat shock proteins derived from tumor cells through TLR4, leading to production of growth factors, which may in turn promote tumor growth via NF-κB signal pathway. Therefore, this signaling cascade may represent a therapeutic target in cancer.

Key Words: Toll-like receptor 4, tumor-associated macrophage, TNF-α, heat shock protein

(*J Immunother* 2010;33:73–82)

Inflammation is central to combat pathogens. Nevertheless, if it fails to proceed through an orderly and timely process, the resulting chronic inflammation may contribute to tumor formation. There is a growing body of evidence to indicate that chronic inflammation is considered to be one of the most important factors contributing to tumor development and progression. Most solid tumors contain many nonmalignant cells, including immune and endothelial cells, which are important in inflammation. Inflammatory cells provide proteases that facilitate tumor invasion and matrix remodeling, along with chemokines, growth factors, angiogenic and lymphangiogenic factors.[1–3] Tumor progression depends, in part, on the density of tumor-associated macrophages (TAMs). There is a positive correlation between TAM abundance and poor prognosis.[4] TAMs are highlighted as not only a major participator but also an important regulator of inflammation. These macrophages actually promote the proliferation and metastasis of tumor cell by secreting a wide range of growth and proangiogenic factors, as well as matrix metalloproteinases.[5] Despite these findings, the molecular mechanisms that allow communication between tumor cells and TAMs remain to be elucidated.

In this regard, nuclear factor-kappa B (NF-κB) is known to be active in macrophages during the induction of inflammation and plays an important role in tumor growth. Several NF-κB – regulated genes in TAMs are essential for tumor growth and metastasis. The NF-κB family is a key player in controlling both innate and adaptive immunity.[6] NF-κB is present in the cytoplasm in association with inhibitory proteins, known as inhibitors of NF-κB (I-κB). After activation by different stimuli, I-κB proteins become phosphorylated, ubiquitylated, and subsequently degraded by the proteasome. The degradation of I-κB allows NF-κB proteins to translocate to the nucleus and bind to their response elements to regulate the transcription of a large number of genes that encode cytokines, chemokines, and antiapoptotic proteins. The strategies for altering the expression and activation of NF-κB in tumor cells and macrophages have been shown to be effective in inhibiting tumor growth.[1,7,8]

The innate immune system recognizes the presence of pathogens through the expression of a family of membrane receptors known as Toll-like receptors (TLRs). TLRs are expressed on a variety of cell types, including immune cells, endothelial cells, cardiac myocytes, and intestinal cells. In this study, we investigated whether TLR4 plays a role in the communication between tumor cells and TAMs. We used wild-type C3H/HeN mice and TLR4-deficient C3H/HeJ mice to study the role for TLR4 in tumor growth. Our results show that mice with functional TLR4 had significantly shorter survival time after tumor inoculation compared with those deficient in TLR4. Moreover, TLR4 signaling also increased proinflammatory cytokine production, tumor sizes, and NF-κB activities in tumor-bearing mice. Our data also show that inhibition of TLR4 signaling in TAMs abrogates their tumor-promoting activity and decreases the secretion of proinflammatory cytokines. These findings provide a new insight into the role of TLR4 on TAMs and the relation between inflammation and tumor.

MATERIALS AND METHODS

Mice and Cells

Male C3H/HeN and C3H/HeJ mice[9] were originally obtained from Charles River Laboratories and the Jackson Laboratory, respectively. Mice at the age of 6 to 8 weeks were purchased from the Laboratory Animal Center of the

Received for publication May 21, 2009; accepted June 18, 2009.

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Grant support: National Science Council grants (NSC 96-2320-B-006-004, NSC 96-3112-B-006-011, and NSC 97-3112-B-006-001), Taiwan.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site, www.immunotherapy-journal.com.

National Cheng Kung University. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the National Cheng Kung University. Murine K1735-M2 melanoma[10,11] and MBT-2 bladder tumor as well as human 293 T cells were cultured in Dulbecco modified Eagle's medium supplemented with 50 μg/mL gentamicin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2. Thioglycollate-elicited peritoneal macrophages were obtained from C3H/HeN and C3H/HeJ mice as previously described.[12] The macrophages were verified by morphologic features and fluorescence-activated cell sorter analysis using macrophages-specific F4/80 (MCAP497, Serotec, Oxford, UK) monoclonal antibody.

Lentiviral Vectors and Transduction of Tumor Cells and Macrophages

The lentiviral expression vector pWPXLLuc was derived from pWPXL where the green fluorescent protein cDNA was replaced with firefly luciferase cDNA obtained from pGL3-Basic vector (Promega, Madison, WI).[13] The elongation factor 1-α promoter was removed from pWPXLLuc at *ClaI* and *SwaI* sites and then replaced by the NF-κB response elements obtained from pGL2-IL8,[14] resulting in pWPXL-NF-κBLuc. The recombinant lentiviruses encoding luciferase were produced by calcium phosphate precipitation as previously described.[13] For transduction of K1735-M2 cells and peritoneal macrophages with the luciferase gene, cells were infected with recombinant lentiviruses carrying luciferase gene under the control of elongation factor 1-α promoter or NF-κB response elements in the presence of 8 μg/mL of polybrene. As the lentiviral vectors do not contain selectable markers, luciferase-expressing stable K1735-M2 clones were identified by monitoring luciferase expression in each isolated clone. K1735-M2/Luc and K1735-M2/NF-κBLuc clones with high-level luciferase expression were used for further studies.

