Roles of toll-like receptors: From inflammation to lung cancer progression (Review)

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Abstract. Lung cancer is among the most common malignant tumors worldwide, and is characterized by a low survival rate compared with other cancers. Toll-like receptors (TLRs) are highly conserved in evolution and widely expressed on immune cells, where they serve an important role in the innate immune system by evoking inflammatory responses. Evasion of immune destruction is an important hallmark in the development of cancer. There is an established association between chronic inflammation and cancer, with TLRs serving important roles in the immune response against tumor cells. Recently, TLRs have been identified on tumor cells, where their activation may orchestrate the downstream signaling pathways that serve crucial functions in tumorigenesis and tumor progression. The present review summarizes the roles of TLRs as sensors on lung cancer cells that regulate lung cancer progression with regard to cell growth and invasion, angiogenesis and cancer stem cell behavior. This aimed to provide theoretical support for the development of therapies that target TLR signaling pathways for the treatment of lung cancer.

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1. Introduction

Lung cancer is among the most malignant tumors worldwide, and in 2012 was reported as the leading cause of cancer-related mortality in men and the second leading cause of cancer-related mortality in women (1). However, there remains to be a lack of effective treatments at present. Lung cancer has a number of subtypes, with approximately 80% of cases belonging to the non-small cell lung cancer (NSCLC) subtype (2). It is reported that 70% of patients are diagnosed with lung cancer at a medium or late stage, which makes treatment more difficult (2). Improved prognosis may be achieved if the lung cancer is identified at an earlier stage. Major risk factors of lung cancer include increasing age, air pollution and smoking (2). The Globocan 2012 cancer report by the International Agency for Research on Cancer identified that 1.8 million new lung cancer cases and 1.6 million lung cancer fatalities occurred in 2012 worldwide, accounting for approximately 19% of all cancer-related mortalities (1,3). In addition to the high rates of morbidity and mortality, lung cancers are also associated with poor prognosis, with the overall five-year survival rate being less than 17% (4). These data demonstrate the need for more effective strategies for the prevention and treatment of lung cancer.

Inflammation, as a crucial biological response to infection and damage, may also cause damage to the body, and persistent inflammation has been identified to be associated with cancer formation (5). Toll-like receptors (TLRs) recognize structurally conserved molecules derived from microbes (6). Upon binding to cognate ligands, TLRs activate downstream signaling pathways that orchestrate inflammatory responses (6). To date, there have been a number of studies on TLRs regarding their potential role in connecting inflammation and lung cancer (7-9); however, information on their exact roles in lung cancer cells, as well as the underlying mechanisms, remains limited. The present review summarizes the roles of TLRs in lung cancer progression with regard to cell growth and invasion, angiogenesis and cancer stem cells (CSCs), with the aim of providing novel insight on targets for the early diagnosis and treatment of lung cancer.

2. TLRs

TLRs are pattern recognition receptors that recognize both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (10). TLRs

are type I single membrane-spanning glycoproteins with an extracellular domain and an intracellular domain. The extracellular domain contains leucine-rich repeat motifs while the intracellular domain contains a highly conserved region termed the Toll/ interleukin (IL)-1 receptor (TIR) domain (6). To date, ten human and twelve murine functional TLRs have been identified (6). TLR1, 2, 4, 5 and 6 are localized on the cell membrane, and primarily detect bacterial PAMPs, while TLR3, 7, 8 and 9 and murine TLR11 are localized in endosomes or lysosomes, and mainly sense viral PAMPs (11). In addition, TLRs including TLR4 may recognize corresponding DAMPs (11).

TLRs exist at the plasma membrane as monomers, and TLR dimerization, either homo-dimerization or hetero-dimerization, is required for signaling (12). Ligand binding and TLR dimerization recruits TIR domain-containing adaptors, including myeloid differentiation factor 88 (MyD88), MyD88 adapter-like, TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adapter molecule, to activate downstream signaling cascades including the inhibitor of nuclear factor-κB kinase (IKK) and mitogen-activated protein kinase (MAPK) pathways (11). In turn these pathways activate nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) transcription factors, respectively, to ultimately upregulate pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and type I interferons (11).

