

Chapter 9

TLR Signaling and Tumour-Associated Macrophages

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Introduction

Macrophages are bone marrow-derived cells that initially circulate as monocytes and subsequently undergo differentiation into tissue resident macrophages. Macrophages support tissues by remodeling, secreting growth and angiogenic factors, and phagocytosing apoptotic cells. Virchow first described the presence of leukocytes in human tumours in 1863 and suggested that cancer originates in sites of chronic inflammation (Balkwill and Mantovani 2001). Wounding of normal tissues results in the production of numerous growth factors, cytokines, and chemokines, all of which recruit and differentiate circulating monocytes to tissues. Tumour cells have been found to produce similar growth factors and cytokines that recruit macrophages to tumours (Condeelis and Pollard 2006). Further, there is an emerging characterization of macrophage activation by tumour-derived ligands of Toll-like receptors (TLRs), a family of transmembrane proteins initially characterized as sensors of infection.

Whereas the role of these tumour-associated macrophages (TAMs) has yet to be clearly characterized, a majority (>80%) of clinical studies correlate an increased number of TAMs with a worse prognosis (Pollard 2004). Several *in vivo* studies have supported these clinical observations: mice deficient in CSF-1 and thus deficient in macrophages (Wiktor-Jedrzejczak et al. 1990), when crossed with mice prone to develop metastatic breast cancer, develop the same numbers of breast tumours but nearly no metastases (Lin et al. 2001). The role that TLR signaling may have in stimulating macrophages to become tumour promoting is not clearly defined. However, given the role that macrophages play in restoring tissue homeostasis such as in the tissue repair response, it is likely that tumour-induced TLR signaling will

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provide macrophages with signals of persistent injury in need of repair. In this chapter, we review the molecular determinants of TLR signaling, the role TLR signaling plays in infection, wound healing, and tumourigenesis and discuss how TLR signaling in TAMs may result in tumour progression.

Toll-Like Receptors

The TLR family of transmembrane proteins is best characterized as pattern recognition receptors (PRR) for sensing conserved pathogen-associated molecular patterns (PAMPs) (Medzhitov et al. 1997). PAMPs are conserved molecular patterns that are universal to all microorganisms within a defined class. Further, PAMPs are present within microorganisms regardless of their pathogenicity. In addition to microbial products, TLRs can also recognize endogenous, host-derived ligands (Jiang et al. 2007; Sims et al. 2010). Ten TLRs have been identified in humans and 12 TLRs in mice (Takeuchi and Akira 2010).

TLRs can be divided into two general groups based on the types of ligands they recognize and their subcellular localization. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are expressed on the plasma membrane and can recognize lipids, lipoproteins, and proteins from exogenous and endogenous sources. In contrast, TLR3, TLR7, TLR8, and TLR9 localize to intracellular compartments including the endoplasmic reticulum, endosomes, lysosomes, and endolysosomes, and they recognize nucleic acids (Kawai and Akira 2010).

TLRs are type I single spanning transmembrane proteins. Their molecular structure is defined by an extracellular or endolysosomal N-terminal leucine-rich repeat (LRR) domain followed by a transmembrane domain and then by a C-terminal cytoplasmic Toll/IL-1 receptor (TIR) domain. Below is a concise description of the known TLRs and their ligands.

TLR2 recognizes PAMPs from a variety of microorganisms including bacteria, fungi, *Mycoplasma*, and viruses by forming heterodimers with TLR1 and TLR6. The TLR1/TLR2 heterodimer recognizes triacyl lipoproteins, whereas the TLR6/TLR2 heterodimer recognizes diacyl lipoproteins.

TLR4 recognizes lipopolysaccharide (LPS), a component of the outer membranes of Gram-negative bacteria, by forming a homodimeric complex with LPS and myeloid differentiation factor-2 (MD2). In addition to bacterial products, TLR4 can recognize components of viral envelopes. TLR4 also recognizes endogenous ligands such as oxidized phospholipids produced during acute lung injury caused by avian influenza (Imai et al. 2008).