Experimental Lung Metastasis Models

In the experimental murine model for pulmonary metastasis, mice were injected with K1735-M2 cells (10^5) admixed with 50 μg lipopolysaccharide (LPS) (Sigma, St. Louis, MO) via the tail vein. In a parallel experiment, mice were injected with 10^5 cells of K1735-M2 cells or MBT-2 cells via the tail vein and survival of the mice was monitored daily. After 20 days, mice were killed, and the lungs were removed, weighed, and histologically examined. To facilitate quantification of the metastatic tumor burden in the lungs, K1735-M2/Luc cells that express luciferase were used to induce lung tumors in C3H/HeN and C3H/HeJ mice. At different time points, the mice were killed, and their lung lysates were assessed for luciferase activity with luciferase reporter gene assay system (Applied Biosystems, Foster City, CA) using a luminometer (MiniLumat LB 9506; Berthold Technologies, Bad Wildbad, Germany). To investigate the effects of macrophages with functional TLR4 signaling on tumor growth, peritoneal macrophages (2×10^5) from C3H/HeN or C3H/HeJ mice were injected at days 1 and 7 into C3H/HeN and C3H/HeJ recipient mice that had been inoculated intravenously (IV) with K1735-M2 cells (10^5) at day 0. The survival of the mice was inspected daily.

Histology, Immunohistochemistry, and Immunofluorescence

Mice were killed at day 20 after tumor inoculation, and their lungs and tumors were formalin-fixed, paraffin-embedded, and sectioned. Sections were subjected to either Harris hematoxylin and eosin staining or immunohistochemical staining with various antibodies. Growth index (mean area of metastasis/total area) of tumor nodules in the lung was quantified at the microscopic level as previously described.[15,16] Tumor angiogenesis and macrophages infiltrating into tumors were assessed by immunostaining as previously described.[11]

To detect the expression of heat shock proteins (HSPs) in K1735-M2 cells, cells were fixed in 10% formalin, permeabilized with cold acetone, and incubated with monoclonal antibodies against HSP60 (24/HSP60, BD Biosciences PharMingen, San Diego, CA), HSP70 (W27, Santa Cruz Biotechnology, Santa Cruz, CA), and HSP90 (F-8, Santa Cruz Biotechnology), and subsequently incubated with rhodomine-conjugated rabbit anti-mouse IgG (KPL, Guildford, UK). Nuclei were counterstained with 4'-6-diamidino-2-phenylindole. For immunofluorescence staining of the macrophages collected from the bronchoalveolar lavage (BAL) fluid, macrophages were fixed in 10% formalin, permeabilized with cold acetone, incubated with rabbit anti-mouse NF-κB p65 antibody (Santa Cruz Biotechnology) at room temperature for 40 minutes, and subsequently incubated with fluorescein-conjugated goat anti-rabbit IgG (KPL). The stained cells were examined under a fluorescence microscope at magnification of $\times 200$.

Immunoblot Analysis and Enzyme-linked Immunosorbent Assay

The protein content in each sample was determined by bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). Proteins from total cell lysates were fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont, UK), and probed with monoclonal antibodies against HSP60 (24/HSP60), HSP70 (W27), HSP90 (F-8), or β-actin (mABcam 8226, ABcam, Cambridge, UK). Horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson, West Grove, PA) was used as the secondary antibody and protein-antibody complexes were visualized by enhanced chemiluminescence system (Amersham). The levels of tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) in the BAL fluid were determined by enzyme-linked immunosorbent assay (R&D, Minneapolis, MN).

Coculture Systems and Conditioned Media

To assess the NF-κB activities in the macrophages and tumor cells in a coculture system, 2×10^4 peritoneal macrophages from C3H/HeN and C3H/HeJ mice transduced with NF-κBLuc were cocultured with K1735-M2 cells (2×10^4). Reciprocally, 2×10^4 peritoneal macrophages from 2 strains of mice were cocultured with K1735-M2/NF-κBLuc cells (2×10^4). The luciferase activities of the cells were determined 16 hours after the coculture. To determine the TNF-α content in the coculture, peritoneal macrophages from C3H/HeN and C3H/HeJ mice were cocultured with K1735-M2 cells that had been preincubated with anti-mouse HSP60, HSP70, or HSP90 neutralizing antibody or with control IgG for 30

minutes at 25°C. After 16 hours, the supernatant was harvested and quantified for TNF-α content by enzyme-linked immunosorbent assay.

The conditioned media (CM) was collected from C3H/HeN and C3H/HeJ peritoneal macrophages that had been seeded on 24-well plates at 2×10^4 cells/well and cultured with or without LPS (100 ng/mL) or polymyxin B (20 ng/mL) for 16 hours. The CM was then preincubated with 10 μg of neutralizing antibody against mouse TLR4 (MTS510, eBioscience, San Diego, CA) or mouse TNF-α (IF3F3D4 IGH 156, Bender MedSystems, Burlingame, CA), or with control IgG (eBioscience) for 30 minutes at 25°C, and then added to the culture of K1735-M2/NF-κBLuc cells stably expressing luciferase under the control of NF-κB response elements. After 16 hours, the luciferase activities of the tumor cells were determined.

Statistical Analysis

The statistical differences in various parameters between different groups were analyzed by Student *t* test. The survival analyses were performed using the Kaplan-Meier survival curve and the log-rank test. Any *P* value less than 0.05 is considered statistically significant.

RESULTS

Deficiency of TLR4 Signaling Enhances Cancer-related Survival

To address the potential role of TLR4 in tumor growth, we first asked whether the absence of TLR4 function affected the survival outcome of tumor-bearing mice. We used a murine metastatic model in which mouse K1735-M2 melanoma cells were injected into C3H/HeJ mice lacking functional TLR4[9] and C3H/HeN mice with normal TLR4 via the tail vein to induce pulmonary metastasis. Because TLR4 is identified as a signaling receptor for LPS derived from gram-negative bacteria, we studied whether LPS treatment affected the survival of TLR4-deficient (C3H/HeJ) and wild-type (C3H/HeN) mice concomitantly administered with tumor cells. C3H/HeN mice injected with tumor cells admixed with LPS had a significantly shorter survival time compared with those injected with only tumor cells (Fig. 1A). Notably, there were no significant differences in the survival between 2 groups of the mice regardless of LPS treatment in C3H/HeJ mice (Fig. 1B). Consistent with a previous report,[17] our results suggest that LPS influenced the survival of tumor-bearing mice through TLR4 signaling. TLR4 recognizes not only bacterial LPS but also host-derived endogenous ligands. To determine whether TLR4 signaling is activated by endogenous ligands from tumor cells, we compared the survival of both strains of mice injected with K1735-M2 cells alone. Figure 1C shows that the survival time of C3H/HeJ mice was dramatically prolonged compared with that of C3H/HeN mice. To further exclude the possibility that the result obtained above could be the result of tumor type specificity rather than a general phenomenon, we used another syngeneic bladder tumor cell line MBT-2 grown in C3H mice to perform the same experiment. Similar results were obtained using the MBT-2 tumor model (Fig. 1D). Collectively, these findings suggest that TLR4 signaling influences the survival of tumor-bearing mice.

