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Innate Immunity-Based Immunotherapy of Cancer

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1. Introduction

The immune system protects against invading pathogens and transformed cells, including cancer. Mammalian immune system is divided into two major categories, i.e., innate and adaptive immunity. Innate immunity consists of cellular and biochemical defense mechanisms that respond in the early phase after harmful events, such as encounters with microbes or transformed cells. The cellular components of innate immunity include dendritic cells (DCs), macrophages and monocytes, polynuclear cells (e.g. neutrophils and mast cells), natural killer (NK) cells, $\gamma\delta$ T cells and natural killer T (NKT) cells. Adaptive immunity consists of T and B lymphocytes and their humoral mediators, including cytokines and antibodies, and achieves excellent antigen specificity by somatic rearrangement of the antigen receptor genes of each lymphocyte lineage; T cell receptor for T lymphocytes and immunoglobulin for B lymphocytes, respectively. Furthermore, another excellent characteristic of adaptive immunity is a “memory system” to maintain antigen-specific lymphocytes in a functionally quiescent or slowly cycling state for many years. The memory system enables host organisms to respond to the second and subsequent exposure to the same or related antigens in a more rapid and effective manner.

Dr. William Coley, an American bone surgeon, is credited with pioneering work in the field of cancer immunotherapy. In the 1890s, he noted that patients with sarcoma who developed bacterial infections after surgery had visible regression of their cancer. At first, Dr. Coley injected live cultures of streptococcus to produce erysipelas in cancer patients and assessed their responses (Bickels et al., 2002; MacCarthy, 2006). He found that the antitumor effects depended upon the toxins of the bacteria, and eventually developed mixed toxins of a Gram-positive and -negative bacterium called Coley toxins (also called “mixed bacterial vaccines”). A compilation of Dr. Coley's clinical observations indicated that in certain tumor

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types, such as soft tissue sarcoma and lymphoma, the response is marked even by today's standards (Tsung & Norton, 2006). Following the pioneering works by Dr. Coley and his daughter, Dr. Helen Coley Nauts, efforts to treat cancer based on the function of the immune system, i.e., immunotherapy, have continued. As with Coley's toxins, the innate immune response against microbes could provoke anti-tumor effects as a secondary response. Although the exact molecular mechanisms of innate immune cells to recognize the components of microorganisms have not been fully understood, the substances such as bacteria-derived materials which evoke tumor immunity have been categorized as 'biological response modifier (BRM)'.

In Japan, several original BRMs have been developed since 1950's. Dr Chisato Maruyama noticed that there were few cancer patients in sanatoriums for tuberculosis or Hansen's disease, and he started research to apply extracts from *Mycobacterium tuberculosis* to cancer treatment. His preparation, named Specific Substance MARUYAMA (SSM, also called the "MARUYAMA vaccine") (Suzuki et al., 1986a; Suzuki et al., 1986b; Sasaki et al., 1990) was composed of deproteinized extracts, and contained lipoarabinomannan, a kind of polysaccharide, as the main component. SSM received much attention from the public as a miracle drug for cancer treatment before being approved by the Ministry of Health and Welfare of Japan at that time. SSM has not been approved to date as a anti-neoplastic drug, but its related preparation, referred to as Z-100 or Ancer, has been approved since 1991 as a drug for radiotherapy associated leucopenia. Two other BRM drugs, krestin (PS-K, polysaccharide-protein complexes extracted from basidiomycetes, *Trametes versicolor*) (Akiyama et al., 1977; Mizushima et al., 1982) and picibanil (OK-432, a lyophilized preparation of attenuated group A *Streptococcus haemolyticus*) (Kai et al., 1979; Kataoka et al., 1979) were approved by the Ministry of Health and Welfare of Japan as anti-neoplastic drugs in 1975. Another example of Japanese BRMs is BCG-CWS, a cell wall skeleton preparation of *Mycobacterium bovis* bacillus Calmette-Guérin, a tuberculosis vaccine strain which is almost nonpathogenic yet retains the immunogenic properties of tuberculosis (Tsuji et al., 2000). BCG-CWS contains a peptidoglycan that is covalently linked to arabinogalactan and mycolic acids (Azuma et al., 1974). Although BCG-CWS has been clinically used for a long time (Hayashi et al., 2009; Kodama et al., 2009), it has not been approved by the Ministry of Health and Welfare of Japan.

One of the characteristics of classical BRMs is that they are crude products which are not fully purified. Their multiple components seem to be important for the induction of efficient anti-tumor effects as seen with Coley toxins.

2. Stimulators of innate immunity

2.1 Toll-like receptors (TLRs)

The cells of the innate immune system recognize infectious agents by receptors for characteristic components of pathogenic microorganisms. The structures of these components are highly conserved and are called pathogen-associated molecular patterns (PAMPs). The receptors for PAMPs, referred to as pattern recognition receptors (PRRs), are germline-encoded and highly conserved across species. The innate immune system detects various classes of pathogens and abnormal cells through PRRs, and serves as a first line of defense against microbes.

Toll, a gene involving to the establishment of dorsal-ventral pattern of *Drosophila* embryo (Hashimoto et al., 1988), other function in immune response against fungus in *Drosophila* adults (Lemaitre et al., 1996). In 1997, a cloning of human homologue of *Drosophila* Toll and its function as an antigen receptor was reported (Medzhitov et al., 1997). At present, 10 molecules have been identified in humans and are referred to as Toll-like receptors (TLRs) (Takeda et al., 2003; Iwasaki & Medzhitov, 2004). The identification of TLRs in mammalian species could give us clues to understand the molecular basis for the early phase events of host defense against microbial infection. In addition, accumulating evidence has shed light on the broad range of their roles in various biological processes. The TLR family is one of the most extensively characterized families of PRRs, and is also regarded as the most rapidly growing research field in immunology.

TLRs are type I transmembrane proteins containing repeated leucine-rich motifs in their extracellular domains similar to such motifs in other PRRs, and have a conserved intracellular motif (i.e. TIR domain, which initiates signal transduction). The typical microbial ligands for TLRs are summarized in Table 1. The initial step of signal transduction from TLRs is mediated through several adaptor molecules, including myeloid differentiation factor 88 (MyD88), Toll receptor-associated activator of interferon (TRIF), MyD88-adaptor-like/TIR-associated protein (MAL/TIRAP) and Toll receptor-associated molecule (TRAM), relayed to the inflammatory pathways involving NF- κ B, Janus kinase (JNK)/p38 kinase and interferon regulatory factor (IRF) 3, 5 and 7. Finally, TLR signal transduction induces various transcripts, including cytokines such as tumor necrosis factor- α (TNF- α) and interferon (IFN)-inducible genes.