TLR5 recognizes flagellin, a component of the bacterial flagellum. In the lamina propria of the small intestine, flagellin stimulates lamina propria dendritic cells (DCs) to induce T cells to differentiate into Th17 cells and Th1 cells and B cells to differentiate into immunoglobulin A-producing plasma cells (Uematsu et al. 2008). TLR11, which is found in mice but not in humans, is closely related to TLR5. It is thought to recognize uropathogenic bacterial components (Zhang et al. 2004). In addition, it can recognize a profilin-like molecule from *Toxoplasma gondii*.

Nucleic acids are recognized by TLR3, TLR7, TLR8, and TLR9. TLR3 recognizes double-stranded RNA (dsRNA) from viruses (Alexopoulou et al. 2001) that are presented within endolysosomes. An analog of dsRNA, polyinosinic:polycytidylic acid (poly I:C), is also recognized by TLR3 and is commonly used to simulate viral infections. Activation of TLR3, as all TLRs that recognize nucleic acids, leads to the production of type I interferons as well as other pro-inflammatory cytokines.

TLR7 and TLR8 recognize single-stranded RNA (ssRNA) as well as the imidazoquinoline analogs of ssRNA within endolysosomes. TLR7 can also detect RNA from bacteria, also within the context of the endolysosome (Mancuso et al. 2009). Unmethylated DNA with CpG oligodeoxynucleotides (cytosine–guanine repeats), commonly found in bacteria and viruses, is recognized by TLR9. In addition to DNA, TLR9 may recognize the crystalline metabolite of malaria, hemozoin (Coban et al. 2005). However, there is some evidence that hemozoin binds to the malaria DNA which can be recognized by TLR9 in the endolysosome (Parroche et al. 2007).

Although the ligand for human TLR10 has not yet been identified, it shares sequence homology with TLR1 and TLR6. In mice, the gene for TLR10 is disrupted by a retrovirus.

TLR Signaling Pathways

Upon recognition of their ligands, TLRs signal for the transcription of genes that allow the stimulated cell to appropriately respond to the stimulus. Specificity of this response is, in part, determined by the combination of adaptor molecules that associate with the C-terminal cytoplasmic domains of TLRs. These adaptors have in common a TIR domain. The five known TIR domain-containing adaptors of TLRs are MyD88 (myeloid differentiation factor 88), TRIF (TIR domain-containing adaptor inducing interferon beta; also known as TICAM-1), TIRAP (also known as MAL), TRAM (TRIF-related adaptor molecule), and SARM (Sterile-alpha and Armadillo motif-containing protein) (O'Neill and Bowie 2007). TLR signaling can be divided into two signaling pathways depending on whether they use either MyD88 or TRIF as adaptor molecules. The downstream targets vary in that MyD88 induces inflammatory cytokines, whereas TRIF induces type I interferons and inflammatory cytokines. TRAM and TIRAP are bridging adaptors that help to recruit and bind TRIF to TLR4 and MyD88 to TLR2, respectively (Takeuchi and Akira 2010). SARM functions as a negative regulator of NF- κ B and IRF activation by blocking TRIF-dependent transcription factor activation (Carty et al. 2006).

MyD88 Signaling

MyD88 was the first characterized TLR adaptor and can signal downstream of all TLRs except TLR3. MyD88 binds to IRAK4 (IL-1 receptor-associated kinase),

which can activate IRAK1 and IRAK2. The IRAKs then associate with the E3 ubiquitin ligase TRAF6 (TNFR-associated factor 6), which with E2-ubiquitin conjugating enzyme complex forms a polyubiquitin chain on TRAF6 (Xia et al. 2009). This polyubiquitin chain can activate kinases such as TAK1 (TGF- β -activating kinase-1) that phosphorylate and thus inactivate the inhibitor of NF- κ B, I κ B α . Free of its inhibitor, NF- κ B can induce the expression of a cascade of pro-inflammatory genes upon translocation to the nucleus. MAPKs (mitogen-activated protein kinases) can also be activated through MyD88 signaling, leading to the induction of pro-inflammatory cytokines.