Deficiency of TLR4 Signaling Reduces Tumor Growth in the Lungs

To study whether TLR signaling influenced the fate of tumor cells, C3H/HeN and C3H/HeJ mice were inoculated via the tail vein with K1735-M2/Luc cells that express luciferase to facilitate quantification of tumor growth, and the luciferase activities in the lungs were determined at

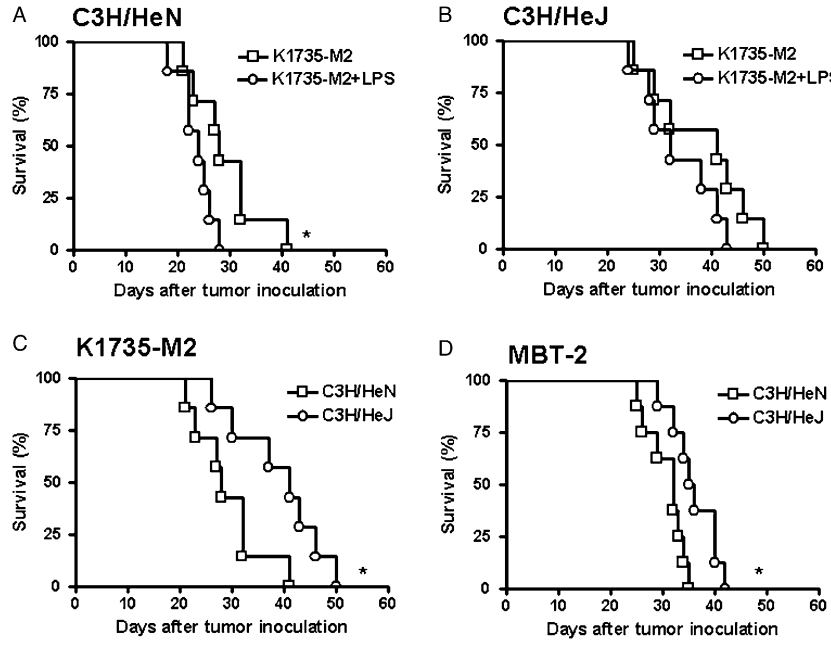


FIGURE 1. Deficiency of TLR4 signaling enhances the survival of tumor-bearing mice. Groups of 7 C3H/HeN (A) and C3H/HeJ (B) mice were inoculated with K1735-M2 melanoma cells (10^5) admixed with LPS (50 μg) via the tail vein. Groups of 7 to 8 C3H/HeN and C3H/HeJ mice were inoculated with 10^5 of K1735-M2 melanoma (C) or MBT-2 bladder cancer cells (D) via the tail vein. Kaplan-Meier survival curves are shown. *$P<0.05$. LPS indicates lipopolysaccharide; TLR4, Toll-like receptor 4.

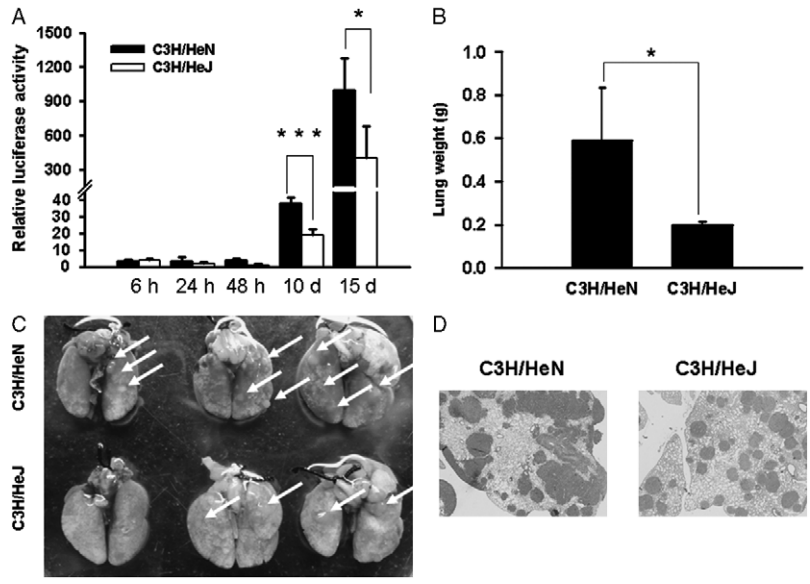


FIGURE 2. Deficiency of TLR4 signaling reduces tumor growth but not initial establishment of pulmonary metastatic foci. C3H/HeN and C3H/HeJ mice were inoculated with K1735-M2/Luc cells (10^5) via the tail vein. A, The mice were killed at various time points, and the amount of luciferase activity in the lung was measured. Each value represents mean ± SD (n = 4). B, At day 20, the wet lung weight of the lungs was measured. Each value represents mean ± SD (n = 5). C, Representative examples of metastatic pulmonary nodules produced 20 days after intravenous injection of 10^5 K1735-M2 cells. Gross appearances of formalin-fixed lungs reveal reductions of numbers and sizes of tumor nodules indicated by arrows in C3H/HeJ mice compared with those in C3H/HeN mice. D, Histologic examination of pulmonary tumor nodules at day 20 after tumor inoculation. Note that H&E staining of 4-μm paraffin-embedded lung tissue sections (40 ×) reveals the presence of larger tumor nodules from C3H/HeN mice compared with those from C3H/HeJ mice. *P<0.05; ***P<0.001. H&E indicates hematoxylin and eosin; TLR4, Toll-like receptor 4.

