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Immunotherapy in Urologic Malignancies: The Evolution and Future of Pattern Recognition Receptors

Jane Lee and Arnold I. Chin
University of California, Los Angeles, USA

1. Introduction

Urologic malignancies, including prostate, bladder, and kidney cancer, have been in the forefront in the use of immunotherapies. However, the tight link between inflammation and cancer can lead to both pro-tumorigenic and anti-tumorigenic effects. Elucidating the crosstalk between immune and cancer cells of the tumor microenvironment will enhance our ability to manipulate the immune system towards generation of an anti-tumor response. Over the last decade, the discovery of pattern recognition receptors of innate immunity has revolutionized the understanding of host-pathogen interactions and shed new light on the mechanisms of existing immunotherapies. In this chapter, we will discuss the role of inflammation in cancer, highlight the current status of immunotherapies in urologic malignancies, review the evolution of pattern recognition receptors, and discuss strategies in harnessing pattern recognition receptors to develop novel therapies.

2. Dual nature of inflammation in cancer

The initial observation associating leukocytes with tumor cells by Rudolf Virchow in 1863 marked the link between inflammation and cancer. Since then, inflammation has been shown to play distinct roles during tumor initiation, promotion, and metastasis. While growing evidence demonstrates the ability of chronic inflammation to initiate tumors, other examples support a role of tumor immune surveillance in cancer elimination. Perhaps the role of inflammation in cancer is analogous to a balance, with scales on opposite sides tightly interdependent. The challenge remains in skewing these inflammatory responses to tip the balance towards an anti-tumor response (Figure 1).

Arguably the cornerstone of anti-tumor immunity rests on the concept of immune surveillance, proposed by Sir Macfarlane Burnet and Lewis Thomas in 1957, whereby the immune system surveys, recognizes, and eliminates developing tumors. Tumor surveillance necessitates recognition of tumor antigens or "altered" self-antigens, and gained acceptance as new models emerged in the field of immunology. This included pre-clinical studies

demonstrating tumor sensitivity to IFNγ treatment *in vivo* and increased carcinogen–induced tumor formation in perforin-deficient mice (Dighe et al., 1994; Russell and Ley, 2002). With the development of mice deficient in recombination activating gene 2 (Rag2), a gene essential in rearrangement and recombination of immunoglobulins and the T cell receptor, more convincing evidence revealed increased spontaneous development of tumors (Shankaran et al., 2001). Indeed, immunocompromised humans have increased risks of developing cancers including those of the bladder, kidney, colon, lung, non-Hodgkin's lymphoma, and melanoma (Dunn et al., 2002). More recently, the concept of tumor surveillance has been modified to incorporate a broader context of immunoediting, which not only encompasses the ability to recognize and eliminate tumors, but also suggests that immunogenicity of tumors can be shaped during tumor development, requiring constant interaction and modulation with the immune system. This was based on studies showing that tumors formed in an immunodeficient host were more immunogenic than tumors from an immunocompotent host. In these series of experiments, increased rejection of tumors generated from Rag2-deficient mice occurred when transplanted into immunocompetent hosts, but not Rag2-deficient hosts, while tumors derived from immunocompetent hosts grew similarly both in immunocompetent and Rag2-deficient hosts (Shankaran et al., 2001).

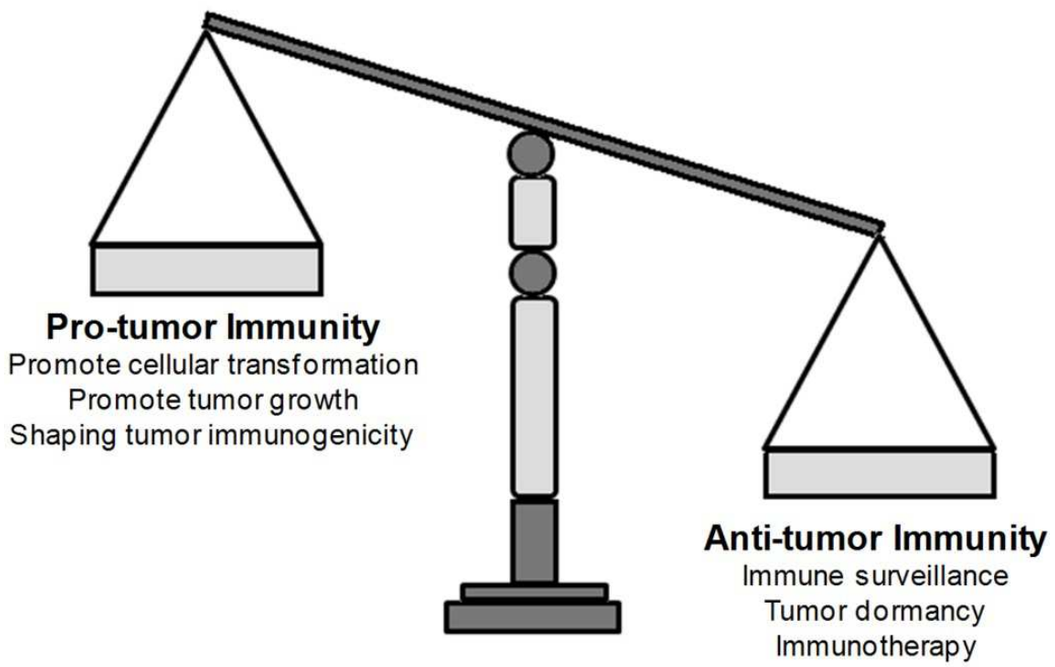

Fig. 1. Balance of Inflammatory Responses.

