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REVIEW

Role of Toll-Like Receptors in Tissue Repair and Tumorigenesis

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Abstract—Toll-like receptors (TLRs) play a critical role in host defense from microbial infection. TLRs recognize conserved molecular structures produced by microorganisms and induce activation of innate and adaptive immune responses. The inflammatory responses induced by TLRs play an important role TLRs not only in host defense from infection, but also in tissue repair and regeneration. This latter function of TLRs can also contribute to tumorigenesis. Here we review recent progress in understanding the role of TLRs in cancer development.

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Infection, inflammation, and injury may converge to increase the risk of cancer in a multitude of ways. Microbial colonization can in some cases promote tumorigenesis. For example, *Helicobacter pylori* and hepatitis C virus (HCV) may occupy host niches that lead to chronic inflammation [1, 2]. Chronic inflammation due either to infection or sterile injury is an important risk factor for cancer [3, 4]. The inflammatory response is well known to play a critical role in all stages of cancer development including initiation, promotion, and progression [5]. In addition, regardless of the origin, whether it be due to infection, inflammation, irritation, or oncogene or DNA-damage associated apoptosis, there is a great deal of cell death and tissue injury associated with cancer. Indeed there are interesting parallels between tumorigenesis, tissue repair, and regeneration [6, 7].

Toll-like receptors (TLRs) are an evolutionarily conserved family of molecules that recognize conserved patterns of microbial structures. In this role, they are critical to mediating host defense strategies to microbial pathogens. In addition, TLRs are important in maintaining tissue homeostasis, such as by repairing injured tissue. TLRs appear to do this by the recognition of microbes and of endogenous signs of cell death and tissue injury. As a transducer of information about microbes and tissue injury TLRs may play a diverse role in the phenomenon of cancer. This will be the topic of this review.

Abbreviations: LPS) lipopolysaccharide; PAMPs) pathogen-associated molecular patterns; PRRs) pattern recognition receptors; TLRs) Toll-like receptors.

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TLRs RECOGNIZE MICROBIAL AND ENDOGENOUS MOLECULAR PATTERNS

TLRs are the best studied of a class of host receptors known as pattern recognition receptors (PRRs). Other PRRs include membrane proteins such as scavenger receptors and C-type lectins, secreted molecules such as acute phase and complements factors, and cytosolic sensors such as NODs, NALPs, NAIPs, and the cytosolic viral nucleic acid receptors RIG-I, MDA-5, and DAI [8-10].

PRRs such as TLRs are best known for their ability to recognize conserved structures of microorganisms originally named PAMPs (pathogen-associated molecular patterns) by Janeway [11]. In actuality, PAMPs are common to all microorganisms regardless of "pathogenicity".

Phylogenetic analysis has revealed that in vertebrates, TLRs group into at least six subfamilies [12] and into three larger families, which seem to correspond to the type of macromolecular ligand recognized (nucleic acid, protein, lipid). TLRs 1, 2, 4, 6, and 10 are involved in lipid recognition, TLRs 5 and 11 recognize proteins, and TLRs 3, 7, 8, and 9 sense nucleic acids, although there are exceptions to this trend. The best characterized microbial ligands for TLRs are lipopolysaccharide (LPS; endotoxin) of gram-negative bacteria (TLR4), bacterial lipoproteins and lipoteichoic acid and fungal zymosan (TLRs 1, 2, 6), bacterial flagellin (TLR5), a profilin-like molecule from the protozoan *Toxoplasma gondi* (TLR11), unmethylated CpG motifs present in DNA (TLR9), double stranded RNA (TLR3), and single

stranded RNA (TLR 7, 8). A growing list of microbial products has been found to activate host cells via TLRs.

In addition to those derived from microbes, there have been numerous reports of the ability of non-microbial factors to stimulate TLRs. These include endogenous (made by the host) and artificial ligands (such as synthesized or natural products used pharmacologically) [13].

One group of endogenous ligands for TLRs is nucleic acids. In this case, the endosomal localization of TLRs 3, 7, 8, and 9 may allow for physiological discrimination between endogenous and microbial nucleic acids [8-10].