different time points after tumor inoculation. As shown in Figure 2A, luciferase activities in the lungs were increased 26-fold and 20-fold at day 15 compared with that at day 10 in C3H/HeN and C3H/HeJ mice, respectively, indicating tumors continued to grow. No quantitative differences in the initial arrest of K1735-M2/Luc cells between C3H/HeN and C3H/HeJ mice were noted. However, a slight increase in the signals was observed in the lungs of C3H/HeN mice compared with those in C3H/HeJ mice at 48 hours. At day 15, the luciferase activity from C3H/HeN mice was 2.5-fold higher than that from C3H/HeJ mice. Accordingly, the wet lung weight of C3H/HeJ mice was ~65% less compared with that of C3H/HeN mice (Fig. 2B). Furthermore, the number and size of tumor nodules in C3H/HeJ mice were significantly smaller than those in C3H/HeN mice (Fig. 2C). Histologic examination confirmed the macroscopic findings (Fig. 2D). Quantitative analysis at the microscopic level also shows that the growth index of tumor nodules was significantly lower in the C3H/HeJ group than in the C3H/HeN group (mean ± SD 0.7883 ± 0.4 vs. 1.20 ± 0.31, n = 6; $P < 0.05$). The lower tumor burden correlated with longer survival time in C3H/HeJ mice, as compared with C3H/HeN mice. Taken together, these results suggest that TLR4 signaling affects tumor growth but not the initial establishment of pulmonary metastatic foci.

TLR4 Signaling Enhances Tumor Cell-induced Lung Inflammation

Clinical and epidemiologic studies suggest an association between inflammatory disorders and tumor.[18] We investigated whether tumor growth in the lungs induced inflammatory response through TLR4 signaling. We compared the levels of total proteins as well as TNF-α and VEGF in the BAL fluid collected from C3H/HeN and C3H/HeJ mice that had been inoculated with K1735-M2 cells. The BAL fluid from C3H/HeN mice contained higher concentrations of total proteins (Fig. 3A), TNF-α (Fig. 3B), and VEGF (Fig. 3C) compared with that from C3H/HeJ mice. These results suggest a potential role for TLR4 signaling in the inflammation and permeability of the lung, which may contribute to the promotion of tumor growth. To define the cell types related to the tumor-promoting effect of an enhanced inflammatory response, we next characterized infiltrating cells in the BAL fluid. The number of infiltrating cells in tumor-bearing mice was significantly increased compared with that in normal mice (Fig. 3D). Of note, the number of macrophages in the BAL fluid from C3H/HeN mice bearing melanoma was increased 1.69-fold compared with that from tumor-bearing C3H/HeJ mice. Immunohistochemical staining using anti-F4/80 antibody also confirmed macrophage infiltration within the tumor tissues from both strains of mice (Supplementary Figure S1, Supplemental Digital Content 1, http://links.lww.com/JIT/A12). Giving that macrophages can promote tumor angiogenesis[19] and that macrophage infiltrates are increased in tumor tissues, we examined the activity of matrix metalloproteinase-9 (MMP-9) by zymography and intratumoral microvessels by immunohistochemistry, respectively. In both strains of mice, tumor-bearing mice produced more MMP-9 and MMP-2 in the tumor site than did normal mice (Supplementary Figure S1, Supplemental Digital Content 1, http://links.lww.com/JIT/A12). Furthermore, tumors from C3H/HeN mice produced more MMP-9 and were more vascularized than tumors from C3H/HeJ mice (Supplementary Figure S1, Supplemental Digital Content 1,

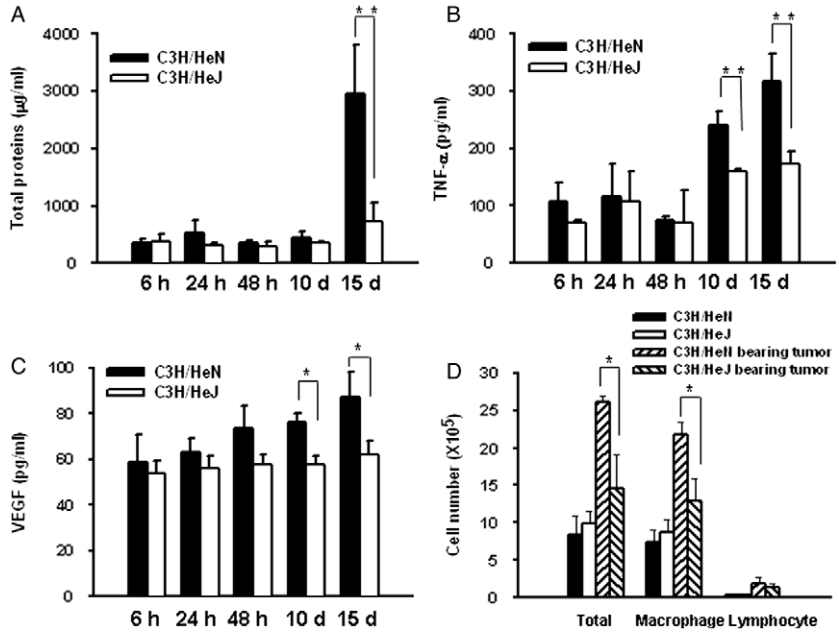


FIGURE 3. TLR4 signaling enhances tumor cell-induced lung inflammation. A–D, Inflammation-related mediators and cell types in the BAL fluid from C3H/HeN and C3H/HeJ mice bearing metastatic melanoma. Both mouse strains were inoculated with K1735-M2/Luc cells (10^5) via the tail vein at day 0. BAL fluid and lung tissues were collected from melanoma-bearing mice at day 20. The levels of total proteins (A), TNF-α (B), and VEGF (C), as well as the numbers of different cell types (D) in the BAL fluid were determined. Each value represents mean ± SD (n = 3 to 4). *$P<0.05$; **$P<0.01$. BAL indicates bronchoalveolar lavage; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