TLR1/TLR2/TLR6	Bacterial lipoproteins and lipoteichoic acid, fungal zymosan
TLR3	Double stranded RNA
TLR4	Lipopolysaccharide (LPS) from Gram-negative bacteria
TLR5	Bacterial flagelin
TLR7/TLR8	Single-stranded RNA
TLR9	Unmethylated CpG motifs in DNA
TLR10	Unknown

Table 1. Typical ligands for human TLRs known as pathogen-associated molecular patterns (PAMPs)

2.1.1 Roles of TLRs in tissue homeostasis

In addition to their role in the host defense system, TLRs have been shown to be involved in various aspects of tissue homeostasis, including tissue repair and regeneration (van Noort & Bsibsi, 2009; Li et al., 2010). TLRs have also been reported to recognize various host-derived endogenous ligands, including cellular proteins such as heat shock proteins (Asea et al., 2002; Dybdahl et al., 2002; Ohashi et al., 2000; Roelofs et al., 2006; Vabulas et al., 2001; Vabulas et al., 2002a; Vabulas et al., 2002b) and high mobility group box1 (HMGB1) (Park et al., 2004; Park et al., 2006), uric acid crystal (Liu-Bryan et al., 2005a; Liu-Bryan et al., 2005b), surfactant protein A (Guillot et al., 2002), and products of the extracellular matrix, such as fibronectin (Okamura et al., 2001), heparan sulfate (Johnson et al., 2002), biglycan (Schaefer et al., 2005), fibrinogen (Smiley et al., 2001), oligosaccharides of hyaluronan and

degraded hyaluronan products (Termeer et al., 2002; Jiang et al., 2005). These endogenous ligands, referred to as damage-associated molecular patterns (DAMPs), are released from dead or dying cells in injured or inflamed tissues, and trigger the activation of TLRs, leading to “sterile inflammation” (inflammation at the site without microbes).

TLRs have a cytoprotective role and prevent tissue injury under stress conditions in the lung and intestine. In bleomycin-induced lung injury, hyaluronan-TLR2/TLR4 interactions were shown to provide signals that initiate inflammatory responses, maintain epithelial cell integrity and promote recovery from acute lung injury (Jiang et al., 2005). In the dextran sulfate sodium-induced intestine injury model, TLR4 and MyD88 signaling have been shown to be required for optimal proliferation and protection against apoptosis in the injured intestine, and activation of TLRs by commensal microflora was shown to be critical for protection against gut injury and associated mortality (Rakoff-Nahoum et al., 2004; Fukata et al., 2006). The injury-promoting effects of TLR4 have been shown in hepatic, renal, cerebral, and cardiac ischemia-reperfusion experiments using TLR4-mutant or -deficient mice (Oyama et al., 2004; Tsung et al., 2005; Tang et al., 2007; Wu et al., 2007). Alcoholic liver injury has been shown to depend on TLR4 and LPS (Bjarnason et al., 1984; Adachi et al., 1995; Uesugi et al., 2001). In the central nervous system, TLRs have been shown to coordinate the protective response to axonal and crush injury of the brain and spinal cord (Babcock et al., 2006; Kigerl et al., 2007; Kim et al., 2007). Thus, TLRs are thought to control inflammation after tissue injury in positive and negative manners.

The involvement of TLRs in tissue/organ regeneration responses is exemplified by liver regeneration after partial hepatectomy (Seki et al., 2005). Regeneration responses consist of multiple biological functions, including cell proliferation, angiogenesis, reconstruction of extra cellular matrix, epithelialization. TLRs have been reported to regulate the compensatory proliferation of parenchymal cells after injury (Rakoff-Nahoum et al., 2004; Tsung et al., 2005; Pull et al., 2005; Campbell et al., 2006; Zhang & Schluesener, 2006), induce cyclooxygenases, chemokines, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (Fukata et al., 2006; Rakoff-Nahoum et al., 2004; Pull et al., 2005), and activate mesenchymal stem cells (Pevsner-Fischer et al., 2007). Thus, TLRs are involved throughout the process of tissue repair and regeneration, indicating their critical role in tissue homeostasis. TLRs may have acquired dual roles in tissue homeostasis during the evolutionary process, i.e. to regulate inflammation and to promote regenerative processes. It seems reasonable that the same molecule mediates two important processes which sequentially take place at the same site; however, accumulating evidence has shown another aspect of TLR roles in carcinogenesis and cancer development.

2.1.2 TLRs and cancer

2.1.2.1 Anti-tumor effects induced by TLR signaling

BRMs derived from biological materials have been used in cancer immunotherapy, however, the detailed molecular mechanisms evoking tumor immunity have been obscure. During the past couple of decades, dozens of immunity-related molecules including PRRs have been identified, and their identifications greatly help us to understand the relationship between innate immunity and cancer. Among the above mentioned classical BRMs, the following TLRs have been shown to mediate their anti-tumor effects; TLR4 for Coley toxin

(Garay et al., 2007) and OK-432 (Hironaka et al., 2006; Okamoto et al., 2006), TLR2/TLR4 for BCG-CWS (Uehori et al., 2005), respectively. TLR agonists mediate anti-tumor activity by multiple mechanisms (Rakoff-Nahoum & Medzhitov, 2008). TLR agonists have been shown to directly kill both tumor cells and ancillary cells of the tumor microenvironment, such as vascular endothelium (Salaun et al., 2006; El Andaloussi et al., 2006; Haimovitz-Friedman et al., 1997; Nogueras et al., 2008). TLR activation may also lead to tumor regression directly or indirectly (TNF-mediated) by increasing vascular permeability (Garay et al., 2007), recruitment of leukocytes, activation of the tumor lytic activity of NK cells and cytotoxic T lymphocytes (CTL), and increasing the sensitivity of tumor cells to elimination mechanisms by effector molecules, such as TRAIL, TNF, and granzyme B/perforin (Smyth et al., 2006; Akazawa, 2007).