3. Relationship between TLRs and lung cancer

In 1863, Rudolf Virchow identified that leukocytes were present in tumor tissue, and thus he hypothesized that there was a link between inflammation and tumor progression (13). Inflammation is an essential immune response to infection or injury. However, persistent infections within the host induce chronic inflammation (14). Innate immune cells, including macrophages, mast cells, dendritic cells and natural killer cells, may repair tissue damage and eliminate invaded pathogenic microorganisms by releasing cytokines, chemokines, matrix remodeling protease, prostaglandin, growth factors, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (15). These above inflammatory mediators are also able to induce genetic and epigenetic changes, such as point mutations in tumor suppressor genes, DNA methylation and post-translational modifications, leading to changes in certain critical pathways required to maintain normal cellular homeostasis, which ultimately leads to the development and progression of cancer (14-17). At present, approximately 18% of cancer cases are caused by infection and chronic inflammation worldwide, and patients with chronic inflammatory diseases have a higher risk of cancer development (8,18). For instance, the risk of lung cancer in patients with asbestosis has been reported to be ten times higher than that in healthy individuals (19).

The expression of TLRs in lung cancer tissues and the interaction between them have been a focus of research in recent years (9,20). TLRs 1-6 and 9 are expressed in airway epithelial cells (7). TLRs in lung cancer cells principally reside on the cell membrane or in the cytoplasm. For instance, TLR5 was identified to be mainly expressed on the membrane of lung cancer cells, TLR4, 8 and 9 were mainly expressed in cytoplasm of the cells, while TLR7 was mainly expressed around the nucleus (21-24).

Previous studies have demonstrated that the expression of TLR4, 5, 7, 8 and 9 in NSCLC was markedly higher than that in normal lung tissue (7,23-26). Among these, the expression of TLR4 was positively correlated with the differentiation degree, stage and metastasis of lung cancer cells (23,25); high expression of TLR5 was significantly associated with improved prognosis in patients with NSCLC (24); while the expression of TLR7 was markedly associated with resistance to neoadjuvant chemotherapy and poor clinical outcomes (26).

Epigenetics may be one of the mechanisms by which TLR gene expression is regulated. In patients with pulmonary tuberculosis, aberrant methylation of certain CpG sites in the TLR2 promoter region has been identified, which was possibly associated with downregulation of TLR2 expression (27). TLR2 CpG promoter hypermethylation has also been noted in non-cystic fibrosis bronchial epithelial cells for the epigenetic control of TLR2 expression (28). DNA methylation of TLR2 may also modulate host innate defense mechanisms and confer increased disease susceptibility, indicated in a chronic *Porphyromonas gingivalis* infection mouse model (29). For TLR3, cis-acting epigenetic control of TLR3 was responsible for impaired transcriptional activation of TLR3 in dendritic cells derived from cord blood when compared with the cells from adults (30). Epigenetic regulation of TLR4 gene expression through histone deacetylation and DNA methylation in intestinal epithelial cells may also be responsible for the maintenance of intestinal homeostasis (31). Furthermore, it has been noted that epigenetic modification of TLR4 was mediated by methyl-CpG-binding domain protein 2 and specificity protein 1 in gastric cancer (32). Regulation of TLRs or TLR signaling pathways by small noncoding RNAs is also a focus of epigenetic research. MicroRNA-21 (miRNA/miR-21), an oncomiR, may establish an important link between cancer and inflammation, as its upregulation has been reported in numerous cancers (33,34) as well as a number of inflamed states, including in allergic airway inflammation (35) and inflamed lung tissue in lipopolysaccharide (LPS)-treated mice (36). Indeed, Sheedy *et al* (37) reported that miR-21 was a negative regulator of TLR4 signaling through targeting of tumor suppressor programmed cell death 4. In addition to miR-21, the cellular miRNA, let-7i, has been observed to regulate TLR4 expression and contribute to cholangiocyte immune responses against *Cryptosporidium parvum* infection (38). In turn, TLRs are also involved in the epigenetic regulation of lung cancer progression. For example, TLR4 activation promoted the expression of the H3K9me1/2 demethylase KDM3A, with KDM3A then binding directly to the forkhead box P3 (Foxp3) promoter and promoting its transcription, thereby inducing the secretion of Foxp3-associated downstream inhibitory cytokines (TGF-β1, IL-35 and HO-1) and ultimately facilitating the immune system evasion of lung adenocarcinoma (39).

TLRs as receptors on immune cells involved in lung cancer progression have been previously reviewed in detail (7,8), thus the present review only recounts TLRs as sensors on lung cancer cells that function in the processes of cell growth, invasion, angiogenesis and CSC behavior.

TLRs are related to the growth of lung cancer cells. Unlimited proliferation capacity and anti-apoptotic mechanisms are hallmarks of cancer cells (40,41). TLRs may promote the growth

of lung cancer cells through proliferative or anti-apoptotic signals (Table I).