http://links.lww.com/JIT/A12). Because activation of NF-κB in TAMs is essential for the production of MMP-9, TNF-α, and VEGF,[20] we further examined the subcellular localization of p65 subunit of NF-κB in the macrophages from the BAL fluid by immunofluorescence staining. The p65 was expressed only modestly at the nuclear periphery and in the cytoplasm of the macrophages from C3H/HeJ mice, whereas it was detected exclusively in the nucleus of the macrophages from C3H/HeN mice (Supplementary Figure S1, Supplemental Digital Content 1, http://links.lww.com/JIT/A12). These results suggest that increased production of proinflammatory cytokines and angiogenic factors in response to tumor cells may be dependent on TLR4 signaling via NF-κB pathway in TAMs.

Activation of NF-κB in Both Macrophages and Tumor Cells is Dependent on TLR4 Signaling

The presence of macrophages in solid tumors suggests that tumors grow at sites of chronic inflammation. NF-κB signaling might play an important role in inflammation and tumor progression.[21] Several NF-κB – regulated genes that encode adhesion molecules, antiapoptotic proteins, and cytokines are essential for tumor growth and invasion.[17] We next investigated whether tumor cells and macrophages influenced each other the NF-κB signaling through TLR4. To assess the activation of NF-κB signal transduction pathway, we performed a reporter assay using K1735-M2 cells and macrophages from C3H/HeN or C3H/HeJ mice lentivirally transduced with the luciferase gene under the control of NF-κB response elements. We cocultured macrophages that expressed luciferase controlled by NF-κB response elements and untransduced K1735-M2 cells to mimic the microenvironment that macrophages infiltrated into tumors. The reporter assay reveals that macrophages from C3H/HeN mice had significantly higher NF-κB activity than did macrophages from C3H/HeJ mice when cocultured with tumor cells (Fig. 4A). Furthermore, we found that the NF-κB activity was elevated in K1735-M2/NF-κBLuc cells when cocultured with macrophages from C3H/HeN mice but not from C3H/HeJ mice (Fig. 4B). These results suggest that functional TLR4 on macrophages plays an important role in mediating the NF-κB transcriptional activities of tumor cells and neighboring macrophages. In this regard, we next examined whether tumor cells and macrophages influenced each other in vivo. K1735-M2/NF-κBLuc cells were injected into the tail vein of C3H/HeN and C3H/HeJ mice, and the luciferase activity of the lungs was determined. Figure 4C shows that luciferase gene expression was higher, indicative of higher NF-κB activity, in the lungs of C3H/HeN mice than that in their C3H/HeJ counterparts, suggesting that functional TLR4 of the host influenced the NF-κB transcriptional activity in tumor cells in vivo. Meanwhile, when 2 strains of mice bearing established K1732-M2 tumors in the lungs received macrophages transduced with luciferase gene driven by the NF-κB response elements via the tail vein, significantly higher NF-κB activity was found at the tumor site in C3H/HeN mice receiving macrophages harboring functional TLR4 from C3H/HeN mice as compared with that in C3H/HeJ mice receiving TLR4-defective macrophages from C3H/HeJ mice (Fig. 4D). Taken together, using reporter assays in vitro and in vivo with lentiviral vectors expressing luciferase controlled by the NF-κB response elements, we demonstrated that the NF-κB transcriptional activities of tumor cells and macrophages influence each other through TLR4 expressed on macrophages.

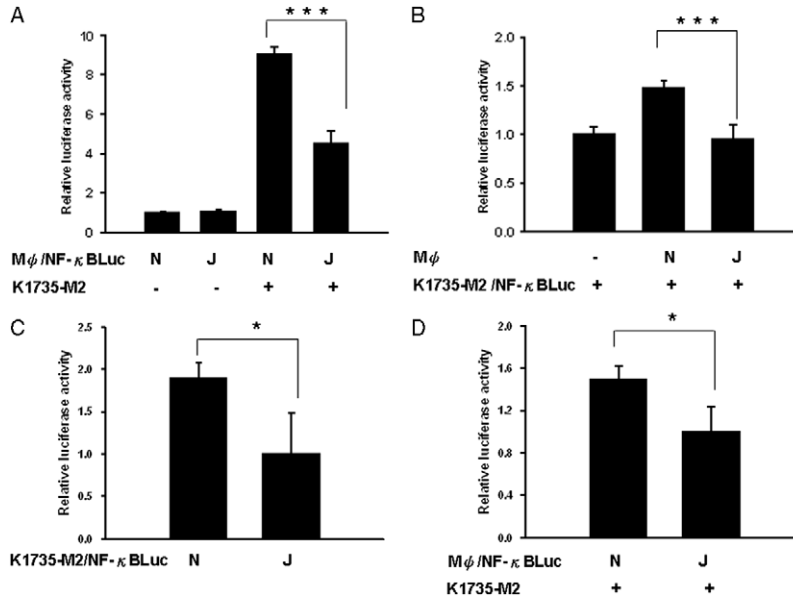


FIGURE 4. Effects of in vitro and in vivo interactions between macrophages and tumor cells on NF-κB activity. NF-κB activities in the macrophages (A) and K1735-M2 tumor cells (B) in the in vitro coculture. A, Peritoneal macrophages (2×10^4) from C3H/HeN and C3H/HeJ mice transduced with NF-κBLuc were cocultured with K1735-M2 cells (2×10^4). B, Macrophages (2×10^4) from C3H/HeN and C3H/HeJ mice were cocultured with K1735-M2/NF-κBLuc cells (2×10^4). The transcriptional activity of NF-κB of the cells was determined 16 hours after coculture. C, Groups of 3 C3H/HeN or C3H/HeJ mice were injected with K1735-M2/NF-κBLuc cells (10^5) via the tail vein and the luciferase activities of the tumors were measured 20 days after tumor inoculation. D, Group of 3 C3H/HeN and C3H/HeJ mice that had been inoculated with K1735-M2 cells (10^5) via the tail vein at day 0 were injected intravenously with 2×10^5 of C3H/HeN and C3H/HeJ macrophages transduced with NF-κBLuc at day 20, respectively. After 1 hour, mice were killed, and their tumors were removed for assessing luciferase activities. Relative luciferase activity is expressed as the percentage of the relative luciferase activity relative to that in the C3H/HeN macrophages cultured alone (A), the K1735-M2/NF-κBLuc cells cultured alone (B), and the tumors from C3H/HeJ mice (C, D).*P<0.05; ***P<0.001. NF-κB indicates nuclear factor-kappa B.