In cancer patients with a growing tumor, immune tolerance between the host immune system and tumor may have already been established. Agonists of TLRs induce intrinsic innate immune responses against microbial infection, leading to adaptive immune responses also to microbial pathogens. At the same time, the triggered innate immune response may also act to break the established tolerance between host and tumor, and induce an adaptive immune response against the tumor, mainly through activated antigen-presenting cell (APC) functions. Breaking the tolerance to tumor self-antigens is a property known as adjuvanticity. The mechanisms by which TLRs induce effective antitumor adaptive immune responses include the uptake, processing and presentation of tumor antigens, enhancement of survival, and induction of costimulatory molecules on professional APCs, induction of Th1 and CTL responses, and the inhibition of regulatory T cell activity (Garay et al., 2007; Smyth et al., 2006; Akazawa et al., 2007).

2.1.2.2 Positive effects of TLRs in cancer development

In the 19th century, Rudolf Virchow, one of the founding fathers of modern pathology, postulated a link between chronic inflammation and cancer (Kluwe et al., 2009). Chronic inflammatory diseases, including inflammatory bowel diseases, hepatitis and *Helicobacter pylori* infection, have been shown to be associated with cancer development (Chen et al., 2007). Although the detailed mechanisms of tumor-promoting effects by chronic inflammation are far from understood, TLRs are likely candidates as mediators of the effects of the innate immune system on carcinogenesis (Kluwe et al., 2009).

The tumor-promoting effects of TLRs have been shown in studies using adoptively transferred tumor models. Lipopolysaccharide (LPS) enhanced tumor growth in a lung metastasis model of mouse mammary carcinoma cells (Pidgeon et al., 1999; Harmey et al., 2002) and mouse colon cancer cells (Luo et al., 2004). Direct injection of *Listeria monocytogenes*, a Gram-positive facultative intracellular bacterium, promoted tumor growth in a subcutaneous tumor model of hepatocarcinoma cells (Huang et al., 2007). In a study using the mouse mammary tumor cell line and its highly antigenic subline D2F2/E2, which stably expresses human ErbB-2, *Salmonella typhimurium* flagellin, a TLR5 ligand, showed complicated effects in the subcutaneous tumor setting; flagellin inhibited tumor growth only in the D2F2/E2 cells when administered 8-10 days after tumor inoculation, but it accelerated tumor growth in both cell lines when administered at the time of inoculation (Sfondrini et al., 2006) suggesting that the status of interactions between the tumor and its microenvironment and/or host immune system are critical for the effects of flagellin.

Furthermore, the combination of flagellin and TLR9 agonist, CpG containing oligodeoxynucleotide (CpG ODN) administered 8-10 days after tumor inoculation, abrogated D2F2/E2 tumor growth.

The involvement of TLR signaling in carcinogenesis has been shown in studies using TLR-deficient or its signaling molecule-deficient mice (Fukata et al., 2007; Rakoff-Nahoum & Medzhitov, 2007; Naugler et al., 2007). TLR4-deficient mice were protected from colon tumorigenesis in an azoxymethan-induced chronic inflammation model, and TLR4 signaling was suggested to be involved in tumorigenesis *via* cyclooxygenase-2 (Cox-2) expression and prostaglandin (PG) E₂ production, leading to the activation of EGFR signaling (Fukata et al., 2007). MyD88 deficiency resulted in decreased colon tumor development in mice with heterozygous mutation of the adenomatous polyposis coli gene (spontaneous model), and also in an azoxymethane-induced colon tumor model (Rakoff-Nahoum & Medzhitov, 2007). Significant reduction of tumor formation was observed in MyD88 or interleukin (IL)-6 deficient male but not female mice in a diethylnitrosamine-induced hepatocellular carcinoma model (Naugler et al., 2007).

A wide range of human cancer cells have been shown to express various TLRs (Sato et al., 2009). For example, nine TLRs are expressed in normal colon epithelial cells, and three TLRs (TLR2-4) are elevated in most colorectal cancer cell lines (Sfondrini et al., 2006). Four TLRs (TLR2-5) are expressed in both normal ovary epithelial cells and ovarian cancer cell lines (Zhou et al., 2009). The significance of the expression of multiple TLRs in a wide range of cancer cells is not fully understood; however, simultaneous activation of multiple TLR types might be able to enhance the biological response, as in the case of synergistic effects by combined TLR ligation reported for cytokine production in DCs (Whitmore et al., 2004; Roelofs et al., 2005; Warger et al., 2006; Gautier et al., 2005; Theiner et al., 2007; Zhu et al., 2008; Krummen et al., 2010) and macrophages (Sato et al., 2000; Ouyang et al., 2007). It is possible that tumor cells express multiple TLRs to recognize various DAMPs in their microenvironment, and thereby enhance the biological process triggered by TLR activation to produce favorable conditions for growth and survival. Furthermore, ligation of TLRs in tumor cells has also been shown to increase the production of immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)- β (Sato et al., 2009), suggesting that tumor cells also utilize the TLR functions to escape from the tumor immunosurveillance.

2.1.3 TLRs as target molecules for drug development

In simple terms, TLR signaling pathway can be facilitated by agonistic agents to enhance the immune response, and inhibited by antagonistic agents to attenuate a hyper-immune reaction. The former can be applied to diseases including cancer and infectious diseases, and the latter to autoimmune diseases, excessive inflammation, and bacteria-induced pathological conditions, such as sepsis. TLR ligands and their related compounds which can bind to TLRs directly are the primary candidates as therapeutic agent. Most of the TLR-targeting drugs under development are agonistic agents, and a few antagonists for TLR4 seems to be developed as therapeutics for sepsis, such as Eritoran (Eisai Co. Ltd, Tokyo, Japan) (Rossignol et al., 2004). These drugs include various types of compounds, including single and double-stranded RNA, CpG ODN, small compounds, etc., but agonistic agents could be roughly divided into two categories in terms of their usage; an immunomodulatory

agent and an adjuvant for vaccination. Agonists of TLRs produce their therapeutic efficacy *via* stimulation of APCs of innate immunity, such as DCs and macrophages, as an initial step, and then their activation/maturation trigger secondary immune responses, including the activation of effector cells of both innate and adaptive immunity, such as NK and T cells; therefore, TLR activation is an indirect stimulus of adaptive immunity. Antigens in vaccines are incorporated by APCs at first, and then presented to T lymphocytes to evoke specific adaptive immune responses. TLR agonists could be one of the best candidates for a vaccine adjuvant, because they are well characterized, and their extensive scientific backgrounds make it possible to produce novel compounds with favorable properties, such as less toxicity, stronger immunostimulatory effects, and longer half life. In the TLR-targeting drugs under development, more than half of compounds seem to be targeting TLR9 (Krishnan et al., 2009; Romagne, 2007), and the reasons for that could be their chemical nature (ready to synthesize and handle) and the economic benefit of DNA and its derivatives in addition to their features as strong inducers of Th1 immune responses.