Interestingly, activation of the immune system to treat cancer predates the understanding of modern immunology and tumor surveillance. Together with reports since the 17th century describing regression of tumors following attacks of erysipelas, the origins of immunotherapy stems from the work of Freidrich Fehleisen in the late 1800's, who inoculated patients with sarcoma using the bacteria causing erysipelas, *Streptococcus*

pyogenes. William Coley, the "father of immunotherapy," began treating cancer patients with inoculation combining *Streptococcus pyogenes* and *Serratia marcesens*. In many instances, injection of the live bacteria induced complete regression of tumors. The use of Coley's toxin continued from 1893 to 1963, largely until the advent of radiotherapy and chemotherapy. In 1943, isolation of lipopolysaccharide as the active component of Coley's toxin and more recently, identification of Toll-like receptor (TLR) 4 as the receptor for lipopolysaccharide, defined the molecular basis for this cancer regression. These findings marked the resurgence in the use of pathogens and pathogen-based components in cancer therapy (Rakoff-Nahoum and Medzhitov, 2009).

However, certain types of inflammation can promote deleterious effects. Although the typical immune response is self-limiting, persistent activation of the immune system may lead to a condition of chronic inflammation (Naugler et al., 2007). Loss of epithelial barrier function with resulting tissue destruction allows the entrance of pathogens and the recruitment of inflammatory cells and mediators. Combined with the persistence of inflammatory signals and the absence of factors that normally mediate resolution of the acute response, it is postulated that chronic inflammation ensues. Chronic inflammation defines many human conditions including chronic gastritis, hepatitis, and atherosclerosis. An epidemiologic association exists between several inflammatory diseases and an increased risk for malignant transformation. Furthermore, infection with a specific pathogen predisposes to the inflammatory disease, suggesting a causative link from pathogen to chronic inflammation to the initiation of cancer. The most clearly defined example is infection with *Helicobacter pylori* resulting in chronic gastritis, peptic ulcer disease, and ultimately gastric carcinoma. In addition, this association is found in the development of hepatitis, cirrhosis, and hepatocellular carcinoma following infection by the hepatitis B and C viruses, in Burkitt's lymphoma in parts of Africa and nasopharyngeal carcinoma in Southeast Asia with Epstein Barr virus, and in the development of cervical carcinoma following infection with certain types of the human papilloma virus. However, the majority of individuals infected with these pathogens do not develop clinical disease, much less the corresponding cancer.

In later stages of cancer, solid malignancies can develop necrotic centers as they outgrow their blood supply, releasing inflammatory mediators such as IL-1 and intracellular components such as heat shock proteins and high-mobility group protein B1 (HMG-B1) (Vakkila and Lotze, 2004). These factors activate recruitment of inflammatory cells such as tumor associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) that facilitate angiogenesis to sustain tumor growth, leading to a cascade of cytokines and chemokines such as TGFβ. In some instances, these inflammatory responses may influence epithelial-to-mesenchymal transition and development of tumor invasion and metastases, while in others, inflammation associated with radiation or chemotherapy may augment anti-tumor immunity (Ghiringhelli et al., 2009; Grivennikov et al., 2010).

The dichotomy between anti- and pro-tumor inflammation may be dictated by the type, location, and timing of the inflammatory response. This may elucidate why certain patients respond to immunotherapy and others do not. Dissecting the composition of the cells within the tumor microenvironment, the cytokines and chemokines involved in autocrine and paracrine signaling cascades, and understanding its molecular mechanisms will be central in

understanding the paradigm on how inflammation influences tumorigenesis. The discovery of Toll-like receptors has provided insight into a molecular basis for antigen recognition and modulation of innate and adaptive immunity, but as you will see, has only widened the dualistic understanding of inflammation and cancer.

3. Components of the tumor microenvironment

The tumor microenvironment consists of a complex milieu of stromal and inflammatory cells, soluble factors, and extracellular matrix, intertwined with tumor cells. Identifying and understanding the regulation of the tumor microenvironment will be critical in designing therapies to inhibit tumor growth and invasion.

3.1 Stroma

The stromal components of the tumor microenvironment include fibroblasts, endothelial cells, and pericytes. Cancer associated fibroblasts (CAFs) provide growth factors, chemokines, and metalloproteinases essential for cellular communication during cancer proliferation and invasion (Bhowmick et al., 2004; Sato et al., 2009). Endothelial cells and pericytes deliver nutrients and oxygen to the cancer cells, allowing their continued growth and survival. The stromal cells along with the extracellular matrix present not only a physical barrier for tumor invasion and metastases, but also a lymphatic and vascular barrier to cancer-specific antibodies preventing immunoconjugates from reaching tumor cells (Yasunaga et al., 2011).