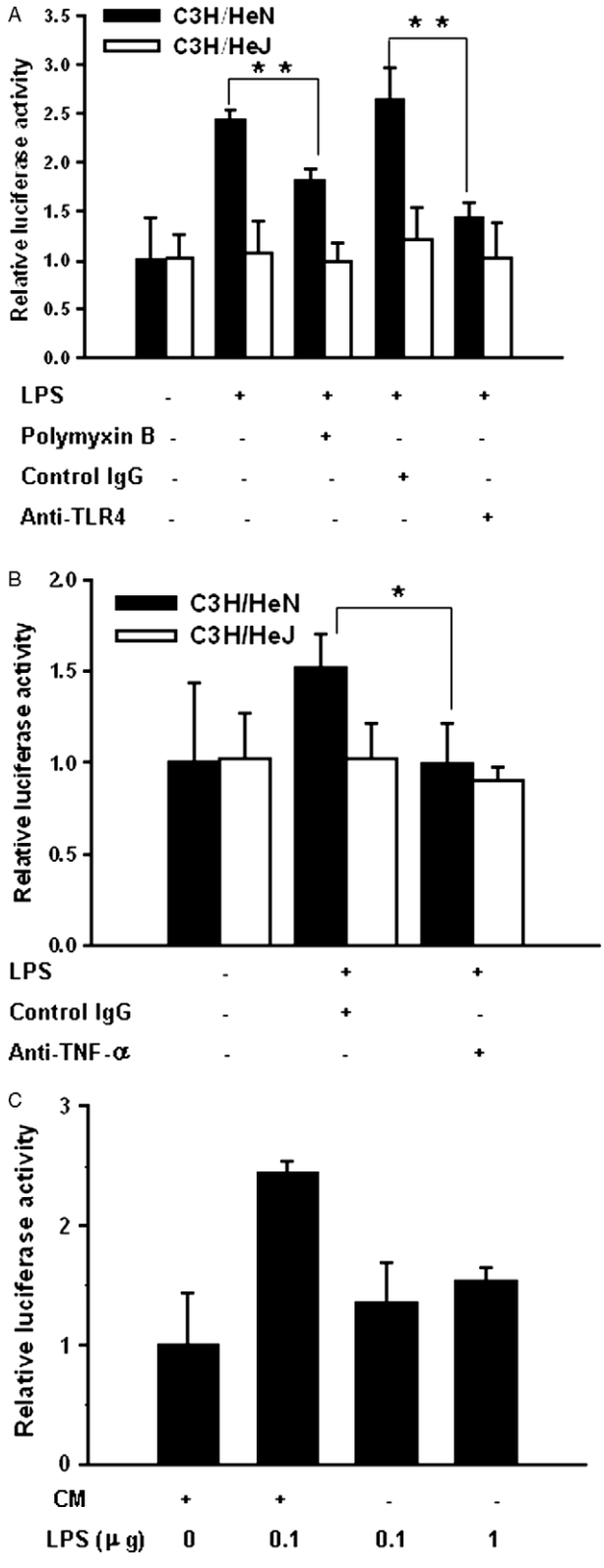
TNF-α Produced by Macrophages Mediates NF-κB Activation in Tumor Cells

To examine how the activation of TLR4 signaling mediates NF-κB – induced tumor growth, we used the CM derived from activated macrophages to analyze the NF-κB transcriptional activity in tumor cells. Figure 5A shows that soluble mediators secreted from macrophages after LPS treatment activated NF-κB responses in tumor cells. Macrophages with functional TLR4 from C3H/HeN mice, when treated with LPS antagonist polymyxin B, dramatically attenuated the ability of the CM to induce NF-κB activation in tumor cells. Similar results were found when neutralizing antibody against TLR4 was used to treat the CM. Notably, the CM derived from C3H/HeJ macrophages had no effects on inducing NF-κB activity in tumor cells upon LPS treatment. Thus, activation of the NF-κB transcriptional activity in tumor cells required macrophages with functional TLR4 signaling. Giving that TNF-α is an important tumor promoter in a variety of experimental animal models[17,21,22] and that the BAL fluid from C3H/HeN mice contained more TNF-α than that from C3H/HeJ mice (Fig. 3B), we next determined whether TNF-α present in the soluble mediators secreted by macrophages mediated NF-κB activation. First, we confirmed the translocation of p65 subunit of NF-κB to the nucleus in macrophages after LPS stimulation, which was accompanied by the secretion of TNF-α and was dependent on functional TLR4 signaling (Supplementary Figure S2, Supplemental Digital Content 2, http://links.lww.com/JIT/A13). The LPS-induced NF-κB activation in tumor cells by the CM from C3H/HeN macrophages was abrogated by treatment with anti – TNF-α neutralizing antibody (Fig. 5B). Of note, the CM from macrophages with defective TLR4 signaling had no effects on the NF-κB response in tumor cells. Although direct incubation with LPS also activated NF-κB in tumor cells in vitro, rather high doses of LPS were required (Fig. 5C). It appeared that the activation of NF-κB in tumor cells depended on TNF-α produced from macrophages, but not the remnant LPS in the CM. These findings suggest that TNF-α produced by macrophages through TLR4 signaling is responsible for NF-κB activation in tumors.

