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REVIEW

TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours

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Toll-like receptor (TLR) agonists are potent activators of innate immune responses, activating dendritic cell (DC) maturation and inflammatory cytokine secretion by innate immune cells and as a consequence they promote adaptive immune response when coadministered with foreign antigens. There is also some evidence from mouse models that TLR ligands can help to break tolerance to self-antigens and promote immune responses to tumour antigens. Therefore, they have been exploited as adjuvants for tumour vaccines or as immunotherapeutics against cancer. Clinical evaluation of TLR agonists has resulted in a licensed immunotherapeutic for basal cell carcinoma, but there have also been disappointing results from clinical trials, with one pharmaceutical company recently halting its clinical programme. A major obstacle to the development of any active immunotherapeutic approach to cancer is the immunosuppressive environment of the growing tumour, including the induction of tolerogenic DCs and regulatory T (Treg) cells, which suppress the development of protective effector T-cell responses. This can be compounded by the use of TLR ligands as immunotherapeutics. A problem with TLR agonists that has not been fully appreciated is that they can generate suppressive as well as inflammatory responses in innate immune cells and can promote the induction of regulatory as well as effector T cells. This is part of a normal mechanism for limiting collateral damage during infection or sterile inflammation, but can constrain their ability to induce protective antitumour immunity, especially in the immune suppressed environment of the tumour. Alternatively, manipulating the TLR-activated innate immune responses to selectively blocking immunosuppressive arm, as well as that induced by the tumour, may hold the key to enhancing their efficacy as tumour immunotherapeutics and as adjuvants for cancer vaccines.
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Introduction

The induction of antitumour immunity through therapeutic vaccination or administration of immune-stimulating agents, such as Toll-like receptor (TLR) agonists, is a promising approach for the treatment of cancer. A significant number of successful preclinical studies in mice have resulted in a number of efficacy trials in humans, and a market product for the treatment of basal cell carcinoma. However, the majority of clinical trials of cancer vaccines and immunotherapeutics have been disappointing, with little or no evidence of tumour regression or remission (Rosenberg *et al*., 2004; Krieg, 2007). This is partly a reflection of the stage of disease of the patients in these trials, but also results from the immunological obstacles faced in generating immune responses in individuals with growing tumours.

The immune system evolved to discriminate self from non-self and this allows it to mount immune responses against infectious agents, without causing damage to host tissues or causing autoimmunity. However, the generation of immunity to tumour is dependant on breaking immunological tolerance and generating effector T-cell responses specific for self-antigens on tumours. This is the first obstacle that must be overcome in developing an effective immunotherapy or therapeutic vaccine against cancer. A second major obstacle is posed by the tumour itself in the form of immune evasion and immune subversion strategies, which in many ways resembles those evolved by pathogens to circumvent protective immune responses of the host. Tumours secrete a range of immunosuppressive molecules that inhibit effector immune responses but also recruit or activate cells of the innate and adaptive immune system that have regulatory activity (Figure 1). These include tolerogenic or regulatory dendritic cells (DCs) and regulatory T (Treg) cells. This can be confounded by the fact that the host can induce regulatory responses to pathogen-associated molecular patterns (PAMPs) as a strategy of feedback control to prevent overactivation of immune responses and collateral damage during infection. Therefore, immunotherapeutic approaches against cancer based on TLR agonists must carefully consider the relative induction of potentially suppressive as well as beneficial effector immune responses.

Immune subversion through regulatory DC induced by tumours

Effective immunological eradication of tumours is dependant on the generation of antitumour $CD4^+$ Th1

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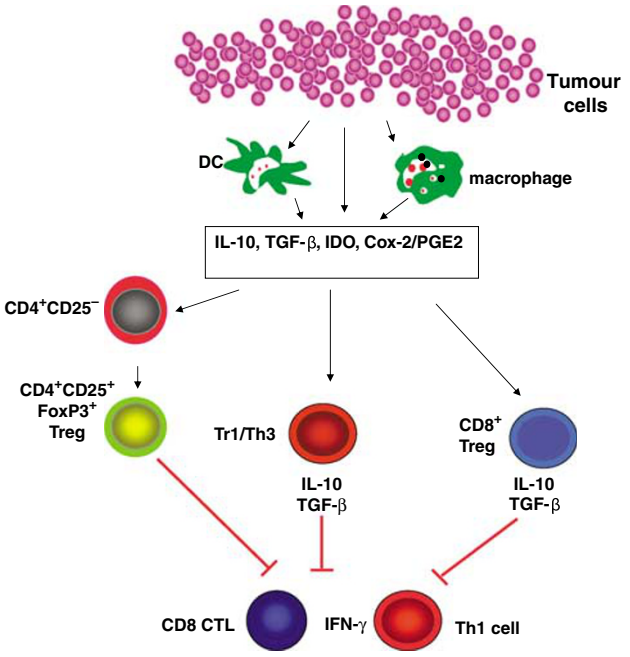