TRIF Signaling

TLR3 and TLR4 can both signal through the adaptor TRIF. Whereas TLR3 can bind directly to TRIF for signaling, TLR4 requires the adaptor TRAM to signal through TRIF. The TRIF-dependent pathway results in the activation of NF- κ B and IRF3. Upon activation, TRIF binds to TRAF6, TRAF3 as well as RIP1 (C-terminal receptor-interacting protein-1) and RIP3. The TRAFs then associate with TRADD (TNFR-associated death domain protein) which, in combination with FADD (FAS-associated death domain-containing protein), can result in the ubiquitination of RIP1 and then the activation of NF- κ B (Ermolaeva et al. 2008; Pobeziinskaya et al. 2008).

TLRs in Host Defense

An evolutionarily conserved role for TLRs is in the host defense against pathogens. The first barriers encountered by most pathogens are surface epithelial cells that line the skin, lungs, gastrointestinal tract, and genitourinary tract. Signaling of TLRs in these tissues can lead to the production and secretion of antimicrobial factors such as α - and β -defensins, cathelicidin, phospholipase A₂, and lysozyme. Moreover, release of these effectors can further enhance the immune response to signal through TLRs. Further, TLR signaling on immune cells such as macrophages and neutrophils leads to the release of the microbicidal compounds, reactive oxygen species, and reactive nitrogen intermediates.

Beyond inducing an acute innate immune response, signaling through TLRs is critical to establishing an adaptive immune response. Antigen uptake with concomitant TLR activation on DCs allows for the discrimination between *self* and *non-self*. One way in which appropriate antigens can be targeted by the adaptive immune system is by their proximity to the TLR ligand (Blander and Medzhitov 2004, 2006a, b). This has been demonstrated through cooccurrence of antigens and TLR ligands in DCs, allowing for efficient presentation of antigen on MHC Class II or through the ligation of an antigen to a TLR ligand enabling an efficient production of antibodies (Palm and Medzhitov 2009). Further, the appropriate immune response

is determined by the general class of pathogen as determined by the detected TLR ligands. For instance, TLR4 recognizes LPS from Gram-negative bacteria, while TLR2 recognizes peptidoglycan from Gram-positive bacteria.

The adaptive immune response can be controlled by TLRs at multiple levels including the control of antigen uptake (West et al. 2004) and presentation (Blander and Medzhitov 2006b) by DCs, the maturation of DCs, cytokine production by antigen-presenting cells, and control of survival of activated T cells. Signaling through TLRs on B cells is crucial for activation, proliferation, immunoglobulin isotype class switching, and maturation during infection as well as immunization (Gerondakis et al. 2007). Further, TLRs can induce antibody production by memory B cells (Bernasconi et al. 2002).

Endogenous Ligands of TLRs

Exogenous, *non-self* molecules were the first identified and characterized TLR ligands. However, more recently, an increasing number of endogenous, *self* ligands have been identified and characterized (Karin et al. 2006; Kawai and Akira 2010). These endogenous ligands have in common their association with either infection or tissue destruction and cell death. Therefore, the ligands are more accurately described as *altered self* rather than *self*. Most of these endogenous ligands are released during tissue damage, such as that observed in tumour progression. The emerging list of endogenous ligands is complicated by the potential contamination of reagents with exogenous, pathogen-associated products. Therefore, biochemical analysis demonstrating direct interaction between endogenous ligands and TLRs will be required to address the skepticism that presently exists. Identification and characterization of endogenous ligands may be especially relevant to TAMs, as many tumours exist in a sterile environment. Therefore, for many tumour types, the TLR ligands that activate TAMs will likely be derived from an endogenous source.