2.1.4 TLR-targeting drugs for cancer

Here, the most advanced two examples of TLR-targeting drug development will be discussed; i.e. agonists for TLR9 and TLR7. A group, so-called antiviral TLRs, including TLR3 and TLR7-9 are predominantly localized intracellularly to endosomal membranes, and recognize nucleic acids and related compounds (Table 1).

2.1.4.1 TLR9 agonists: CpG ODN

TLR9 detects the unmethylated CpG dinucleotides prevalent in bacterial and viral DNA but not in vertebrate genomes (Krieg, 2007). TLR9 has been shown to have the narrowest expression profile among all the TLRs; among resting immune cells, B cells and plasmacytoid DCs (pDCs) seem to exclusively express TLR9 (Iwasaki & Medzhitov, 2004). pDCs are extremely important cells in host defense as they produce most of the type I IFN that is made in response to viral infection and that is essential to control viral replication and to promote the development of an immune response to eradicate infected cells and prevent recurrence (Liu, 2005). One of the most notable features of TLR9 activation is that strong Th1 responses are triggered. In synthetic CpG ODN used *in vivo* applications, including animal experiments and clinical trials, native phosphodiester backbones are replaced partially or fully with phosphorothioate backbones to improve stability.

In animal experiments, significant anti-tumor effects of TLR9 agonists have been demonstrated in a variety of settings; not only in monotherapy (Lonsdorf et al., 2003; Baines & Celis, 2003), but also in combination therapy with monoclonal antibody therapy (Buhtoiarov et al., 2006; Daftarian et al., 2004; Davila et al., 2003; Dercamp et al., 2005; Guiducci et al., 2005; Vicari et al., 2002; Wooldridge et al., 1997), cytokines, chemokines and related factors (Chaudhry et al., 2006; Ishii et al., 2003; Merad et al., 2002; Okano et al., 2005), TLR3 and TLR5 ligands (Sfondrini et al., 2006; Whitmore et al., 2004), EGFR-related signaling and angiogenesis inhibition (Damiano et al., 2006), radiotherapy (Mason et al., 2005), surgery (Ohashi et al., 2006; Weigel et al., 2003), cryotherapy (den Brok et al., 2006), and chemotherapy (Balsari et al., 2004; Pratesi et al., 2005; Taieb et al., 2006; van der Most et al., 2006; H. Wang et al. 2006; X.S. Wang et al., 2005; Weigel et al., 2003). TLR9 has been thought to be the only TLR for which a systemically administered specific agonist has

shown substantial evidence of anti-tumor activity in human clinical trials (reviewed in Krieg, 2007). CPG 7909 (also called PF-3512676), a CpG ODN with a fully modified phosphorothioate backbone, has been thought to be one of the most promising drug candidates for cancer therapy.

Unfortunately, it has been announced that the sponsor pharmaceutical company made the decision to terminate phase III clinical trials of CPG 7907. In these phase III clinical trials, the efficacy of CPG 7909 had been tested in combination with gemcitabine/cisplatin or paclitaxel/carboplatin in patients with advanced non-small-cell lung cancer.

2.1.4.2 TLR7 agonists: Imiquimod

TLR7 recognizes single-stranded RNA, and acts as a potent activator of innate immune responses to viral infections (Diebold et al., 2004; Heil et al., 2004). Other than the natural ligands, several distinct classes of low-molecular-weight compounds have been shown to effectively and selectively activate this receptor (Hemmi et al., 2002; Lee et al., 2003). Imiquimod (also called Aldara, R-837, and S-28463), a member of the imidazoquinoline family, has antiviral activity in guinea pigs infected with herpes simplex virus (Harrison et al., 1988), and against arbovirus and cytomegalovirus (Akira & Hemmi, 2003). The first report on the anti-tumor activity of imiquimod was published in 1992, using subcutaneous tumor models of mouse colon carcinoma and mouse sarcoma and a lung metastasis model of mouse Lewis lung carcinoma (Sidky et al., 1992); however, the mechanism of imiquimod action as TLR7 agonist was totally unknown at that time. Subsequently, anti-tumor effects of imiquimod have been shown to be mediated by the induction of cytokines, including IFN- γ and IL-12 (Hemmi et al., 2002), the enhancement of the activation of tumor antigen-specific CTLs in vaccination studies (Prins et al., 2006; Rechtsteiner et al., 2005), and the activation of a myeloid DC subset with cytotoxic activity (Stary et al., 2007). Because of the intracellular localization of TLR7, small molecules such as imiquimod might have an advantage in terms of cellular uptake (Schön & Schön, 2008).

Imiquimod is a success story of TLR-targeting drugs. A topical cream formulation of imiquimod has been approved to treat actinic keratosis and external genital warts, and its additional indication for the treatment of superficial basal cell carcinoma was approved by the United States Food and Drug Administration in 2004. At present, 20 clinical oncology trials with imiquimod are ongoing (Maruyama et al., 2011). In these clinical trials, new target cancers are included such as basal cell carcinoma of different types/stages, melanoma, cancers of the breast, ovary, uterine cervix, and lung, glioblastoma, neuroblastoma, rhabdomyosarcoma, and osteogenic sarcoma. In addition, imiquimod is used as a vaccine adjuvant in 10 out of 20 trials. Vaccines in these clinical trials include DC-based vaccines, peptides or proteins of tumor associated antigens, human papilloma virus related DNA vaccines (Maruyama et al., 2011). Thus, imiquimod has already been established as a therapeutic product, and is under development as a vaccine adjuvant.