TLR4 is among the most characterized TLRs that have been identified to be related to the growth of lung cancer cells. TLR4 activation by LPS stimulation, a TLR4 ligand, induced a time- and dose-dependent increase in the proliferation of human lung adenocarcinoma cell line A549 (42). This effect of LPS on A549 cells could be reproduced in a mouse model with subcutaneous injection of A549 cells, whereby LPS treatment prior to cell injection enhanced tumor growth in the implanted tumors (42). A knockdown model of TLR4 significantly suppressed cell proliferation and induced apoptosis in A549 cells, and also suppressed tumor growth *in vivo*. This was potentially mediated by the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway (25). Zhang *et al* (43) isolated primary human lung cancer cells from fresh tumor tissue and identified a potential role of a TLR4/ROS/miR-21 pathway in aiding LPS-induced outgrowth of lung cancer cells. In addition to LPS stimulation, ligation of TLR4 by high mobility group box-1 (HMGB1) protein, another ligand of TLR4, inhibited apoptosis in Lewis lung cancer cells by upregulating the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and downregulting the pro-apoptotic protein Bcl-2-associated X protein in a time-dependent manner, which was attributed to the PI3K/AKT and extracellular regulated protein kinase (ERK)1/2 signaling pathways (44). Notably, TLR4 activation may also induce resistance of human lung cancer cells to apoptosis induced by TNF-α or TNF-related apoptosis-inducing ligand, mediated by the NF-κB signaling pathway (45). Further experiments have suggested that TLR4 on lung cancer cells mediates resistance of the cells to death induced by cytotoxic T lymphocytes, and promotes tumor growth *in vivo* (46). TLRs other than TLR4 have also been implicated in tumor growth. For instance, ligation of TLR2 followed by auto- or paracrine signaling by endogenously synthesized IL-8 was involved in lipoteichoic acid-induced proliferation of the NSCLC cell lines A549 and H226 (47). TLR7 or TLR8 ligations in lung cancer cells resulted in activation of NF-κB and upregulation of Bcl-2 expression, which were associated with increased cell survival and resistance to apoptosis (21). Accordingly, TLR7 stimulation has been identified to favor the growth of A549 cells in non-obese diabetic/severe combined immunodeficient mice and induce resistance to chemotherapy (26). Similarly, TLR9 agonist CpG oligonucleotides (CpG-ODNs) could markedly stimulate cell cycle entry and increase the proliferation of human lung cancer cell line 95D, which was accompanied by the selective upregulation of cyclin-dependent kinase 2, which was critical for cell cycle progression (48). However, the converse effect of TLRs has also been reported. For instance, inhibition of TLR5/MyD88/NF-κB signaling enhanced tumor growth in mouse xenografts of A549 lung cancer cells *in vivo*, whereas activation of the TLR5 signaling pathway inhibited the growth (49). Therefore, enhancing the survival pathways and alleviating the apoptotic pathways mediated by TLRs may favor the growth of lung cancer cells.

TLRs are involved in angiogenesis in lung cancer. The formation and development of vascular networks are strictly controlled processes that have both physiological and pathophysiological

significance. The formation and development of cancer depends on the growth of a capillary vessel network, as this ensures a supply of oxygen and nutrition to the tumor tissue (50). TLRs, particularly TLR4 and 9, have been identified to be associated with tumor angiogenesis (Table I). In recent years, the vascular endothelial growth factor (VEGF) pathway has gained improved understanding regarding its role in angiogenesis (51). In particular, a study identified that activation of the TLR4/NF-κB signaling pathway enhanced the expression of VEGF, while inhibition of the pathway disrupted endothelial tube arrangement, blood capillary formation, endothelial sprouting and thus vascular growth (52,53). This was also confirmed in a mouse model, whereby intraperitoneal injection of LPS increased angiogenesis in BALB/c mice bearing 4T1 lung metastases, due to the increased vascular permeability (54). In addition, TLR4 ligation in human lung cancer cells promoted production of the proangiogenic chemokine IL-8 through the p38 MAPK signaling pathway, which served a positive role in the regulation of vessel formation (45). In addition to TLR4, TLR9 has been implicated in the angiogenic process, although its role is controversial. One study identified that TLR9 agonist CpG-ODNs significantly enhanced the secretion of IL-10, an important factor in tumor angiogenesis, in 95D cells (55,56). By contrast, another study observed that TLR9 agonist immunomodulatory oligonucleotides inhibited micro-vessel formation and tumor growth synergistically with epidermal growth factor receptor inhibitors in NSCLC (57). Thus, the exact role of TLR9 in angiogenesis requires further investigation.