Intracellular-Derived Ligands

Intracellular-derived endogenous ligands include nuclear proteins and heat shock proteins. HMGB1 (high-mobility group box 1) is a nuclear protein that can bind chromatin that is released during cell necrosis or inflammation (Scaffidi et al. 2002; Wang et al. 1999). HMGB1 has been reported to be recognized by a variety of TLRs including TLR2, TLR4, and TLR9 (Sims et al. 2010). In models of ischemia–reperfusion and septic shock, HMGB1 is proinflammatory. Neutralizing antibodies against HMGB1 in the TLR4^{-/-} background are both protective in the ischemia–reperfusion model, suggesting that endogenous, non-pathogen-derived proinflammatory signaling is mediated through TLR4 (Tsung et al. 2005b). Further, other HMGB proteins – namely HMGB1, HMGB2, and HMGB3 have been found to function as universal sentinels for nucleic acids that can activate a variety of PRRs including TLRs, NLRs (NOD-like receptors), and RLRs (RIG-I-like receptors)

(Yanai et al. 2009). As a DNA-binding protein, HMGB1 can bind both host- and pathogen-derived DNA. When the HMGB1–nucleic acid complex binds the RAGE (receptor for advanced glycation end products) receptor, it is endocytosed and presented to TLR9, where it leads to the activation of DCs and B cells (Tian et al. 2007). How the release of HMGB1 is regulated, whether through a non-classical secretory lysosomal pathway or during necrosis, has not yet been determined. However, the release of HMGB1 was found to be dependent on the cytosolic NLR, NLRP3, and its adaptor ASC (apoptotic speck-like protein containing a caspase recruitment domain) but independent of caspase-1 in a lung infection model (Willingham et al. 2009). Whether there is a physiologic correlate to controlled HMGB1 release has yet to be determined. However, HMGB1 is highly expressed in a variety of solid tumours including colon cancer, breast cancer, melanoma, prostate cancer, and pancreatic cancer. Further, this elevated expression of HMGB1 is associated with tumour progression and metastasis (Ellerman et al. 2007). One consequence of TLR signaling in TAMs by endogenous ligands was suggested in a report of the induction of apoptosis in activated T cells by TAMs that were stimulated by unidentified tumour-derived TLR4 ligands (Liu et al. 2010).

Heat-shock proteins including Hsp60, Hsp70, Hsp90, and gp96 have been reported to activate macrophages and DCs through TLR2 and TLR4. However, heat-shock proteins have also been shown to bind exogenous ligands for TLRs such as LPS and lipoproteins. Therefore, it is unclear whether the effects of these molecular chaperones is direct or through contamination (Tsan and Gao 2009).

TLR Ligands Induced by TLR Signaling

TLR signaling by PAMPs leads to the production of antimicrobial products. In a possible feed-forward mechanism of signal amplification, two of these products have been reported to signal through TLRs. β -Defensin-2 is a pore-forming antimicrobial protein secreted by mucosal epithelium in response to TLR signaling. However, it was also suggested to directly activate immature DCs to express costimulatory molecules and undergo maturation through TLR4, leading to an adaptive immune response (Biragyn et al. 2002). Cathelicidin (LL37) is an antimicrobial peptide that is secreted by keratinocytes and neutrophils upon TLR stimulation. In addition to its antimicrobial properties, LL37 can bind with host DNA or RNA released from necrotic cells and, upon phagocytosis of this aggregate, signal through TLR9 and TLR7, respectively (Ganguly et al. 2009; Lande et al. 2007).

A cellular byproduct of infection by the H5N1 avian influenza virus and from aspiration of acidic solutions that leads to TLR signaling is oxidized phospholipid. In a murine model of lung infection, both TLR4^{-/-} and TRIF^{-/-} mice were protected from the acute lung injury resulting from the avian flu infection, suggesting that this pathway is critical in the detection of this form of oxidative damage (Imai et al. 2008). Of note, all forms of acute lung injury examined including SARS (severe acute respiratory syndrome), pulmonary anthrax, monkey pox, and *Yersinia pestis* infection resulted in significantly elevated levels of oxidized phospholipids.