2.1.5 Perspectives: TLRs as double-edged swords in cancer therapy

TLRs have dual important roles in mammals; host defense from infection and maintaining tissue homeostasis by regulating inflammatory and tissue repair responses to damage. Antitumor effects of TLR agonists, including classical BRMs, depend on the former role of

TLRs in the immune systems. TLRs initiate innate immune responses, bridge the response from innate to adaptive immunity, and lead to the elimination of transformed cancer cells; however, cancer cells themselves utilize the latter role of TLRs in tissue homeostasis for their growth and survival by means of a variety of strategies; suppression of host immune responses by the production of various immunosuppressive factors, promotion of cell growth and angiogenesis, induction of cytoprotective and anti-apoptotic factors. These findings suggest that TLR signaling acts as a double-edged sword in cancer therapy. Administration routes and doses may be critical issues to develop TLR-targeting drugs for cancer treatment. Systemic administration of TLR agonists may include the risks of promoting growth and survival of tumor. On the other hand, local application, especially transcutaneous or intradermal administration could be a promising modality to lower the risks. In addition, the dose of the TLR agonist may be an important issue. It has been suggested that exceptionally high doses of TLR agonists appear to have an antitumor effect, whereas low doses of TLR agonists promote tumor growth (Kluwe et al., 2009). It is critical to know how we can dominantly use the unilateral antitumor edge of the TLR sword. Furthermore, another point to be tested in clinical studies may be the combinatorial usage of agonists for different types of TLRs, such as TLR4 agonist plus TLR9 agonist, as tested in animal experiments. As mentioned above, the mixed nature of classical BRMs seems to contribute to efficient antitumor effects. The evidence on the role of TLRs in tissue homeostasis has been obtained mainly from the studies focused on TLR2 and TLR4. For a better understanding of the role of TLRs in tissue homeostasis, carcinogenesis, and tumor progression, more research data on other types of TLR are necessary.

2.2 Fms-like tyrosine kinase 3 ligand (FL)

2.2.1 Cloning of FL as a ligand for fms-like tyrosine kinase-3 (Flt3-R)

FL is a transmembrane or soluble protein and is expressed by a variety of cells including hematopoietic and bone marrow stromal cells. In synergy with other cytokines and growth factors, FL stimulates proliferation and development of a wide range of hematopoietic cells including stem cells, myeloid and lymphoid progenitor cells, DCs and NK cells (Drexler & Quentmeier, 2004; Lyman & Jacobsen, 1998). FL was cloned as a ligand for Flt3-R, a tyrosine kinase receptor belongs to the class III receptor tyrosine kinase family structurally related to macrophage colony-stimulating factor receptor (c-fms) and to mast stem cell factor (also known as steel factor, stem cell factor, and c-kit ligand) receptor (c-kit) (Matthews et al., 1991; Rosnet et al., 1991a; Rosnet et al., 1991b). Lyman et al. isolated a murine Flt3L cDNA by screening an expression library with a fusion protein consisting of the extra cellular domain of the Flt3-R (Lyman et al., 1993). Hannum et al. purified FL from conditioned medium of a murine thymic stromal cell line using an affinity column made with Flt3-R extracellular domain (Hannum et al., 1994). Subsequently, a human counterpart of a murine FL was cloned using a mouse cDNA as a probe (Hannum et al., 1994; Lyman et al., 1994b). The mouse and human FL proteins are 72% identical at the amino acid level with a greater homology in the extracellular region than in the cytoplasmic domain.

Multiple isoforms of FL have been reported for mouse and human; the first one is a transmembrane protein which is a predominant isoform in human (Hannum et al., 1994; Lyman et al., 1993; Lyman et al., 1994b), and is converted to biologically active soluble form

by proteolytic cleavage (Lyman et al., 1993). The second biologically active isoform, the most abundantly found in mouse (Lyman et al., 1995b), is a membrane bound form which lacks a normal transmembrane structure but has a hydrophobic amino acid stretch in N-terminus (Hannum et al., 1994; Lyman et al., 1995a). The third isoform identified in mouse and human is a soluble form generated by alternative splicing (Lyman et al., 1995a, Lyman et al., 1995b).

2.2.2 Biological activity of FL

It is known that FL is interactive between different species, mouse and human FL can be fully active on cells expressing either the mouse or human Flt3-R (Lyman et al., 1994a). Although physiological importance for the ubiquitous expression is not clear, FL is widely expressed in murine and human tissues, the transcripts have been detected almost every tissue tested (Hannum et al., 1994; Lyman et al., 1994b; Lyman et al., 1995a). In contrast to FL, its receptor Flt3-R seems to have a relatively limited expression profile. Flt3-R expression in hematopoietic system appear predominantly restricted to the progenitor/stem cell compartment (Drexler et al., 2004).

FL stimulates proliferation and development of a wide range of hematopoietic cells including hematopoietic stem cells, myeloid and lymphoid progenitor cells (reviewed in Drexler et al., 2004). In this section, effects of in vivo administered FL on DCs and NK cells will be discussed.

2.2.2.1 Effects of FL on DC development

DCs express CD45 and arise from BM progenitor cells; evidence suggests that DCs are derived from myeloid and lymphoid progenitor cells (Caux et al., 1995; Peters et al., 1996). Early studies of mouse in vivo experiments identified that FL as a cytokine that could affect DC proliferation (Maraskovsky et al., 1996; Pulendran et al., 1999). In vivo administration of FL in mouse results in a dramatic increase in the number of myeloid- and lymphoid-derived functional DC in BM, spleen, thymus, peripheral blood, and other tissues indicating an absolute increase in functionally mature DC (Maraskovsky et al., 1996). In contrast, polyethylene glycol-modified granulocyte-macrophage colony-stimulating factor (GM-CSF) into mice only expands the myeloid-related DC subset (Pulendran et al., 1999).

In human, two distinct classes of blood DC subsets, CD11c⁺ immature DCs and CD11c⁻ pDCs with different morphologies, phenotypes, and functional properties, have been identified (Liu, 2001). Blood CD11c⁺ DCs are considered to be myeloid derived, whereas pDCs, also known as type 1 interferon-producing cells as mentioned in section 5.1, are considered to be lymphoid related (Liu, 2001). Administration of FL to healthy volunteers significantly expands the number of circulating CD11c⁺ and CD11c⁻ DC subsets and DC precursors (Maraskovsky, 2000).

2.2.2.2 Effects of FL on NK cells

FL administered to mice increased number of NK cells (Brasel et al., 1996; Shaw et al., 1998). Brasel et al. reported that daily subcutaneous injection of recombinant FL (10 µg) for 10 days resulted in 2.5-fold increase in NK cell absolute number in spleen (Brasel et al., 1996). Shaw et al. also showed daily intraperitoneal administration of recombinant FL resulted in

increase in the absolute number of CD3⁻ NK1.1⁺ NK cell especially in spleen and liver (Shaw et al., 1998). Similar effect of FL on NK expansion was reported by He et al. using intravenous injection of a plasmid expression vector for FL cDNA encoding extra cellular domain (He et al., 2000). These in vivo effects of FL on NK expansion was supported by the evidence obtained from mice lacking FL in which a marked deficiency of NK cells in the spleen was noted (McKenna et al., 2000). The ability of FL to expand NK cells in vivo has been confirmed by subsequent studies (Guimond et al., 2010; Péron et al., 1998; Smith et al., 2001).