There is a growing list of candidate endogenous ligands that have been reported to stimulate TLR expressed on the cell surface (namely TLRs 2 and 4). These include many heat shock proteins including those associated with the mitochondria (Hsp60 and 70) [14-18], with endoplasmic reticulum (gp96) [19], and other members of this family (Hspb8 and αA crystallin) [20], high mobility group box 1 (HMGB1) [21, 22], uric acid crystals [23, 24], surfactant protein A [25], and various products of the extracellular matrix such as fibronectin [26], heparan sulfate [27], biglycan [28], fibrinogen [29], oligosaccharides of hyaluronan [30], and hyaluronan breakdown fragments [31-33].

A number of agents used pharmacologically have been shown to activate TLR-dependent signaling. These include the murine TLR4 agonist Taxol, a compound isolated from the evergreen plant *Taxus brevifolia* (Pacific yew) [34-36], and synthetic ligands of TLR7 such as imiquimod [37], R848 [37], and loxoribine [38].

TLR SIGNALING

TLRs localize to different subcellular locations that seem to correspond to the chemical nature of the ligands they recognize. In general, lipid and protein recognition TLRs are at the plasma membrane, while nucleic acid sensing TLRs are found in endocytic compartments. Upon ligation, TLRs form homo- and heterodimers and transmit signals throughout the cell via TIR–TIR homotypic binding with one or a combination of four TIR-containing adaptor proteins—MyD88, TRIF/TICAM-1, TIRAP/MAL, and TRAM/TICAM-2/TIRP. All TLR (in addition to IL-1, -18, and -33R), except for TLR3 (which exclusively signals through TRIF), signal through a bottleneck and use MyD88 as an adaptor [39].

Signaling transduction from TLRs occurs via a number of physical and biochemical events of which reversible covalent modifications via phosphorylation and ubiquitination are paramount. All TLR use IRAK and TRAF family members as upstream components. This signaling converges on intermediate level kinases such as TAK-1 and TBK1/IKKi leading to signal diversification and amplification and activation of MAP kinase (JNK, p38, and ERK), IRF (notably IRF 3, 5, and 7), and NF-κB pathways [10]. In addition, TLR signaling leads to the activation of PI3K and Akt [40-42], which may be important in regulation of glycogen synthase kinase 3 (GSK3) [41, 42] and β-catenin [42, 43].

ROLE OF TLRs IN TISSUE REPAIR AND REGENERATION

In addition to their role in mammalian host defense from deleterious microbial infection, TLRs are also involved in various aspects of mammalian homeostasis such as development, the recognition of cellular and tissue injury, and tissue repair and regeneration.

As mentioned above, Toll was originally identified in Drosophila as a maternally derived factor necessary for dorsal–ventral axis formation of the developing zygote [44, 45]. Later in Drosophila development, Toll is important in the regulation of axon pathfinding of motoneurons and acts to inhibit synaptic initiation [46]. In addition, while a direct role for TLRs has not been discerned, LPS recognition has been shown to be a crucial mediator of organogenesis in the host–microbial symbiosis between the Hawaiian bobtail squid *Euprymna scolopes* and *Vibrio fischeri* [47] and in intestinal development in zebrafish (*Danio rerio*) [48, 49].

Recent evidence has pointed to a role of TLRs in mammalian development and homeostasis [50, 51]. In the brain, TLRs have both been shown to play a role in the regulation of proliferation and differentiation of neurons in the adult hippocampus [52] and in the inhibition of neurite outgrowth [53]. In the colon, steady-state (in the absence of overt infection or injury) activation of TLR is important for epithelial barrier function such as through the strengthening of tight junctions and induction of cytoprotective factors [54-56]. Epithelial maintenance at the steady-state also occurs in the lung through basal inhibition of apoptosis via signaling of TLRs 2 and 4 [31].

TLRs are crucial in the response to tissue injury and subsequent tissue repair and regeneration. TLR signaling has been demonstrated to be critical for maintaining tissue integrity and repairing damaged tissue in models of chemical, radiation, and infectious colonic injury [54, 57-60]. TLR signaling is required for liver regeneration after partial hepatectomy [61, 62] and also protection from bleomycin- and hyperoxic-induced lung injury [31, 63]. In the central nervous system, TLRs orchestrate the protective response to axonal and crush injury of the brain and spinal cord [64-66].