HSPs Activate Macrophages Through TLR4

The results presented above show that tumors grew faster in C3H/HeN mice than in C3H/HeJ mice, and that K1735-M2 cells could induce NF-κB activation in macrophages. Because a number of endogenous ligands are known to bind and stimulate TLR4 signaling,[23,24] we hypothesized that endogenous ligands derived from tumor cells could activate macrophages. The fact that HSPs can use TLR4 to mediate NF-κB activation and induce the production of proinflammatory cytokines raises the question of whether HSPs can also represent endogenous ligands for TLR4.[25–28] Using immunoblot analysis and immunofluorescence staining, we found that K1735-M2 cells expressed HSPs, including HSP60, HSP70, and HSP90 (Fig. 6A). To examine whether endogenous HSP-induced signaling cascade was mediated through TLR4 signaling pathway, we cocultured macrophages from C3H/HeN or C3H/HeJ mice with K1735-M2 cells that served as ligands

and quantified the production of TNF-α. Macrophages treated with LPS served as the positive control for the assay. To confirm whether the HSPs from K1735-M2 cells were indeed involved in the signaling through TLR4, we pretreated K1735-M2 cells with neutralization antibodies against HSP60, HSP70, or HSP90, before performing the coculture experiment. As shown in Figure 6B, pretreatment of K1735-M2 cells with these antibodies significantly reduced the production of TNF-α by C3H/HeN macrophages. Of note, neutralization of HSP70 or HSP90 resulted in greater abrogation of TNF-α production than did neutralization of HSP60. In marked contrast, K1735-M2 cells had no effects on TNF-α production by C3H/HeJ macrophages after any treatments. The levels of TNF-α in the supernatant of K1735-M2 cells cultured alone after any treatments were below the limit of detection (32 pg/mL), indicating the main source of TNF-α produced in the coculture was activated macrophages. Taken together, these results show that HSPs from tumor cells represent the endogenous ligands that dictate the TLR4-dependent inflammatory response in macrophages.



Functional TLR4 Signaling on Macrophages Enhances Tumor Growth

We next determined whether functional TLR4 on macrophages could stimulate tumor growth in TLR4-deficient mice by adoptive transfer of macrophages from C3H/HeN mice into C3H/HeJ recipient mice bearing experimental metastatic melanoma. Macrophages harvested from C3H/HeN and C3H/HeJ mice were injected IV into the C3H/HeN or C3H/HeJ recipients. Tumor-bearing C3H/HeJ mice to which had been adoptively transferred macrophages from C3H/HeN mice had reduced survival time, as compared with those receiving macrophages from C3H/HeJ mice (Fig. 6C). By contrast, transfer of macrophages from C3H/HeJ or C3H/HeN mice to C3H/HeN recipients had no effect on mouse survival (Fig. 6D). Taken together, these results suggest that functional TLR4 on macrophages can enhance tumor growth and decrease survival time in the experimental metastatic tumor models.

DISCUSSION

The expression of TLR4 on tumor cells was reported to play a role in immune surveillance and facilitate tumor growth and chemoresistance.[29] TLR4 initiates signals through the sequential recruitment of myeloid differentiation

FIGURE 5. TNF-α secreted by macrophages mediates NF-κB activation in tumors. A, Activation of NF-κB in tumor cells depends on the functional TLR4 of macrophages. B, Activation of NF-κB in tumor cells is TNF-α – dependent. Peritoneal macrophages from C3H/HeN and C3H/HeJ mice were cultured and treated with LPS (100 ng/mL) or LPS plus polymyxin B (20 ng/mL) for 16 hours. The CM was collected, preincubated with anti-TLR4 or anti – TNF-α antibody, or with control IgG, and added to the culture of K1735-M2/NF-κBLuc cells for 16 hours. C, LPS is not a direct activator of NF-κB in tumor cells. K1735-M2/NF-κBLuc cells were treated with or without the CM collected from LPS-treated C3H/HeN macrophages in the presence or absence of LPS (0.1 or 1 μg/mL) for 16 hours. The transcriptional activity of NF-κB was determined by the luciferase reporter assay and is expressed as the percentage of the relative luciferase activity relative to that in the tumor cells treated with the CM from untreated (A, B) and from LPS-treated (C) C3H/HeN peritoneal macrophages. Each value represents mean ± SD (n = 4). *P<0.05; **P<0.01. CM indicates conditioned media; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