TLRs are associated with the invasion of lung cancer cells. Metastasis is the spread of cancer cells from a primary site to a secondary site via the blood or lymphatic system. Metastasis of lung cancer is a malignant marker and a primary reason for poor patient prognosis and in some cases mortality. The five-year survival rate in early-stage NSCLC is 50-70%, though drops to 2-5% for patients with cancer that has spread distally (2). As aforementioned, vessel formation is conducive to the invasion of lung cancer cells. In addition, TLRs may promote the invasion of lung cancer cells by other mechanisms (Table I). A previous study indicated that TLR3 and TLR4 stimulated respectively by polyinosinic-polycytidylic acid and LPS promoted the autophagy of lung cancer cells, mediated by the adapter protein TRIF, which in turn generated a series of corresponding cytokines, including IL-6, monocyte chemotactic protein 1, macrophage inflammatory protein3-α and matrix metalloprotein-2 (MMP-2), resulting in migration and invasion of the lung cancer cells (58). TLR4 activation with heat-inactivated *Escherichia coli* (*E. coli*) increased gross hepatic metastases of H59 cells (a Lewis lung adenocarcinoma subline) *in vivo*, and induced H59 cell migration *in vitro*; however, when the p38 MAPK or ERK1/2 signaling pathway was inhibited, the adhesion of H59 cells to the extracellular matrix decreased, thus causing a decrease in the migratory ability of the lung cancer cells (59). A similar result was obtained in mouse Lewis lung cancer cells, with the migratory ability of cells significantly increased following LPS stimulation (60). Furthermore, activation of TLR4 by HMGB1 has been reported to induce migration and invasion of A549 and H838 lung cancer cells by activating integrin αvβ3/focal adhesion kinase through the TLR4/NF-κB pathway (61). Conversely, knockdown of

Table I. Roles of TLRs in lung cancer progression.

TLR activated	Models	Effect on lung cancer progression	Pathways or molecules involved	(Refs.)
TLR4	A549, Mouse model	Favor proliferation	PI3K/AKT	(25,42)
TLR4	Primary human lung cancer cells	Favor proliferation	ROS/miR-21	(43)
TLR4	Lewis lung cancer cells	Favor apoptosis	Bax, Bcl-2, PI3K/AKT, ERK1/2	(44)
TLR4	A549, H1299	Favor apoptosis	NF-κB	(45)
TLR2	A549, H226	Favor proliferation	IL-8	(47)
TLR7/8	A549, SK-MES, H1355	Favor cell survival	NF-κB, Bcl-2	(21)
TLR7	A549	Favor growth and induce resistance to chemotherapy	/	(26)
TLR9	95D	Favor proliferation	CDK2	(48)
TLR5	A549	Inhibit growth	NF-κB	(49)
TLR4	Mouse model	Favor angiogenesis	VEGF	(54)
TLR4	A549, H1299	Favor angiogenesis	p38 MAPK	(45)
TLR9	95D	Favor angiogenesis	IL-10	(55,56)
TLR3/4	A549, H460	Favor migration and invasion	TRIF	(58)
TLR4	H59	Favor migration	p38 MAPK, ERK1/2	(59)
TLR4	Lewis lung cancer cells	Favor migration	TGF-β1, IL-10	(60)
TLR4	A549, H838	Favor migration and invasion	Integrin αvβ3/FAK	(61,25)
TLR9	95D,	Favor invasion	MMP-2	(62)
TLR9	H460, 95D 95C	Favor growth and metastasis	PI3K/AKT	(63,64)
TLR3	SUM190	Favor stemness	β-catenin NF-κB	(71)
TLR4	MCF-7, T47D	Favor stemness	POU5F1 SOX2 NANOG	(72)
TLR7	HCCLM3, MHCC97-H	Decrease stem cell no.	IKK/ NF-κB	(73)
TLR4/9	Caco-2, HT29, HCT116	Favor stemness	Nod1/Rip2	(74)
TLR4	Mouse model, human specimen	Decrease CSC self-renewal	RBBP5	(75)

TLR, toll-like receptor; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; ROS, reactive oxygen species; miR, microRNA; Bcl-2, B-cell lymphoma 2 protein; Bax, Bcl-2-associated X protein; ERK, extracellular signal-regulated protein kinase; NF-κB, nuclear factor-κB; IL, interleukin; CDK2, cyclin-dependant kinase 2; p38 MAPK, p38 mitogen-activated protein kinase; TRIF, toll/ IL-1 receptor-domain-containing adapter-inducing interferon-β; TGF, tumor growth factor; FAK, focal adhesion kinase; MMP, matrix metalloprotein; POU5F1, POU domain, class 5, transcription factor 1; SOX2, sex determining region Y-related HMG box2; Nod1/Rip2, nucleotide-binding oligomerization domain-containing protein 1/ receptor-interacting protein 2; RBBP5, retinoblastoma binding protein 5.