The repair and regeneration of tissue after injurious insult is a complex process. However, the first generation of information from this nascent field suggests that TLRs may be important in many stages of this process. In providing pro-survival signals and in preventing apoptosis, TLRs may dictate the threshold of injury and cellular death, thereby limiting the extent of damage to initial

injury. In addition, signaling induced after injury, such as the TLR-dependent production of prostaglandins (via COX-2 regulation) in tissue stroma [67, 68] or the up-regulation of anti-apoptotic factors may provide signals to keep both differentiated and progenitor cells within the tissue alive. This may be particularly important for regeneration after primary injury and subsequent to inflammation.

After injury, the regeneration process begins. Regeneration entails the restoration of lost cell populations, tissue architecture, blood supply, and innervation amongst others. Depending on the organ and nature of injury, TLRs have been demonstrated to be critical for the orchestration of many of these events. In the colon, liver, and central nervous system, TLRs regulate the compensatory proliferation of parenchymal cells after injury [54, 58, 61, 62, 69]. TLR signaling repositions tissue resident prostaglandin secreting stromal cells to activate colonic epithelial progenitors at the base of regenerating crypts [68]. TLR-derived signals are likely to regulate processes such as angiogenesis and tissue remodeling by the induction of cyclooxygenases, chemokines, VEGF, and matrix metalloproteinases [54, 58, 67] and by activating mesenchymal stem cells [70]. In addition to acting on resident cells, TLRs have been shown to be central in the recruitment of leukocytes and other ancillary cells in the context of non-immune injury [71].

What is activating TLRs in their role in tissue homeostasis? Depending on the context, both microbial and endogenous ligands activate TLRs. At sites colonized by the indigenous microflora, such as the colon, microbial-derived ligands stimulate TLR-dependent tissue homeostasis during the steady-state and upon injury [54, 58]. Signals from the indigenous flora may also be active at the liver due to the presence of microbial ligands for TLR in entero-hepatic circulation [72]. TLRs may also be activated by microbial ligands during infectious injury or by endogenous ligands such as those liberated from necrotic cells such as HMGB1 [73, 74] or extracellular matrix components as a consequence of non-infectious injury or repair [75].

TLRs AND CANCER

Anti-cancer effects of TLRs: immunotherapy and host defense from infection. A role of microbes in mediating an anti-tumor effect can be dated to the XVIII century when Deidier observed a positive correlation between infection and remission of malignant disease [76]. Evidence that microbial products, rather than infection per se, may have an effect against tumors came at the end of the XIX century when Coley found that repeated injections of a mixture of bacterial toxins from the gram positive bacteria *Streptococcus* and the gram negative bacteria *Serratia marcescens* served as efficient anti-tumor therapeutic agent [77]. Shear and Turner later revealed LPS to be the "hemorrhage producing fraction" of Coley's toxin responsible for its anti-tumor effect [76], suggesting that the long appreciated anti-tumor effect of Coley's toxin can be attributed to stimulation of the host via TLRs.

The anti-tumor activity of other microbial derived therapeutics over the years can be linked to their ability to activate TLRs. A lyophilized preparation of group A streptococcus known as OK-432 [78], which has been shown to elicit therapeutic effects for the treatment of cervical, gastric, and oral squamous cell carcinoma [79-81], was recently shown to stimulate TLR4 [82, 83]. For 30 years, BCG (a potent activator of TLR2- and 4-dependent signaling [84, 85]) has been used as an effective treatment of bladder cancer via intravesicular injection of the mycobacteria [86].

Administration of purified ligands for TLRs has been shown to have potent anti-cancer effects against established tumors in both mice and humans [76, 87]. These effects have been demonstrated via both local (at the site of the tumor) and systemic delivery [87]. Systemic administration of LPS has been used in phase II clinical trials for the treatment of colorectal and lung cancer [88] and leads to tumor regression when directly injected into adoptively transferred tumors [89]. For the latter, a similar result has been demonstrated for the injection of flagellin [90]. Local applications of ligands for TLR7/8, such as imiquimod, are being studied as treatments for skin cancer and may also be effective when administered systemically for chronic lymphocytic leukemia [91-93]. The most studied (and perhaps most promising) TLR ligand used for its anti-tumor effects is the TLR9 activator CpG, which is under study for the treatment of brain, lymphoma, skin, and renal cancer [87, 94].