In contrast to the mouse findings in vivo, few are available on the effect of FL on human NK cells in vivo. In a phase I clinical trial, FL was subcutaneously injected into cancer patients including Hodgkin disease, non-Hodgkin lymphoma, or advanced-stage breast cancer after autologous hematopoietic cell transplantation (Chen et al., 2005). Injection of FL was safe and well tolerated and significantly increased blood DC subsets without affecting other mature cell lineages including NK cells (Chen et al., 2005). According to the findings obtained, the activity of FL alone to induce cellular expansion is higher in DCs than that in NK cells. It appears necessary to combine FL with other cytokines to induce effective NK cell expansion in vivo especially in human.

2.2.3 Anti-tumor effects evoked by FL

The finding that FL administration resulted in dramatic expansion of DCs led to the studies to test anti-tumor effect of FL. Early study demonstrated that FL alone induced the regression of methylcholanthrene (MCA)-induced fibrosarcoma (Lynch et al., 1997). Subsequently, anti-tumor effect of systemically administered FL has been demonstrated in a lot of syngeneic murine tumor models of melanoma, lymphoma (Esche et al., 1998), Lewis lung carcinoma (Chakravarty et al., 1999), liver metastasis (Péron et al., 1998), mesothelioma (Fernandez et al., 1999), breast cancer (Chen et al., 1997), prostate cancer (Ciavarrá et al., 2000). The anti-tumor effect of FL has been shown also in rat syngeneic colon cancer model (Favre-Felix et al., 2000) and xenograft model of human ovarian cancer (Silver et al., 2000). In these tumor models, NK cells seem to have a critical role in the anti-tumor effect induced by FL administration, because depletion of NK cells resulted in abrogation of anti-tumor effect (Fernandez et al., 1999; Péron et al., 1998; Silver et al., 2000). However, the anti-tumor effects induced by FL have also been shown to be T-cell mediated in several mouse models (Chen et al., 1997; Lynch et al., 1997). Fernandez et al. reported that in mice with MHC class I-negative tumors, adoptively transferred- or FL-expanded DCs promoted NK cell-dependent anti-tumor effects (Fernandez et al., 1999). Kelly et al. demonstrated that primary rejection of MHC-class I negative-CD70 expressing RMA-S tumor cells by NK cells efficiently evoked the subsequent development of tumor-specific cytotoxic and T helper type 1 responses to the parental MHC class I-sufficient RMA tumor cells (Kelly et al., 2002). Activated NK cells can facilitate adaptive immune responses via induction of DC maturation by secretion of cytokines and a cell-cell contact (Walzer et al., 2005). These findings suggest that interaction between NK cells and DCs induces not only anti-tumor effects by NK cells, but also adaptive anti-tumor immunity.

At present, limited information are available on clinical studies using FL administration. Marroquin et al. reported that FL administration resulted in a 19-fold increase in DC

number in the peripheral blood of patients with melanoma and renal cancer, however, DC generated in vivo appeared only partially activated. This partial activation might account for the lack of enhanced immune responses to melanoma antigens and absence of clinical response in the patients even in combination with antigen immunization (Marroquin et al., 2002). Rini et al. reported that administration of FL either alone or in combination with interleukin-2 (IL-2), although capable of inducing expansion of circulating myeloid and plasmacytoid DCs in patients with metastatic renal cell carcinoma, lacks significant clinical activity (Rini et al., 2002). The above mentioned report by Chen et al. showed that FL administration significantly increased the frequency and absolute number of myeloid DCs, plasmacytoid DCs, and monocytes in the patients with Hodgkin disease, non-Hodgkin lymphoma, or advanced-stage breast cancer after autologous hematopoietic cell transplantation (Chen et al., 2005). In the clinical studies mentioned here, expected clinical responses were not obtained.

The clinical application of DCs for cancer vaccines, however, has been moderately successful (Rosenberg et al., 2004). In the clinical procedure to prepare DC vaccines, monocytes harvested from cancer patients by leukocyte apheresis are cultured in the presence of cytokines to generate DCs. The generated DCs are matured in vitro, treated with tumor antigens such as peptides and tumor lysates, and then injected into patients. The course to prepare DC vaccines needs a lot of times, efforts, and costs. If the in vivo expansion of DCs by FL could be induced effectively and appropriately, it is possible that there is no need to generate DC vaccines ex vivo. Furthermore, ability to expand NK cells is another important point in the clinical application of FL. In the clinical trial mentioned above (Chen et al., 2005), administration of FL to cancer patients did not result in apparent expansion of NK cells. Combination of FL with other cytokines like IL-15 may be the promising strategy to expand human NK cells in vivo (Yu et al., 1998).

2.3 Alpha-galactosylceramide (α -GalCer)

2.3.1 Isolation, identification, and synthesis of α -GalCer

Glycosphingolipid compounds, named agelasphins, were isolated from an extract of the Okinawan marine sponge, *Agelas mauritianus*, by Kirin Pharmaceuticals, and some of them were found to possess anti-tumor activity in mice (Natori et al., 1994; Tsuji, 2006). Because of the low contents of agelasphins in the marine sponge, and the difficulty of their scale-up synthesis, the structure of agelasphins was modified, and a novel compound, α -GalCer (also called KRN7000) was synthesized (Kobayashi et al., 1995). This glycolipid was shown to bind non-classical MHC molecule CD1d of human and mice (Gansert et al., 2003; Taniguchi & Nakayama, 2000), and activate natural killer T (NKT) cells of both species in vitro and in vivo when it is presented on CD1d molecule (Brossay et al., 1998; Burdin et al., 1998; Kawano et al., 1998; Spada et al., 1998). To date, α -GalCer has been the most extensively studied ligand for CD1d molecules and stimulant for NKT cells.

2.3.2 NKT cells

NKT cells are a unique subpopulation of T cells which play important role in regulating immune responses by bridging innate and adaptive immune systems. The term 'NKT cells'

was first used in 1995 (Makino et al., 1995) to define a subset of mouse T cells that share some characteristics with NK cells, particularly expression of the NK1.1 marker (Nkrp1c or CD161c). At present, the term NKT cells is well accepted and broadly applied to mice, humans, and other species (Godfrey et al., 2004). NKT cells express an antigen-specific T cell receptor (TCR), but unlike with conventional T cells that detect peptide antigens presented by MHC molecules, NKT cells recognize lipid antigens presented by CD1d molecule. NKT cells have been shown to involve wide spectrum of disorders including infectious diseases caused by bacteria, parasites, and virus, autoimmunity, and cancer (Terabe & Berzofsky, 2008).