3.2 Inflammatory cells

Tumor-associated macrophages (TAMs) constitute the majority of infiltrating cells in the microenvironment (Jinushi et al., 2011). TAMs are classified into M1 and M2 types similar to Th1 and Th2 $CD4^+$ T cells, with M1 macrophages favoring pathogen elimination and M2 macrophages associated with angiogenesis and tissue remodeling (Balkwill and Mantovani, 2001). The most potent of antigen-presenting cells, dentritic cells (DCs), process and present antigens on their surface in context with major histocompatibilty complex class I (MHC) and class II molecules, to interact with $CD8^+$ T lymphocytes and $CD4^+$ T helper cells respectively. These are divided into myeloid DCs and plasmacytoid DCs, characterized by production of type I interferons. Natural killer cells (NKs) of innate immunity eradicate cells by inducing cytotoxicity through the release of perforin and granzyme that target the cell to destruction by apoptosis, while NKT cells share similarities with T cells, with recognition of lipid and glycolipid antigens. A subset of early myeloid cells termed myeloid derived suppressor cells (MDSCs) has the ability to suppress NK, NKT, and T cell responses, marked by production of L-arginine and upregulation of nitric oxide synthase 2 (Dolcetti et al., 2008).

Tumor infiltrating lymphocytes (TILs) represent the adaptive arm of immunity and include cytotoxic $CD8^+$ T cells (CTLs), B lymphocytes, and $CD4^+$ T helper cells, including Th1, Th2, and Th17 cells typically associated with autoimmunity. T regulatory (Treg) cells, characterized by the expression of the forkhead box P3 transcription factor (Foxp3), along with MDSCs, may play an important role in immune tolerance, regulating the immunosuppressive environment of cancer and posing as a barrier to successful immunotherapy.

3.3 Cytokines and chemokines

Cytokines and chemokines provide autocrine and paracrine signaling and play a critical role in shaping the tumor microenvironment. These include cytokines that favor development of anti-tumor immunity include IL-12, IFNα, and IFNγ, and those that enhance immune suppression such as IL-10, IL-17, and TGFβ or tumor progression such as IL-1 or IL6 (Grivennikov et al., 2010). Chemokines of the CC and CXC family secreted by tumors and infiltrating leukocytes, recruit inflammatory cells to the tumor microenvironment. This network of cytokines and chemokines plays an active role in regulating communication between the tumor, stroma, and inflammatory cells. Together, they have shown to influence tumor survival, growth, and epithelial-to-mesenchymal transition (EMT).

4. Immunotherapy in urologic malignancies

The incidence of urologic malignancies with bladder, kidney, and prostate cancer comprise almost 40% of cancer in men and almost 23% of all cancers in the United States, according to statistics provided by the 2010 American Cancer Society. Remarkably, in each of these malignancies, a Food and Drug Administration (FDA) approved immunotherapy exists (Figure 2). The following section briefly discusses the approved therapies and the strategies utilized.

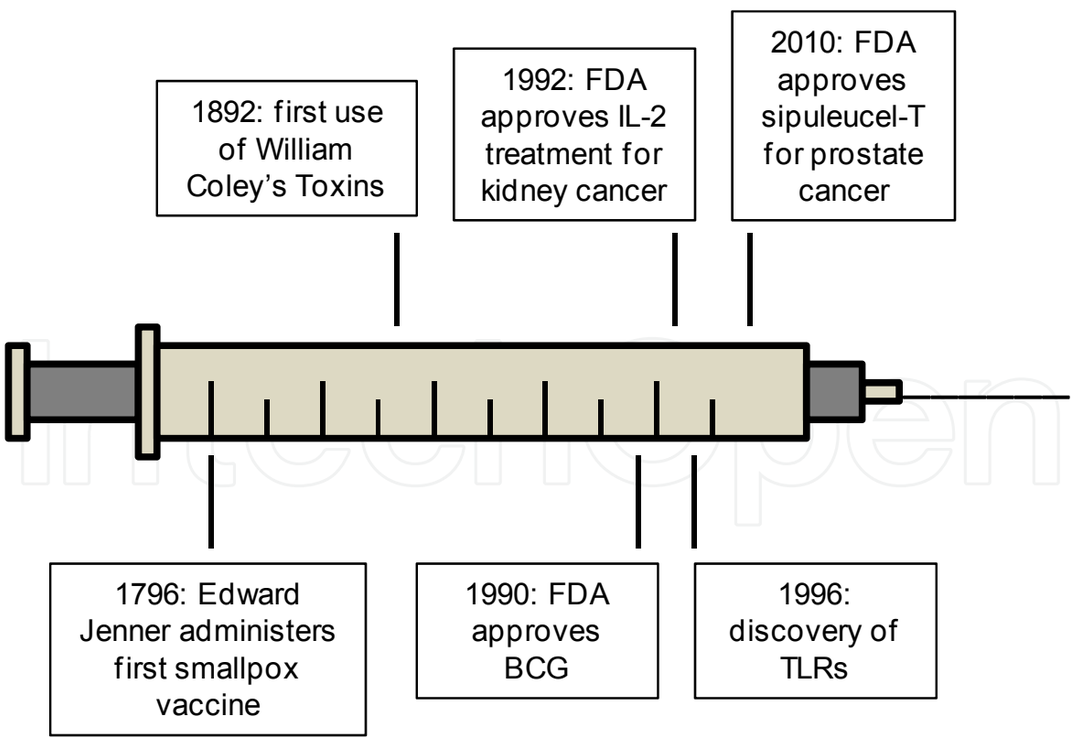

Fig. 2. Use of Immunotherapies in Bladder, Kidney, and Prostate Cancer.