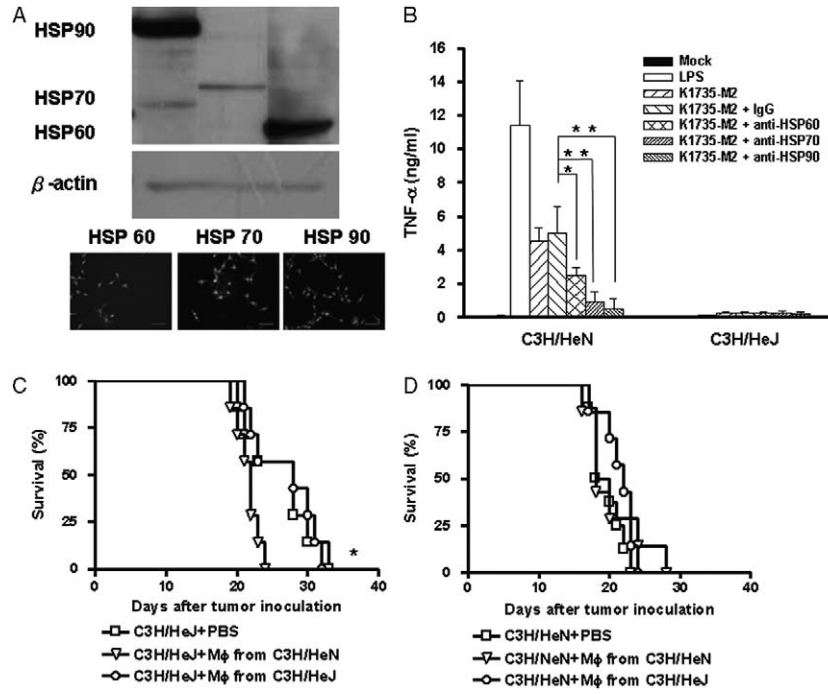


FIGURE 6. A, HSPs are endogenous ligands of TLR4. A, K1735-M2 cells express HSP60, HSP70, and HSP90, as determined by immunoblot analysis and immunofluorescence staining. B, The endogenous HSPs of tumor cells induce the production of TNF-α via TLR4. K1735-M2 cells (2×10^4) that had been pretreated with anti-mouse HSP60, HSP70, or HSP90 neutralizing antibody or with control IgG were cocultured with macrophages (2×10^4) from C3H/HeN or C3H/HeJ mice for 16 hours. Macrophages treated with LPS (100 ng/mL) and cultured alone served as the positive control. The CM was harvested and quantified for TNF-α content by ELISA. Each value represents mean ± SD (n = 4). *P<0.05; **P<0.01. C ands D, TLR4 of macrophages influences the survival of tumor-bearing mice. Groups of 8 C3H/HeJ (C) and C3H/HeN (D) mice that had been inoculated with 10^5 K1735-M2 cells via the tail vein at day 0 were adoptively transferred with macrophages (2×10^5) from C3H/HeN or C3H/HeJ mice at days 1 and 7 or with PBS. Kaplan-Meier survival curves are shown. *P<0.05. CM indicates conditioned media; ELISA, enzyme-linked immunosorbent assay; HSP, heat shock protein; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

protein 88 (MyD88), which in turn activates downstream mediators, such as NF-κB, leading to the activation of genes encoding proinflammatory cytokines. The MyD88 signaling pathway downstream of TLR4 was demonstrated to induce hepatocarcinogenesis and intestinal tumorigenesis.[30,31] TLR4-MyD88 signaling may function upstream of NF-κB in cells involved in inflammation-associated cancer. NF-κB is a multifunctional transcription factor that affects tumor growth and metastasis.[8,17] Increases in the NF-κB activity in the tumor microenvironment result in chronic inflammation and a substantial protumorigenic effect. Consistent with previous reports,[8,17,20,32] we found that the effector cells involved in enhancing tumor growth appear to be TAMs, which are recruited to the tumor sites and produce inflammatory cytokines to activate NF-κB in tumor cells. Although tumor cells express or release factors that are potent macrophage activators, some endogenous ligands, such as HSPs, have thus far been implicated in interaction with the cells of the immune system. In this study, we identified HSPs derived from tumors to be TLR4 ligands. HSPs are highly conserved cellular proteins. The primary function ascribed to HSPs is molecular chaperones by binding nascent polypeptides to assist proper folding and assembly of proteins. Although HSPs are found in different intracellular compartments, some have been found to be expressed on the cell surface or present in the extracellular compartment.[26,33] HSPs can interact with cells of the immune system and exert immunoregulatory effects.[27,28,34] Here, we demonstrate that HSPs derived from tumor cells play important roles in stimulating macrophages in a TLR4-dependent fashion. HSP70 and HSP90 significantly influenced the production of TNF-α by macrophages. Interestingly, HSP60 seems to play a minor role in macrophage activation compared with HSP70 and HSP90. The differential effects of HSPs may be attributed to the differences in their distribution on the cell surface and the amount of secreted HSPs. In conclusion, overexpression of HSPs in tumor cells increases tumor growth and inflammation. Thus, targeting HSPs represents an emerging strategy for cancer therapy.[35,36]

In the present study, we demonstrate that TLR4 signaling promotes tumor growth in experimental melanoma metastatic models. There has been previous report on a modulatory role for TLR4 in chronic inflammation and tumorigenesis.[37] Bauer et al[37] showed that TLR4 reduces lung permeability, inflammation, and tumor formation, which is contradicted by our results. The discrepancies between the 2 studies may result from the differences of experimental designs. Bauer et al used butylated hydroxytoluene (BHT) to induce lung injury and inflammation, which then led to promotion of lung tumorigenesis. Actually, tumors did not develop in BHT-treated C3H mice. In our tumor models, we used murine cancer cell lines grown in syngeneic C3H mice to establish tumors. The

inflammatory response induced by BHT reported by Bauer et al was observed in the early phase, whereas in our animal model, the pulmonary inflammation was induced by tumors per se and our observations were carried out in the late phase when the tumor microenvironment had established, which attracted many infiltrating cells. In different experimental models, TLR4 may play different roles in tumor growth. Our studies suggest that NF-κB pathway affects not only tumor cells but also TAMs. The activation of NF-κB pathway in tumor cells prevents the death of tumor cells and enhances their invasion capability. The activation of NF-κB pathway in TAMs stimulates the production of inflammatory cytokines and angiogenic factors in the tumor mass. The proinflammatory cytokines, such as TNF-α, promote the survival and proliferation of tumor cells.[38,39]

In this study, we have shown a link between TLR4 signaling, macrophages, and tumor growth. The expression of HSPs in tumor cells leads to the activation of TAMs through TLR4 signaling. The activation of NF-κB signaling in response to TLR4 signaling in TAMs produces growth factors, such as TNF-α and VEGF, which promote tumor growth and progression. Activation of the innate immune system, particularly through TLR4, may stimulate tumor growth. Therefore, this study may offer an attractive therapeutic strategy that combines conventional cancer therapeutics with immunomodulatory drugs.

ACKNOWLEDGMENTS

The authors thank D. Trono (School of Life Sciences and Frontiers in Genetics, National Center for Competence in Research, Swiss Federal Institute of Technology, Lausanne, Switzerland) for generously providing lentiviral vectors (pWPXL, psPAX2, and pMD2.G) and M. A. Fiedler (Division of Pulmonary Medicine, Children's Hospital Research Foundation, Cincinnati, OH) for kindly providing pGL2-IL8 plasmid.

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