Accumulating several lines of evidences which obtained from the late 80's to 90's had been integrated into the establishment of novel immune cell lineage 'NKT cells' (Bendelac et al., 1997; Bendelac et al., 2007; Godfrey et al., 2004; Godfrey et al., 2010; Macdonald., 2007; Taniguchi et al., 2003; Terabe. & Berzofsky, 2008). However, an ambiguous definition of NKT cells has caused confusion in our understanding of their biological roles. At present, it has been proposed to classify NKT cells into two types (Godfrey et al., 2010).

Type I NKT cells, also called invariant NKT cells or iNKT cells, are defined by their expression of the canonical invariant TCR α chain of V α 14J α 18 in mouse (V α 24J α 18 in human) with a limited number of TCR β chains of V β 8, V β 7, and V β 2 in mouse (V β 11 in human). Type I NKT cells are detected with α -GalCer-loaded CD1d tetramers, however, other CD1d restricted T cells exist (Godfrey et al., 2004; Godfrey et al., 2010; Terabe & Berzofsky, 2007). On stimulation with α -GalCer, type I NKT cells produce a large amount of TH1 (IFN- γ) and Th2 (IL-4 and IL-13) cytokines. Although type I NKT cells have NK-like cytolytic activity, they are considered to be regulators of immune responses because they rapidly produce large amount of both Th1 and Th2 cytokines in autoimmune diseases, infectious diseases, and cancer. Another subset of CD1d-restricted NKT cells which does not respond to α -GalCer, seems to recognize a range of hydrophobic antigens including sulfatide (Jahng et al., 2004), lysophosphatidylcholine (Chang et al., 2008), and small aromatic (non-lipid) molecules (Van Rhijn et al., 2004). This NKT subset, called 'type II NKT cells', is present in humans and mice, and has been shown to be more heterogeneous than type I in both TCR α and TCR β chain usage (Godfrey et al., 2004; Godfrey et al., 2010; Terabe & Berzofsky, 2007; Terabe & Berzofsky, 2008). In this chapter, the findings on type I NKT cells and their ligands will be discussed hereafter.

2.3.3 Anti-tumor effects of α -GalCer

Originally, a mother compound of α -GalCer, agelasphins, was selected from several glycosphingolipids extracted from marine sponge depending on their anti-tumor effects in mouse tumor models (Natori et al., 1994). Anti-tumor effect caused by α -GalCer injection has been shown in mouse tumor models (Kobayashi et al., 1995; Motoki et al., 1995; Morita et al., 1995). Subsequently, protective roles of NKT cells stimulated by α -GalCer were confirmed by liver and lung metastasis models in mice using B16 melanoma cells and Lewis lung carcinoma cells, respectively (Kawano et al., 1998). A complete inhibition of B16 melanoma metastasis in the liver was observed when α -GalCer-pulsed DCs were injected 7 days after transfer of tumor cells to mice where metastatic nodules were already formed (Toura et al., 1999). In addition, long-term administration of α -GalCer inhibited primary tumor formation in the tumor models of MCA-induced sarcoma, mammary carcinomas in Her-2/neu transgenic mice, and spontaneous sarcomas in p53 deficient mice (Hayakawa et al., 2003).

Anti-tumor effects of α -GalCer depend on the production of IFN- γ from activated type I NKT cells (Terabe & Berzofsky, 2007). Crowe et al. showed that IFN- γ production by NKT cells was essential for protection against tumor, and perforin production by effector cells, but not NKT cells, was also critical in MCA-induced sarcoma models (Crowe et al., 2002). Smyth et al. reported that both NK cells and NKT cells were essential and collaborate in host protection from MCA-induced sarcoma (Smyth et al., 2001), and sequential production of IFN- γ by NKT cells and NK cells was essential for the antimetastatic effect of α -GalCer in B16 melanoma metastasis models (Smyth et al., 2002).

In addition to the essential role of IFN- γ , IL-12 was shown to be critical for the anti-tumor effect of NKT cells. Kitamura et al. demonstrated that production of IFN- γ by NKT cells in response to α -GalCer required IL-12 produced by DCs and direct contact between NKT cells and DCs through CD40-CD40 ligand interactions (Kitamura et al., 1999). Hayakawa et al. showed that both CD28-CD80/CD86 and CD40-CD40 ligand costimulatory pathways are essential for α -GalCer-induced IFN- γ production by NKT cells (Hayakawa et al., 2001). Furthermore, administration of α -GalCer induced not only innate immune response, but also adaptive immune response. A single dose of α -GalCer rapidly stimulated the full maturation of DCs in NKTcell-dependent manner, and this maturation accounted for the induction of combined Th1 CD4⁺ and CD8⁺ T cell immunity to a coadministered antigen (Fujii et al., 2003).

Taken together, anti-tumor effects of α -GalCer are thought to be evoked by the following sequential processes; α -GalCer presented on CD1d of DCs is recognized by NKT cells, and up-regulates CD40 ligand on NKT cells. Activated DCs by CD40 and CD28 costimulatory pathways produce IL-12, and activate NKT cells by IL-12. IFN- γ produced by fully activated NKT cells trigger DC maturation, activation and recruitment of NK cells, Th1 CD4⁺ and CD8⁺ T cell leading to direct anti-tumor effects.

2.3.4 Clinical trials using α -GalCer in cancer patients

At present, a number of clinical trials of α -GalCer have been conducted in multiple institutes, and their results have been published since 2002. Early study of phase I clinical trial in which α -GalCer was injected intravenously reported that α -GalCer was well tolerated in cancer patients over a wide range of doses (Giaccone et al., 2002). Phase I studies of α -GalCer-pulsed DCs have been conducted in patients with various solid tumors and myeloma (Chang et al., 2005; Dhodapkar et al., 2004; Ishikawa et al., 2005; Nieda et al., 2004; Okai et al., 2002; Uchida et al., 2008). However, none of these clinical trials reported significant efficacy against tumors.