4.1 Bladder cancer

Bladder cancer incidence ranks the 4th and 9th most prevalent in men and women, respectively, in the United States. Since its first therapeutic instillation in the bladder by Jean B. deKernion in 1975 for melanoma and later by Alvaro Morales for urothelial cancer, intravescical instillation of *bacillus calmette-guerin (BCG)*, an attenuated strain of *Mycobacterium bovis*, has demonstrated to be more effective than chemotherapy and is the standard intravesical treatment for non-muscle invasive bladder cancer and carcinoma *in situ*, garnering FDA approval in 1990. In the landmark trial, *BCG* administration in nine patients with a history of recurrent urothelial carcinomas reduced recurrences from a pre-treatment rate of 22 recurrences amongst the nine patients within 77 months, to just one during 41 months following therapy (Morales et al., 1976). *BCG* immunotherapy induces a local inflammatory response recruiting macrophages, DCs, T cells, NK cells, and neutrophils (Saint et al., 2001). Elevated cytokines including IL-6, IL-10, IL-12, IFNγ, and TNFα have been reported in patients following intravesical *BCG* (de Reijke et al., 1996). *BCG* can bind fibronectin on urothelial cells and more recently has been shown to mediate its effector functions through activation of TLR2 and TLR4 (Rakoff-Nahoum and Medzhitov, 2009; Ratliff, 1991; Tsuji et al., 2000).

BCG treatment can lead to significant morbidity including debilitating arthritis or sepsis. Efforts to increase its efficacy and decrease toxicity led to co-administration of *BCG* with IFNα, first recognized as an effective intravesical treatment in 1988 (Torti et al., 1988). Pre-clinical studies established a synergy between *BCG* and IFNα, with clinical trials demonstrating efficacy and safety using combinatorial administration of low-dose BCG and IFNα-2b with improved side effect profiles (Bazarbashi et al., 2011; Stricker et al., 1996; Torti et al., 1988). Currently, this combination has been used in *BCG* refractory patients with an additional 25% response rate (Gallagher et al., 2008).

4.2 Kidney cancer

As the 7th and 8th leading site of new cancer cases in men and women in the United States respectively, renal cell carcinoma (RCC) is relatively resistant to chemotherapy and radiotherapy. Reports of spontaneous regression following cytoreductive nephrectomy suggested an immunological basis of disease initiated from the primary tumor. The use of cytokine therapy has made important impacts in its treatment. This includes high dose IL-2, which garnered FDA approval in 1992 for metastatic RCC following a review of 225 patients in seven phase II trails, with complete responses occurring in 10%-20% of patients (Fyfe et al., 1995). IFNα, although currently not FDA approved for this indication, has shown efficacy for melanoma as well as for metastatic RCC. A landmark trial on the benefits of nephrectomy in 120 metastatic RCC patients undergoing IFNα -2b therapy revealed that IFNα with cytoreductive nephrectomy resulted in a median survival of 11.1 months over IFNα alone with a median survival of 8.1 months (Flanigan et al., 2001). IL-2 is a potent T cell activator, while IFNα induces T cell activation, upregulates MHC class I and II, and augments NK cells. In the age of targeted therapies to various tyrosine kinases, cytokine therapy remains the only curative therapy for metastatic RCC.

4.3 Prostate cancer

Prostate cancer remains the leading incidence of cancer in men, and the second highest cause of cancer death in men in the United States. Following hormone ablation for metastatic disease, patients inevitably develop castrate-resistant prostate cancer (CRPC), with options limited to systemic chemotherapy. The approval of the first in class cell-based vaccine for prostate cancer in 2010, sipuleucel-T, ended the search for an immunological treatment for prostate cancer that began decades earlier. Sipuleucel T combines *ex vivo* patient-derived DCs with a fusion of the tumor antigen prostatic-acid phosphatase and GM-CSF. In a phase III trial on 127 men with CRPC, median survival of those treated with sipuleucel-T was 25.9 months compared to 21.4 months for placebo, with generation of PAP-specific T cell immunity (Small et al., 2006).

5. Inflammation in urologic malignancies

An emerging theme in cancer is how the inflammatory composition of the tumor microenvironment influences cancer prognosis and overall patient survival. This has been demonstrated in breast cancer, where the ratio of $CD68^+$ macrophages to $CD8^+$ T cells, $CD4^+$ to $CD8^+$ T cells, or Th2 to Th1 $CD4^+$ T cells have all independently correlated with survival (Kohrt et al., 2005). In colon cancer, infiltration of $CD8^+$ T cells, CD45RO, and $Foxp3^+$ Tregs predicts overall survival better than grade and stage (Galon et al., 2006; Salama et al., 2009).

In human bladder cancer patients, elevated numbers of $CD8^+$ T cells in TILs have predicted greater disease-free and overall survival (Sharma et al., 2007). However, negative regulators have been linked with more aggressive cancers, including $CD4^+CD25^+Foxp3^+$ Tregs and cytokines important in their development such as TGFβ (Loskog et al., 2007). These suppressive effects may lead to T cell anergy and ineffective cytotoxic responses, questioning the functionality of infiltrating $CD8^+$ T cells. A similar observation exists in kidney and prostate cancer. In advanced renal cell carcinoma patients, elevated levels of Tregs are present in peripheral blood, with IFNα treatment resulting in inhibition of both $CD4^+$ T lymphocytes and Tregs (Tatsugami et al., 2010). Increased circulating $CD4^+$ and $CD8^+$ Tregs have been linked in human prostate cancer, while a murine model demonstrated tolerization of $CD8^+$ T cells (Anderson et al., 2007; Kiniwa et al., 2007; Miller et al., 2006; Sfanos et al., 2008).