TLR4 in A549 cells significantly suppressed cell migration and invasion (25). In addition, stimulation of lung cancer 95D cells with TLR9 ligand significantly elevated secretion of IL-1α and IL-8, as well as the expression of C-X-C chemokine receptor type 4, intercellular cell adhesion molecule-1 and MMP-2, which ultimately contributed to the invasion of the 95D cells; however, the invasive ability of the 95D cells was suppressed by an inhibitor of TLR9 ligand (62). Consistent with these findings, *in vivo* studies have also demonstrated a direct positive effect of TLR9 signaling on lung cancer metastasis, which was conferred by the PI3K/AKT pathway (63,64).

TLRs are associated with CSC behavior. CSCs make up a small population of cancer cells, and possess self-renewal and differentiation abilities that promote malignancy and, ultimately, treatment resistance and cancer relapse (65,66). Chronic inflammation has been identified as one of the major factors involved in CSC expansion and tumor progression (67). CSCs constitutively exhibit higher NF-κB activation, which increases stemness in cancer cells (68-70). The activation of NF-κB has been attributed to ligation of the TLR signaling pathway (Table I). For instance, TLR3 has been identified to promote a phenotypic switch in breast cancer cells towards a CSC phenotype, which induced tumor growth (71). Further experiments have observed that simultaneous activation of β-catenin and NF-κB signaling pathways triggered by TLR3 stimulation was required for enhanced CSC phenotypes (71). For TLR4, it has been reported that HMGB1 released by autophagic cancer-associated fibroblasts in the breast cancer microenvironment activated TLR4 on luminal breast cancer cells to facilitate the expansion of breast CSCs and the maintenance of their stemness (72). Contrasting with the tumorigenic role, TLR7 activation with the agonist imiquimod exhibited inhibitory effects on cell proliferation and mammosphere formation in hepatic cells and stem cells, and decreased stem cell number. These effects were achieved through the TLR7/IKK/NF-κB/IL-6 signaling pathway (73). Commensal *E. coli* has been identified as a major protagonist of microbe-induced colorectal oncogenesis (74). In a previous study, repression of the microbe sensing pathway TLR/MyD88 with simultaneous activation of the nucleotide-binding oligomerization domain-containing protein 1/ receptor-interacting protein 2 (Nod1/Rip2) pathway was responsible for the acquisition of stem-like properties in bacteria-infected intestinal cancer cells, which resulted in expansion of a tumorigenic CSC population marked by enhanced malignancy traits, long term self-renewal capacity and robust tumorigenic ability (74). Similarly, Alvarado *et al* (75) reported that glioblastoma CSCs downregulated TLR4 to evade immune suppression, and that activation of downstream TLR signaling pathways may reduce tumor growth and disrupt CSC self-renewal by repressing the expression of the transcription factor retinoblastoma binding protein 5 (RBBP5) (76).

4. Conclusion

The roles of TLRs in immunity have been widely studied. Based on current findings, TLRs as sensors on lung cancer cells may favor the cell growth, angiogenesis and invasive abilities of lung cancer cells and regulate the behavior of CSCs, as detailed in Table I. However, their roles and fundamental mechanisms in lung cancer progression need to be further elucidated. The development of novel treatments that target the TLR pathways may be promising therapeutic strategies for alleviating lung cancer progression. To date, a number of TLR agonists have been approved or are in clinical trials as cancer therapeutics. For instance, Bacillus Calmette-Guérin (BCG), an agonist of TLR2 and 4, has been approved for the treatment of superficial bladder cancer (77); TLR4 agonist monophosphoryl lipid A has been approved for use in the Cervarix vaccine as an adjuvant for the prophylaxis of human papillomavirus-associated cervical cancer (78); and TLR7 agonist imiquimod has been approved as a therapeutic for superficial basal cell carcinoma (79). Although TLRs agonists are promising therapeutics for certain types of cancer, combining the TLR agonist with the current treatments, including radiation and chemotherapy, may be of greater benefit, given that they can exert potent immunostimulatory effects *in vivo* (80). For instance, the TLR9 agonist CpG-ODN plus chemotherapy is currently under phase III trials for NSCLC and under phase I trials for colorectal cancer (80); and BCG combined with FDA-approved immunostimulatory monoclonal antibody ipilimumab is under phase I trials for melanoma (81). Thus, identification of compatible TLR agonists may be a promising direction for drug development in the future.

Acknowledgements

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