Extracellular Matrix Ligands

Altered extracellular matrix glycoproteins are another class of endogenous ligands of TLRs. Biglycan is a proteoglycan that binds collagen and is expressed in the extracellular matrix. Biglycan was found to induce pro-inflammatory cytokines in a TLR2- and TLR4-dependent manner (Schaefer et al. 2005). Further, biglycan-deficient mice were more resilient to shock induced by LPS or zymosan, and this was associated with lower levels of circulating TNF α . These findings suggest that release of biglycan from the extracellular matrix through stresses such as inflammation may result in a pro-inflammatory feed-forward mechanism.

Similar to biglycan, hyaluronan, a glycosaminoglycan, is a major constituent of the extracellular matrix. Hyaluronan is also a cell wall component of the bacteria *Streptococcus* groups A and C as well as *Pasturella multocida* (Jiang et al. 2007). Upon tissue damage or inflammation, small fragments of hyaluronan can be recognized by TLR2 and TLR4 to produce pro-inflammatory cytokines. Signaling through these TLRs may be important in tissue repair as TLR2- and TLR4-deficient mice challenged with bleomycin-induced sterile lung injury had greater lung pathology and a higher mortality rate than wild-type mice. The converse corollary experiment in which lung-specific overexpression of hyaluronan is protective of sterile injury supports the hypothesis that TLR signaling via hyaluronan is protective of the lung epithelium (Jiang et al. 2005). These findings suggest that the physiologic correlate of these systems may be that with small deviations from homeostasis, signaling by endogenous ligands through TLRs may be used to restore tissue integrity, whereas in a more severe infection with increased tissue damage, this mechanism may lead to immunopathology.

Versican, a proteoglycan of the extracellular matrix, was identified as the active component of media conditioned by Lewis lung carcinoma that led to the induction of pro-inflammatory cytokines by macrophages (Kim et al. 2009). The induction of these cytokines was determined to be through TLR2/TLR6 signaling. Further, mice deficient in TLR2 or its adaptor, MyD88, had a higher rate of survival and fewer metastases than the wild-type mice. In this study, direct binding of versican to TLR2 but not TLR4 was demonstrated. Whether the versican produced by Lewis lung carcinoma is altered in a way to make it detectable by TLRs is not known.

TLRs and Tissue Repair

TLR signaling has been found to have significant roles in tissue regeneration and repair (Kluwe et al. 2009; Rakoff-Nahoum and Medzhitov 2009). The process of tissue repair requires the clearance of damaged tissues, repopulation of lost cell types, reestablishment of the tissue vasculature and innervation, and remodeling of the overall tissue architecture.

TLR ligands can be categorized into exogenous and endogenous categories, based on from where they are derived. As such, upon damage of tissue, signaling

through TLRs can be mediated by exogenous and endogenous ligands. Damage of epithelial surfaces, including the skin and intestine, compromise the integrity of these barriers, allowing for exposure to TLR ligands from microorganisms whether commensals or pathogens. In contrast, damage to internal organs that do not have surfaces colonized by microorganisms results in the release of stress signals, which can relay the nature and degree of tissue damage through TLRs.