The balance in TILs towards a suppressive state suggests a major role of antigen tolerance in tumorigenesis. Current strategies aimed at targeting these negative regulatory populations include monoclonal antibody therapies against the CD28 family of co-receptors CTLA-4 or PD-1, with an anti-CTLA-4 monoclonal antibody ipilimumab recently approved by the FDA in 2011 (Mangsbo et al., 2010; May et al., 2011). The signals that program the composition of the tumor microenvironment and the ability to alter individual components to favor a cell-mediated anti-tumor immunity will be an important future direction.

6. Pattern recognition receptors

Charles Janeway first proposed the idea of germline-encoded pattern recognition receptors (PRRs) of innate immunity that recognized conserved motifs of microbial origin termed

pathogen-associated molecular patterns (PAMPs). These evolutionarily conserved receptors found throughout the animal kingdom activate the innate arm of immunity as well as direct adaptive immunity. Humans and microbes exist in direct interaction. In an environment with constant exposure to microbes, the host immune system is challenged to discern between benign flora and potential pathogens, and to initiate an appropriate immune response. The innate immune response initiated immediately upon pathogen entry mediates components such as macrophages, neutrophils, NK cells, alternative complement proteins, and other anti-microbial molecules. Recognition of pathogens in innate immunity utilizes germ line-encoded proteins, without the generation of lasting immunity. In addition to phagocytosis and killing of pathogens, innate immune cells synthesize and secrete a broad range of inflammatory mediators and cytokines that regulate systemic responses to infection, recruit additional white blood cells to sites of inflammation, and importantly, dictate the nature of the adaptive response. In contrast, the adaptive response, mediated by lymphocytes and their effector functions, requires several days to develop. Adaptive immunity has the ability to generate antigen-specific receptors in T cell receptors and immunoglobulins through somatic cell DNA rearrangement, and to elicit lasting immunity through development of memory cells.

The PRR superfamily now includes the family of Toll-like receptors (TLRs), cytosolic NOD-like receptors (NLRs) and RIG-I-like receptors, and membrane-bound C-type lectin receptors (CLRs) (Elinav et al., 2011; Kawai and Akira, 2011). In addition to host defense, PRRs may also play a major role in tissue repair and maintenance of tissue homeostasis, and emerging evidence suggests a role in cancer. In the following section, we will discuss the most well characterized family of Toll-like receptors and their role in tumor surveillance and cancer therapy.

6.1 Toll-like receptors signaling

TLRs are best defined in their host defense role through their ability to recognize PAMPs, leading to enhanced uptake of microorganisms, generation of reactive oxygen and nitrogen intermediates, and recruitment of leukocytes to the area of inflammation (Kawai and Akira, 2011; Modlin and Cheng, 2004). TLRs also shape the induction of adaptive immunity through activation of APCs by upregulation of co-stimulatory molecules CD80 and CD86. Currently, 10 human and 12 murine TLRs have been identified with PAMPs ranging from lipopolysaccharide (LPS) found in gram-negative bacterial walls recognized by TLR4, peptidoglycan and lipoprotein from gram-positive bacteria specific to TLR2 in conjunction with TLR1 or TLR6, double stranded RNA produced by many viruses for TLR3, single stranded RNA by TLR7 and TLR8, unmethylated CpG motifs with TLR9, and flagellin for TLR5 (Table 1). More recently, endogenous ligands termed danger-associated molecular patterns (DAMPs), including heat-shock proteins, the chromatin component HMG-B1, surfactant, protein A, fibronectin, heparan sulfate, fibrinogen, hyaluronan, and other components of injured cells, have also been identified suggesting a role for this receptor family in inflammatory responses resulting from tissue damage, such as lung injury or ischemic-reperfusion injury, or during tumor growth and necrosis (Rakoff-Nahoum and Medzhitov, 2009).

TLRs contain multiple leucine-rich repeats in the extracellular domain, and an intracellular Toll/IL-1R/Resistance (TIR) domain conserved in all TLRs (Kawai and Akira, 2011).

Proximally, the TIR interacts with other TIR domain adaptor proteins including recruitment of myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor protein (TIRAP/Mal), which initiate a signaling cascade to the serine kinase IL-1R-associated kinase (IRAK) to tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6), activating transforming growth factor-β-activated protein kinase 1 (TAK1). This results in activation of downstream transcription factors including NF-κB, MAP kinases, Jun N-terminal kinases, p38, ERK, and interferon regulator factors (Modlin and Cheng, 2004).

Toll-like receptor	Ligand(s)	Localization
TLR-1	Lipoprotein - bacteria	Membrane
TLR-2	Lipoprotein - bacteria; Heat-shock protein 70 - endogenous	Membrane
TLR-3	Double-stranded RNA - virus	Endosome
TLR-4	Lipopolysaccharide - gram-negative bacteria; Heat-shock protein 60/70 - endogenous	Membrane
TLR-5	Flagellin - bacteria	Membrane
TLR-6	Lipoprotein - bacteria	Membrane
TLR-7	Single-strand RNA - virus	Endosome
TLR-8	Single-strand RNA - virus	Endosome
TLR-9	CpG-containing DNA - bacteria and virus	Endosome
TLR-10	Unknown	Membrane
TLR-11	Urogenic bacteria	Membrane

Table 1. Human Toll-like Receptors and Known Ligands (So and Ouchi, 2010).

Although most TLRs utilize the MyD88 pathway, TLR3 and TLR4 interact with the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) also known as Toll-like receptor adaptor molecule 1 (TICAM-1) to activate a MyD88-independent pathway leading to IRF3 activation and production of type I interferons. TLR3 has been implicated in NK cell activation, and while MyD88-dependent pathways largely regulate CTL induction, NK activation requires MyD88-independent pathways (Akazawa et al., 2007; Alexopoulou et al., 2001; Guerra et al., 2008).