It is clear administration of TLR agonists mediate anti-tumor activity by a multitude of mechanisms. As noted above, and especially in high doses, TLR ligation leads to apoptosis of cells. TLR agonists have been shown to directly kill both tumor cells and ancillary cells of the tumor microenvironment such as vascular endothelium [95-98]. TLR activation may also lead to tumor regression by directly or indirectly (TNF-mediated) increasing vascular permeability [76], recruiting of leukocytes (such as macrophages) involved in resolving tumor, direct and indirect activation of the tumor lytic activity of NK- and cytotoxic T-cells, and increasing the sensitivity of tumor cells to assisted killing such as via TRAIL, TNF, and granzyme B/perforin [99, 100].

The best appreciated role of TLR in cancer therapy has come from taking advantage of the function of TLRs in stimulating the adaptive immune response against microbial pathogens. These studies have sought to break tolerance to tumor self-antigens and induce anti-tumor effector immune responses by using TLR ligands as adjuvants (or even alone in TLR monotherapy) in cancer vaccines, as targets of gene therapy, and in raising anti-tumor

antigen-specific T cells *in vitro* for adoptive transfer [87]. The mechanisms by which TLRs induce effective anti-tumor adaptive immune responses include uptake, processing, and (cross)-presentation of tumor cells by dendrite cells, increased survival of dendrite cells, induction of co-stimulatory markers on professional antigen-presenting cells, induction of Th1 and CTL responses, and the inhibition of regulatory T cell activity [87, 101, 102].

Most studies have used exogenous TLR agonists to induce anti-cancer T cell responses that are very hard to induce under physiologic (endogenous) circumstances. However, one recent study has suggested a more physiologic role of TLRs in inducing anti-tumor T cell responses [73]. This study reported that the anti-tumor efficacy of numerous chemotherapeutic agents to mice with established, adoptively transferred tumors was dependent on TLR-4 and MyD88 [73]. The authors suggest that this phenomenon is due to the activation of TLR4 and induction of anti-tumor T cell immunity by HMGB1 released from dying tumor cells due to chemotherapy [73].

The most physiological role that TLRs play against cancer may be in preventing infection by microbial pathogens associated with the development of cancer. TLRs have been shown to be important in the recognition of microbial pathogens such as Epstein–Barr virus [103], hepatitis B and C virus [104-106], human papilloma virus [107], and *H. pylori* [108], all of which are important etiologic agents of human cancer. Functional TLR responses (in addition to those of other microbial PRRs) are likely to be important in whatever natural resistance humans have to these pathogens, and perhaps more importantly, in inducing protective immune response for cancer prevention by vaccines.

TLR as a positive regulator of cancer. A first indication that TLR stimulation may have a positive role in tumorigenesis came from reports demonstrating that TLR ligands augment the growth of adoptively transferred tumors [90, 109-112]. These models have been used to study the role of TLR agonist administration in a number of processes that augment carcinogenesis. Using a model of intravenous injection of a spontaneously metastasizing mammary adenocarcinoma cell line, it has been shown that systemic LPS administration increases both tumor migration and invasion to secondary sites from the bloodstream and angiogenesis at these sites [110]. In a similar model, but using a colonic adenocarcinoma cell line, intraperitoneal injection of LPS has been shown to increase proliferation and decrease apoptosis of metastatic tumors [111].