Various models have demonstrated that TLR signaling can either prevent or promote tissue injury. In models of sterile lung injury using bleomycin, signaling through TLR2 and TLR4 was found to be protective of the lung epithelium. The endogenous ligand in this model was determined to be the extracellular matrix proteoglycan hyaluronan. When TLR signaling induced by hyaluronan was blocked with inhibitory peptides, the increased lung pathology was similar to that seen in the TLR2/TLR4 deficient mice (Jiang et al. 2005). In an analogous system, mice sterilized of their gut flora demonstrated increased intestinal injury after treatment with dextran sulfate sodium (DSS) compared with wild-type mice. Further, this increased DSS-induced intestinal damage was similar to that seen in MyD88 deficient mice, indicating that signals from the commensal bacteria is important in tissue preservation (Rakoff-Nahoum et al. 2004). One mechanism by which TLR signaling may result in tissue protection is the delivery of anti-apoptotic signals to cells, preventing cell loss. This may allow TLR signaling to establish the threshold of tolerable injury and cell death. Downstream products of TLR signaling such as cyclooxygenase-2 provide a signal for progenitor cells to proliferate, restoring the damaged tissue.

In other models of tissue injury, signaling through TLRs has been associated with increased tissue pathology. Ischemia followed by reperfusion results in inflammation and oxidative stress of the affected tissue. In various models of ischemia–reperfusion including liver, kidney, heart, and brain, TLR4-deficient mice are relatively protected (Oyama et al. 2004; Tang et al. 2007; Tsung et al. 2005b; Wu et al. 2007). The cells in which TLR signaling is critical for this pathology vary. For instance, in the liver, the abrogation of TLR4 signaling in hematopoietic cells is protective (Tsung et al. 2005a), whereas in the kidney, the abrogation of TLR4 signaling in parenchymal cells is protective (Zhang et al. 2008). HMGB1 is an endogenous TLR ligand that is upregulated in both liver and kidney after ischemia–reperfusion. Antibodies that bind and deplete HMGB1 after liver ischemia–reperfusion are protective of subsequent tissue damage. Further, the depletion of HMGB1 in mice deficient in both TLR2 and TLR4 does not confer any additional protection from tissue pathology (Tsung et al. 2005b). This suggests that the protection conferred in the TLR2 and TLR4 deficient mouse model is due to the loss of signaling downstream of TLR ligand HMGB1. Two extracellular matrix proteins known to be endogenous TLR ligands, biglycan and hyaluronan, are induced by ischemia–reperfusion (Wu et al. 2007), but their role in mediating tissue injury has yet to be determined.

TLR signaling also promotes tissue regeneration in several models. After resection of part of the liver, MyD88-deficient mice exhibit delayed regeneration of the liver (Seki et al. 2005). Although the ligands for TLRs leading to this regenerative effect of MyD88 signaling are not known, it is thought they may be from intestinal commensal bacteria as mice grown in germ-free conditions have similarly impaired

liver regeneration after resection. Models of skin wound healing have also demonstrated that MyD88 deficient mice heal at a slower rate with less granulation tissue and fewer vessels within granulation tissue of the wound (Macedo et al. 2007).

TLRs in Cancer and Tumour-Associated Macrophages

Correlations between inflammation and cancer have been made over the past several hundred years. In some instances, it has been observed that infections correlate with the remission of tumours. Deidier observed that tumours resolved after patients developed venereal infections in the eighteenth century (Rakoff-Nahoum and Medzhitov 2009). In contrast, Virchow suggested in the nineteenth century that sites of chronic inflammation are the origin of some cancers (Balkwill and Mantovani 2001). Experimental models have demonstrated evidence supporting both of these observations, with TLR signaling leading to either the regression or the promotion of various tumour types. Further, genetic association studies have linked polymorphisms within various TLR loci (TLR1, TLR2, TLR3, TLR4, TLR6, and TLR10) to an increased risk of prostate, breast, colorectal, and nasopharyngeal cancers (El-Omar et al. 2008; Rakoff-Nahoum and Medzhitov 2009). Whether these TLR variants display a gain or loss of function remains to be determined. Although the cell types in which TLR signaling is relevant to disease outcomes have not firmly been established in all model cases, there is increasing evidence that TLR signaling in macrophages has a profound impact on tumour progression.