6.2 Toll-like receptors in activation and regulation of inflammatory responses

Predominantly expressed on innate immune cells such as macrophages, DCs, and plasmacytoid DCs, recognition of PAMPS by TLRs leads to activation of transcription

factors leading to production of inflammatory target genes such as cell cycle regulator genes c-myc and cyclin D1, cell survival genes bcl-xL, angiogenesis factors including VEGF, inflammatory cytokines such as IL-1, IL-6, and IL-8, type I interferons, chemokines, and T cell co-stimulatory molecules. These signals are crucial elements in the coordination of the host innate immune responses leading to recruitment of neutrophils, natural killer cells, and induction of antimicrobial peptides, resulting in killing of pathogens. Activation of TLRs ultimately dictate the nature of adaptive responses through dendritic cell maturation and the development of CTLs (Modlin and Cheng, 2004).

While stimulation of TLRs induces robust inflammatory pathways, negative regulatory mechanisms exist to balance immune activation to prevent chronic inflammation and autoimmunity. This includes decoy receptors, intracellular or transmembrane regulators, control of TLR expression, or caspase-dependent apoptosis of TLR-expressing cells (Kobayashi et al., 2002; Liew et al., 2005; Liu and Zhao, 2007). Activation of suppressor pathways through induction of cytokines IL-10, IL-27, and cells such as Tregs or MDSCs, may pose a significant barrier in antigen tolerance during tumor surveillance, reflected by increased numbers of suppressor cells in cancer patients (van Maren et al., 2008). Several lines of evidence support a critical role of TLRs in manipulating these suppressor cell populations. Multiple TLRs, including TLR2, TLR4, and TLR8 are expressed on the surface of Tregs, and may have a direct regulatory role with suppression of human prostate tumor infiltrating $CD8^+$ Treg cells following activation of TLR8 (Liu and Zhao, 2007). TLR9 activation has been shown to inhibit Tregs through IL-6 produced by DCs, although reports also show a TLR9-mediated induction of IL-10 and thus activation of Tregs (Jarnicki et al., 2008; Pasare and Medzhitov, 2003). In an autochthonous prostate cancer model, TLR3 activation increased infiltration of tumor infiltrating T and NK cells, and suppressed splenic Tregs, suggesting the ability of TLR activation to selectively modify the tumor microenvironment (Chin et al., 2010). The relationship between TLRs and MDSCs is less clear, but a recent study showed that TLR9 activation may inhibit MDSCs in a murine model (Ostrand-Rosenberg and Sinha, 2009; Peng et al., 2005; Zoglmeier et al., 2011). Collectively, these studies suggest that selective activation of TLRs may not only increase tumor infiltration of cytotoxic T and NK cells, but may also inhibit specific types of suppressor populations.

6.3 Toll-like receptors on tumor cells

In addition to immune cells, a broad variety of epithelial cells including colon, ovarian, bladder, kidney, and prostate express various TLRs. Although the endogenous role of TLRs on epithelial cells is unclear, it may stem from regulation of tissue growth and repair. Activation of TLRs in various tumor lines and models has shown both evidence of tumor reduction and cancer progression (Maruyama et al., 2011). In prostate and kidney cancer cell lines, TLR3 activation has been shown to induce apoptosis, while TLR9 has been shown to promote prostate cancer invasion, and IL-8 and TGFβ production *in vitro* (Di et al., 2009; Ilvesaro et al., 2007; Paone et al., 2008; Taura et al., 2010). In bladder cancer lines, elevated expression of TLR2-4, 5, 7, and 9 was detected in non-muscle invasive tumors, with decreased expression in muscle invasive tumors (Ayari et al., 2011).

The role of TLRs on epithelial cells needs to be clarified. What is the impact of TLR expression on epithelial cells during tumor initiation, growth, and response to

immunotherapies? In human population studies, a sequence variant in a 3'-untranslated region of TLR4 as well as polymorphisms in the TLR gene cluster encoding TLR1, 6 and 10, and the downstream signaling mediator IRAK1 and IRAK4 confer increased prostate cancer risk (Lindstrom et al., 2010). However, the contribution of these TLR signaling components is unclear. In order to distinguish the role of TLRs on epithelial cells versus stromal or immune cells, tissue specific models will need to be examined.

6.4 Toll-like receptors in immune surveillance

The evidence of TLRs in mediating immune surveillance is based on tumor growth in knockout models of TLRs and their signaling adaptors, with studies supporting tumor promoting as well as suppressing effects. Exogenous administration of TLR ligands may not truly demonstrate a role of tumor surveillance and may enhance host immunity above physiologic levels. In support of a role of TLRs in tumor surveillance, mice deficient in TLR3 and TLR9 show increased growth of subcutaneously implanted prostate cancer, while deficiency in the negative regulatory adaptor molecule IRAK-M impairs growth of implanted tumor cells (Chin et al., 2010; Xie et al., 2007). Supporting a role in tumorigenesis, MyD88 mediates tumor initiation in a mouse model of spontaneous intestinal tumorigenesis and diethylnitrosamine-induced hepatocellular tumors (Naugler et al., 2007; Rakoff-Nahoum and Medzhitov, 2007; Xie et al., 2007). These opposing effects are confounded by the tumor origin, tumor model used, and potential contribution of TLRs on tumor cells, and further studies will need to explore this important issue.