Recently, the research team of Chiba University in Japan reported the summary of their clinical trials (Motohashi et al., 2011). The research team has been well known by their pioneering works on α -GalCer, and has been conducted multiple clinical trials with enthusiasm (Ishikawa et al., 2005; Kunii et al., 2009; Kurosaki et al., 2011; Motohashi et al., 2006; Motohashi et al., 2009; Uchida et al., 2008; Yamasaki et al., 2011). The team conducted the α -GalCer-NKT cell-based clinical trials in patients with non-small cell lung cancer and head and neck squamous cell carcinoma (Motohashi et al., 2011). Alpha-GalCer-pulsed APCs seemed to produce better clinical outcomes in the patients with head and neck squamous cell carcinoma than in those with non-small cell lung cancer. The cells were

injected into nasal submucosa of patients with head and neck squamous cell carcinoma, whereas intravenous injection was performed in the non-small cell lung cancer patients. Furthermore, the anti-tumor effect of α -GalCer-pulsed APCs seemed to be augmented by the combined adoptive transfer of α -GalCer-activated NKT cells. Thus, clinical efficacy of α -GalCer-based immunotherapy may depend on the tumor type and treatment settings. Further efforts are necessary to develop an effective treatment modality.

3. Conclusion

In cancer patients with a growing tumor, immune tolerance between the host immune system and tumor may have already been established. Agents mentioned in this chapter induce inherent innate immune responses intrinsically against microbial infection. The anti-tumor effect *via* activation of innate immune system may depend on the action to break the established tolerance between host and tumor leading to adaptive immune responses (Figure.1). In other words, the activation of innate immune system acts like as a ‘reset’

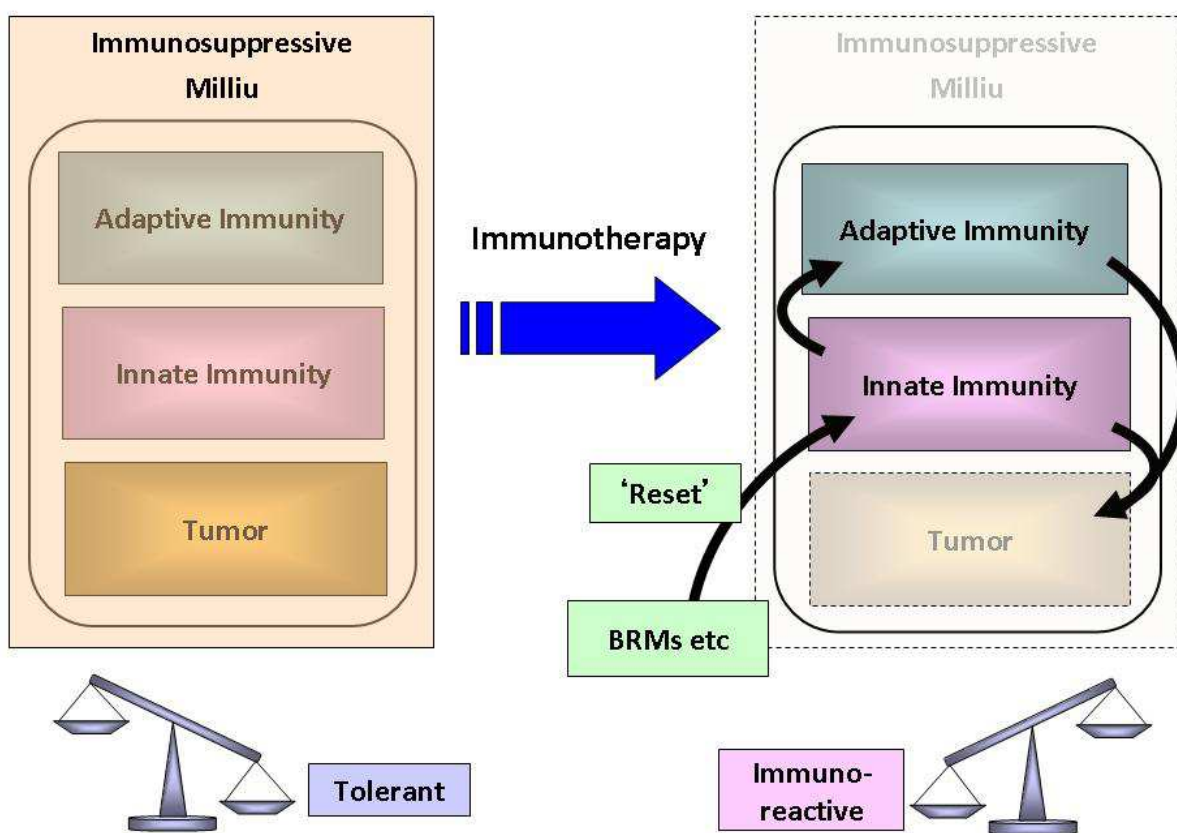


Fig. 1. In the cancer patients with a growing tumor, immune tolerance between host immune system and tumor may have already been established. Stimulants of innate immunity such as biological response modifiers (BRMs) or ligands for toll-like receptors induce inherent innate immune responses intrinsically against microbial infection. The anti-tumor effect *via* activation of innate immune system may depend on the action to break the established tolerance between host and tumor leading to adaptive immune responses.

button' in cancer patients to restart the immune responses. Administration of cytokines such as FL which induce expansion of immune cells can potentiate subsequent immune responses, and it may be the ideal pretreatment for cancer patients with immunosuppressive status. With regards to TLRs agonist, FL, and α -GalCer, the efforts to develop them as vaccine adjuvant have already been started. At present, the efficacy of a prophylactic vaccination against human papillomavirus has been established in the prevention of cervical cancer. Although tough and long-lasting works and huge research fund are required to perform clinical trials, one of the most promising immunotherapeutic modalities for cancer may be cancer prevention. Above mentioned three agents may be excellent vaccine adjuvants to enhance tumor immunity in cancer prevention. Further research efforts are required to establish more effective immunotherapy which will also achieve a better quality of life for cancer patients.

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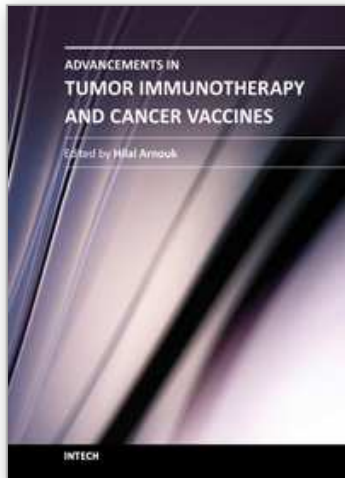
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Harnessing the potential of the human body's own immune system to attack malignant tumor cells has been the goal of many scientific investigators in recent years, with advances in cancer biology and immunology enabling cancer immunotherapy to become 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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