TLR Signaling Causes Tumour Regression

In the late nineteenth century, William Coley read and observed that postoperative infections after resection of tumours often lead to a better clinical outcome. To determine whether this was due to live microorganisms or their products, Coley generated a combination of killed Gram-positive and Gram-negative bacteria, which included *Streptococcus pyogenes* and *Serratia marcescens*, respectively (Hennessy et al. 2010). Intratumoural injection of these “toxins” was observed to have antitumour responses (Hallam et al. 2009). In the mid-twentieth century, Shear and Turner determined that LPS was the active antitumour component in Coley’s toxins (Rakoff-Nahoum and Medzhitov 2009). Therefore, TLR signaling likely mediated these original observations of antitumour response to TLR ligand injections.

Proof for the antitumour efficacy of TLR ligands comes in the form of two agents approved by the US Food and Drug Administration for the treatment of cancers. First, the Bacillus Calmette-Guerin (BCG) vaccine, a live attenuated strain of *Mycobacterium bovis*, is approved for the treatment of primary and relapsed superficial transitional cell bladder carcinoma. The use of this agent is mucosal via intravesicular administration into the bladder. The effect of the BCG vaccine may be through

TLR2 and TLR4 signaling as abrogation of signaling through these TLRs either by knockout or by blocking antibodies inhibited the maturation of and proinflammatory cytokine production by myeloid DCs in model systems (Uehori et al. 2003).

Second, imiquimod is an imidazoquinoline analog of ssRNA that signals through TLR7. Imiquimod is approved by the US Food and Drug Administration for the treatment of superficial basal cell carcinomas and actinic keratoses (intraepidermal neoplasm of dysplastic keratinocytes). Imiquimod induces maturation and proinflammatory cytokine production in DCs and leads to a Th1 antitumour lymphocyte response (Clark et al. 2008; Schon and Schon 2008). TLR7 is also expressed on macrophages and keratinocytes. The critical responsive cell(s) by which this TLR7 ligand acts has yet to be characterized.

TLR signaling from endogenous ligands has recently been determined to have a profound impact on the clinical response to chemotherapy and radiation therapy (Apetoh et al. 2007). Treatment of solid tumours often includes chemotherapy and/or radiation therapy. The efficacy of these therapies has been attributed to their direct elimination of tumour cells. However, an adaptive immune response stemming from these destructive therapies was determined to affect clinical outcome. In mice and humans, dying tumour cells treated with chemotherapy or radiotherapy release the nuclear protein HMGB1, which activates the TLR4–MyD88 pathway in DCs leading to tumour antigen-specific T-cell immunity. The activation of TLR4 by HMGB1 is essential for efficient cross-presentation of tumour antigens and effective antitumour response. Further, a cohort of breast cancer patients carrying a sequence polymorphism of TLR4, which is hypo-responsive to LPS, was found to relapse more quickly after chemotherapy or radiation therapy than the wild-type allele (Apetoh et al. 2007).

Various TLR ligands are in clinical trials for the treatment of cancer, infections, and autoimmune diseases. Currently, CpG-based oligonucleotides that target TLR9 are in clinical trials for the treatment of non-small cell lung cancer (Krieg 2008). Other TLR9 ligands are in clinical trials for the treatment of non-Hodgkin's lymphoma as well as prostate and colorectal cancer (Hennessy et al. 2010). Specific to the effect of TLR signaling in TAMs, injection of M13 bacteriophage into an ectopic tumour model of melanoma resulted in a MyD88-dependent shift from the alternatively activated M2 phenotype to the classically activated M1 phenotype. This shift to the M1 phenotype correlated with an increase production of proinflammatory cytokines and an influx of neutrophils into the tumour (Eriksson et al. 2009).