In vivo administration of TLR ligands may be protumorigenic due to action on both the tumor cells themselves and accessory cells in the tumor microenvironment. Numerous studies have demonstrated that both primary and tumor cell lines express TLRs. *In vitro* stimulation of tumor cell lines leads to both increased survival and proliferation of tumor cell lines. Notably, isolated plasma cells from patients with multiple myeloma were shown to express an increased repertoire of TLRs compared to plasma cells from healthy donors [113, 114]. Stimulation of these multiple myeloma cells with various TLR ligands led to increased proliferation in part due to autocrine secretion of IL-6 [113, 114]. Such a direct effect of TLR ligation has been demonstrated by knocking down endogenous expression of TLRs in tumor cell lines before adoptive transfer [112, 115]. In these studies, the growth promoting effect of TLR4 on a TLR4-expressing colon cancer cell line occurred independently of exogenous administration of LPS [115], while the positive effect of TLR2 on *in vivo* hepatocellular carcinoma cell line growth was due to intratumoral administration of *Listeria monocytogenes* [112].

In vivo administration of TLR ligands has also been shown to enhance the growth of adoptively transferred tumor cell lines by acting on host cells. TLR4-dependent signaling in the recipient was shown to be responsible for LPS-induced tumor growth by the increasing the levels of circulating TNF, which led to the up-regulation of NF-κB anti-apoptotic factors such as Bcl-Xl, cIAP1, and cIAP2 in tumor cells [111].

While we have gained an appreciation of the role of TLRs in enhancing the progression of adoptively transferred tumor cells, we know very little about the role of TLR signaling in tumor progression as it occurs for "naturally" arising tumors in their tissue microenvironments.

However, two recent reports have demonstrated that TLRs are involved in the development of tumors as they arise in their natural microenvironment. In a murine model of liver carcinogenesis induced by injection of the mutagen diethylnitroseamine, MyD88-dependent signaling has been shown to be critical for tumorigenesis [116]. In this model mutagenesis precipitated by diethylnitroseamine leads to the initiation of hepatocytes. Recent work has suggested that the response of stromal cells such as tissue resident macrophages to the cell death of initiated hepatocytes is critical to proliferation and expansion of initiated cells and tumor promotion [117]. This promotion is the result of the NF-κB dependent production of inflammatory mediators such as IL-6 upon recognition of necrotic hepatocytes by tumor stroma [116, 117]. In this setting, MyD88 signaling was demonstrated to be responsible for the activation of NF-κB in response to hepatocyte cell death and the production of factors (such as IL-6) responsible for promotion of initiated hepatocytes [116].

MyD88 has also recently been shown to be crucial to tumor promotion in the ApcMin/+ and azoxymethane (AZO) model of spontaneous (ApcMin/+) and carcinogen-induced (AZO) intestinal tumorigenesis [118]. ApcMin/+ mice are heterozygous for a mutant allele of the tumor suppressor APC [5]. After a loss of heterozygosity at the APC locus, small foci of initiated intestinal epithelial cells form macroscopic tumors. This is a

process dependent on factors derived from epithelial cells such as matrix metalloproteinase (MMP) 7 and the tumor microenvironmental stroma such as COX-2 [119-121]. ApcMin/+ mice deficient in MyD88 showed both fewer and smaller tumors compared to ApcMin/+ wild type mice (Rakoff-Nahoum, 2007, #970). This did not appear to be due to an effect of initiation by MyD88, as a loss of heterozygosity as measured by the frequency of microadenomas did not depend on MyD88. Rather, it seems that MyD88 regulated the expression of many positive regulators of tumor promotion such as COX-2, MMP7, and cPLA2 [118], which are important in many aspects of tumor growth [119-122].

Microbial infections are reported to be responsible for 15-20% of the global burden of cancer [123]. Many microbes encode adaptation factors that increase their fitness by dysregulating host cell checkpoints and mediating oncogenic transformation. However, in addition to this, the response of the host to the microbe, such as the induction of inflammation, is likely to play an important role in tumorigenesis. Thus the recognition of microbes via innate immune pattern recognition receptors may prove to be important links between microbes, inflammation, and cancer. Such a role of microbial pattern recognition receptors in driving the growth of tumors is paradoxical to the original connection between the host response and microbial products in tumor growth made by Coley in 1893 [77]. However, five years later, Coley published his empirical observations relating injury and trauma to cancer [124]. In their role in tissue repair and homeostasis, innate pattern recognition receptors such as TLRs may be a point of convergence of infection, injury, inflammation, and cancer.

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