7. Toll-like receptors in human immunotherapy

The role of TLRs in cancer therapy harnesses the exogenous use of synthetic TLR agonists to enhance host immunity. Despite pre-clinical evidence supporting anti-tumor responses as well as facilitating tumor promotion, the use of TLR agonists have a significant clinical importance and a promising future. Most clinical trial designs focus on the adjuvant properties of TLRs, predominantly by stimulating APCs through upregulation of co-stimulatory molecules such as CD80 and CD86 (Medzhitov et al., 1997). In addition to activation of adaptive immunity, effector functions include increase recruitment of innate immune cells such as NK, NKT, γδT cells, modulating the cytokine milieu, and direct cytotoxicity of tumor cells (Figure 3). Overcoming immune suppression is a major obstacle for successful immunotherapy and TLR activation may suppress Tregs and MDSCs to break antigen tolerance in conjunction with activation of adaptive immunity (Pasare and Medzhitov, 2003). More recently, strategies have adopted the use of TLR agonists with tumor antigens for the development of cancer vaccines.

Freund's complete adjuvant (FCA) has been the most common adjuvant for antibody production, produced in a water-in-oil emulsion containing heat-killed mycobacterial cells (Stewart-Tull, 1996). TLR2 and TLR4 play a crucial role in the recognition of FCA, which has increased antibody responses crucial for delayed-type hypersensitivity reactions over Freund's incomplete adjuvant lacking mycobacteria (Azuma and Seya, 2001). *BCG* has been used for over three decades as intravesical therapy in bladder cancer and mediates its function through TLR2 and 4 pathways as well. Recent trials utilizing components of mycobacterial cell walls rather than live bacteria may have similar efficacy while reducing toxicity (Chin et al., 1996). In fact, activation of TLRs using synthetic PAMPs reduces tumor

growth in pre-clinical models in bladder and prostate cancer. With the impact of IFNα in kidney cancer patients, it is likely that TLR activation may play an important role in kidney cancer in particular with TLRs that activate type I interferons such as TLR3.

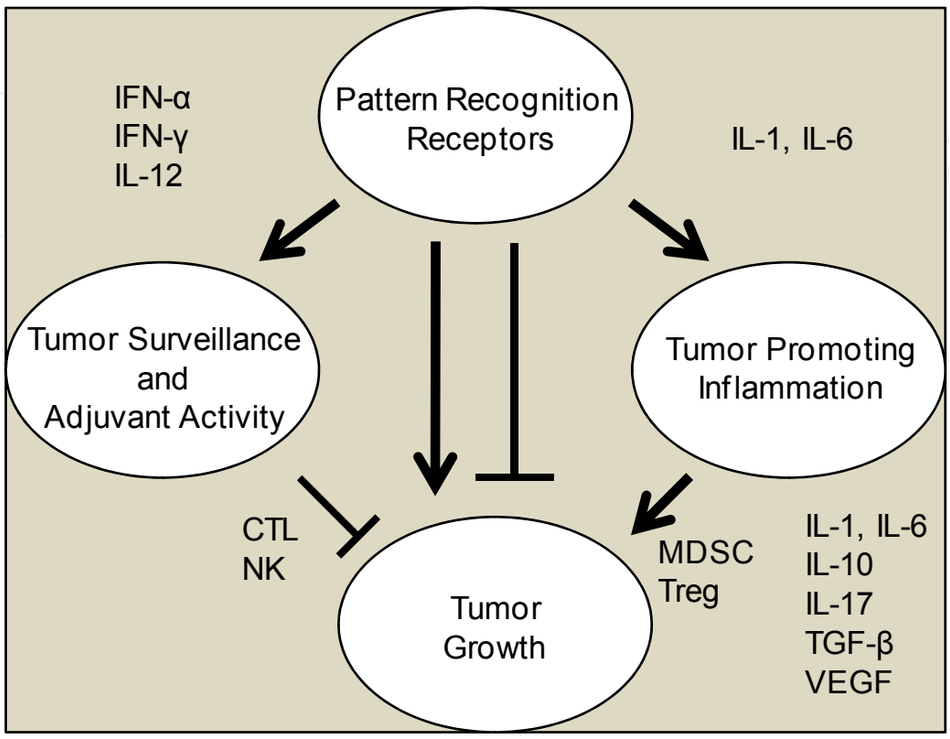

Fig. 3. The Direct and Indirect Influences of Pattern Recognition Receptors on Tumor Growth.

Although many TLRs share common signaling pathways, it is evident that ligation of different TLRs will induce unique gene expression profiles that translate to specific effector functions (Doyle et al., 2002). As supported by the wide variation in pre-clinical responses, the effector functions and resulting tumor response by TLR activation may change based on tumor type, location, dose, and timing. In the near future, perhaps activation of specific TLRs can be tailored to augment a desired tissue-specific effector function that partners with a particular vaccine.

To date, three TLR agonists have been used in clinical trials, all recognizing nucleic acids for receptors expressed on endosomal membrances. The only approved agonist, the single-stranded RNA analogue imiquimod specific for TLR7, showed activity in murine colon cancer and sarcoma models, inducing IFNγ and IL-12 to activate CTLs and myeloid DCs (Maruyama et al., 2011). Initially used clinically for actinic keratosis and genital warts, imiquimod has show activity against superficial basal cell carcinoma and received FDA approval in 2004.