TLR Signaling Promotes Tumourigenesis

In contrast to the relatively protective effects resulting from TLR signaling described above, numerous studies have revealed that TLR signaling can conversely lead to tumour progression and metastasis. In a murine model of breast cancer metastasis, 4T1 mammary adenocarcinoma cells injected intravenously resulted in increased angiogenesis, vascular permeability, and tumour cell invasion in the group receiving

intraperitoneal LPS compared with the control groups (Harmey et al. 2002). Analogously, in a murine model of colon carcinoma metastasis, CT26 colon adenocarcinoma cells injected intravenously developed an increased number of tumour nodules and lung weights in the group receiving intraperitoneal LPS than the control mice. In this case, TLR-signaling was dependent on a supportive cell type such as a TAM. This is likely because NF- κ B signaling within the tumour cells which was found critical to the anti-apoptotic effects was observed in the wild-type but not the TLR4 mutant mice (Luo et al. 2004).

Whereas the initial studies on the tumour-promoting effects of TLR signaling used isolated TLR ligands, more recent studies have studied the effect of bacterial infection on tumourigenesis. The clearest example of how bacterial infection can promote tumourigenesis is the association of chronic infection with *Helicobacter pylori* and the development of gastric cancer. To study the role of infection on tumour progression, *Listeria monocytogenes*, a Gram-positive facultative intracellular bacterium, was injected into ectopically implanted H22 hepatocellular carcinoma cells. The *Listeria* survived in larger but not smaller tumours. Further, *Listeria* increased growth of these tumours, which could be abrogated with silencing of TLR2 but not TLR4 expression (Huang et al. 2007).

MyD88 signaling is crucial for tumour progression in both spontaneous $Apc^{min/+}$ model and the azoxymethane chemical carcinogenesis model of intestinal tumourigenesis. In the first model, $Apc^{min/+}$ mice lack one copy of the adenomatous polyposis coli (APC) tumour suppressor gene. Therefore, upon loss of the working copy of the gene, development of a focus of atypical epithelia begins. $Apc^{min/+}$ MyD88^{-/-} mice had fewer and smaller polyps than $Apc^{min/+}$ mice; however, the number of microadenomas was not different between the $Apc^{min/+}$ MyD88^{-/-} mice and the $Apc^{min/+}$ mice. Similarly, in azoxymethane-induced carcinogenesis, the incidence of tumour formation was decreased in the MyD88^{-/-} mice. Therefore, MyD88 signaling is essential for tumour progression but not initiation (Rakoff-Nahoum and Medzhitov 2007).

TAMs are associated with a worse clinical outcome in a majority of tumour types. Yet, few tumour-derived TLR ligands that activate TAMs to promote tumour progression and metastasis have been identified and characterized. One clear example was the product of a biochemical screen of macrophage-activating factors secreted by tumours. The extracellular matrix protein versican produced by Lewis lung carcinoma cells induced the secretion of IL-6 and TNF α by macrophages through TLR2/TLR6 signaling. Further, direct binding of TLR2 to versican was demonstrated. Abrogation of signaling through TLR2/TLR6 and MyD88 resulted in increased survival and fewer metastases in a murine model of metastatic lung cancer (Kim et al. 2009).

Conclusions

Tumour progression has long been associated with inflammation. In particular, increased macrophage density in solid tumours as well as lymphomas has been associated with a poorer prognosis. Inherent to cancer is the loss of normal tissue

architecture, which may provide signals or perpetual tissue stress and damage. Cancers have been compared with wounds that do not heal (Dvorak 1986) and they both have significant overlap in their gene expression signatures (Chang et al. 2004). Among the signals for tissue damage are endogenous TLR ligands. As macrophages have the physiologic role of restoring tissue integrity and homeostasis after tissue damage, cancers exploit them as a rich source of growth factors and angiogenic factors (Pollard 2009). Future therapeutic opportunities to modulate the progression of cancer will come from understanding the signals produced by tumours, and the pathways they educate macrophages into trophic TAMs. In addition, early studies suggest that TLR signaling may enable the production of an acquired immune response and, therefore, a more durable remission after treatment. Dissecting and modulating both the trophic and acquired immune responses from TLR signaling will be challenging but will have great therapeutic promise.

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