Unmethylated CpG oligodeoxynucleotides (ODN) found in bacterial and viral DNA has been used in phase I-III trials against multiple malignancies including kidney, breast, melanoma, and lymphomas (Krieg, 2008). Ligands for TLR9 have been grouped into three

different classes based on their roles in activating the immune system. A-class CpG ODN (CpG-A) stimulate type I interferon production by plasmacytoid dendritic cells, activating natural killer cells and IFNγ (Krug et al., 2001); B-Class CpG ODNs (CpG-B) induce B cell and monocyte maturation, leading to B cell proliferation with little pDC activation; and C-Class CpG ODNs (CpG-C) mediate signaling pathways of both CpG-A and CpG-B (Rothenfusser et al., 2004). Although pre-clinical trials demonstrate that TLR9 activation potently induces Th1 responses, NK activation, stimulation of cytokines TNFα, IL-12, and IFNγ, and induces a strong $CD8^+$ T-cell response, clinical trials have not yielded robust results (Valmori et al., 2003). This may be in part due to different expression of TLR9 in murine models with broad expression in myeloid DCs, plasmacytoid DCs, macrophages, and B cells, with expression limited to pDCs and B cells in humans.

To address this disparity, combinatorial strategies attempt to enhance the activity of CpG ODN, with the addition of alum, emulsigen, and polyphosphazenes (Malyala et al., 2009). Other strategies include inhibition of p38 that may enhance T cell activation or through blockade of CTLA-4 or PD-1 (Mangsbo et al., 2010; Takauji et al., 2002). These combinatorial strategies will be increasingly important in promoting synergic responses to augment host immunity, while unhinging negative regulatory factors.

TLR3 ligand polyriboinosinic:polyribocytidylic acid (poly(I:C)), a synthetic analog of double-stranded RNA, has demonstrated to be a promising adjuvant for immunotherapy. Studies have reported poly(I:C) as an effective inducer of inflammatory cytokines, dendritic cells, and macrophages, leading to subsequent activation of natural killer cells. While poly(I:C) has proven effective in inhibiting tumor metastasis and prolonging survival in animal models, the drawback exists in its inability to efficiently penetrate the cell membrane in order to bind to its cognate receptor. The development of stabilized compounds, including polyICLC, has been used in phase II studies against gliomas (Butowski et al., 2009). A recent phase I trial against multiple malignancies including advanced bladder cancer utilized a novel vaccine approach combining a human chorionic gonadotropin-β antigen fusion protein with adjuvants poly ICLC and the TLR7/8 agonist resiquimod (Morse et al., 2011). This orchestration of TLR-based adjuvant activation with tumor antigen stimulation is promising and utilizes the ability of TLRs for cross antigen presentation, allowing extracellular antigens to be processed and presented by class I MHC (Oh and Kedl, 2010).

8. Therapeutic design and conclusion

Urologic malignancies comprise 23% of all cancers in the United States, excluding basal skin cancer. Immunotherapeutic approaches in urologic malignancies broadly encompass cytokine-based, bacteria-mediated, and cell-based vaccine therapies. This demonstrates the immunological sensitivity of urologic malignancies and opens avenues to develop novel strategies. Clearly the composition of inflammation in the tumor microenvironment influences tumor growth, metastases, and overall survival. Toll-like receptors play important roles in host defense against pathogens, and tissue homeostasis and repair in response to tissue damage. Mounting evidence suggests that TLRs can recognize endogenous antigens released from tumors and mediate tumor immune surveillance. Furthermore, exogenous activation of TLRs can alter the tumor microenvironment and

induce adaptive immunity, influencing the response not only to immunotherapies, but also potentially to radiation, chemotherapy, and targeted therapies.

Understanding the specificities of various TLRs will be critical, as will be determining the timing of agonist stimulation, dose, and tissue specificity. Exploring the potential of other PRR families in cancer is clearly an open field. Similarly, challenges in modulating immunity to prevent antigen tolerance or an inappropriate response will need to be addressed. By incorporating activation of distinct PRR and PRR signaling pathways, specific components of the tumor microenvironment may be modulated to augment cell-mediated immunity. Combining the ability of PRRs to regulate suppressor cells, novel vaccine strategies may overcome antigen tolerance. At the same time, caution needs to be exercised to understand direct PRR effects on tumors and development of pro-tumorigenic immunity.

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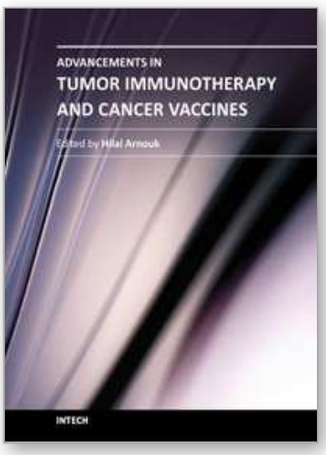

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Harnessing the potential of the human body's own immune system to attack malignant tumor cells has been the goal of many scientific investigators in recent years, with advances in cancer biology and immunology enabling cancer immunotherapy to become a reality. World-class bench and clinical researchers have joined forces to collaborate and review current developments and trends in cancer immunology for the purposes of this book, and the result is a promising review of contemporary clinical treatments. In each chapter the authors present the scientific basis behind such therapeutic approaches, including cancer vaccines with special focus on prostate cancer, melanoma and novel approaches utilizing both innate and adaptive immune responses.

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Phone: +385 (51) 770 447
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