Figure 1 Immunosuppressive effects of growing tumours. Tumours secrete a range of immunosuppressive molecules or induce their production by dendritic cells (DCs) and macrophages, which in turn promote the differentiation of inducible regulatory T (Treg) cells and the peripheral conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+Foxp3^+$ natural Treg cells. These Treg cells suppress effector $CD4^+$ Th1 and $CD8^+$ CTL against the tumour.

cells, which in turn promote the recruitment of tumour-specific $CD8^+$ cytotoxic T-lymphocytes (CTLs) (Knutson and Disis, 2005). The aim of a successful immunotherapy against cancer is to break self-tolerance and provoke tumour-specific immune response in the host (Smyth *et al*., 2001). One of the most promising immunotherapeutic approaches to amplify tumour-specific Th1 and CTL responses has been to specifically target DCs (Gilboa, 2007). In addition to acting as professional antigen-presenting cells, DC play a pivotal role in directing T-cell responses (Reis e Sousa, 2004). Activation of DCs with TLR agonists, either as components of killed bacteria or attenuated virus or as exogenous adjuvant, is the underlying basis of many successful prophylactic vaccines against infectious diseases in man. However, immunization with tumour antigens with or without adjuvants that activate DC has had limited success in cancer patients (Livingston, 1989; Rosenberg *et al*., 2004). One of the reasons for this is believed to be the immunosuppressive environment created by the growing tumour (Figure 1), which can directly influence DCs infiltrating the tumour or in the draining lymph node (Pinzon-Charry *et al*., 2005).

For DCs to act as efficient antigen-presenting cells for the induction of T-cell responses, they must differentiate from an immature state, where they function in uptake and processing of antigen in tissues, to the mature DC, with upregulated major histocompatibility complex and co-stimulatory molecules, which migrate to the lymph node and present antigens to naive T cells (Reis e Sousa, 2004). However, tumour-infiltrating DCs have been shown to be immature and tolerogenic and ineffective at priming T-cell responses *in vivo* (Vicari *et al*., 2002; Dercamp *et al*., 2005). A number of factors secreted by tumours can directly interfere with DC maturation. Tumour-associated cytokines, such as vascular endothelial growth factor and interleukin (IL)-6, inhibit maturation of DCs (Gabrilovich *et al*., 1996). IL-8 interferes with chemotaxis prohibiting movement of the DC to the lymph node and IL-10 inhibits the ability of DCs to stimulate T-cell proliferation (Fricke and Gabrilovich, 2006). Prostaglandin E2 (PGE2) secreted by tumour cells acts on a number of cell types to suppress their function, including DCs, T cells and B cells. Most tumour cells are found to express elevated levels of cyclooxygenase-2 (Cox2), which can contribute to transformation of the cells. Cox-2 is responsible for the synthesis of the prostanoid PGE2 from arachadonic acid, and PGE2 released into the tumour site interferes in multiple ways with DC function, including reducing the expression of cell-surface major histocompatibility complex class I and II molecules and the co-stimulatory molecules, CD80 and CD86, inhibiting antigen presentation as well as reducing the ability of the DC to secrete IL-12, while increasing IL-10 production (Natarajan *et al*., 2003). PGE2 can also upregulate expression of indoleamine 2-3 dioxygenase (IDO) in DC. Tumour cells and DC have been found to express elevated levels of this enzyme. IDO metabolizes tryptophan and the resulting metabolites, such as kynurenine and picolinic acid, can inhibit proliferation of activated T cells (Terness *et al*., 2002; Braun *et al*., 2005).

DC immunotherapy for cancer

The immature and tolorogenic state of DCs infiltrating tumours (Vicari *et al*., 2002; Bellone *et al*., 2006) and in the draining lymph node may in part explain the failure of many immunotherapeutic approaches aimed at inducing tumour-specific T-cell responses in cancer patients. The development of protocols for expansion of DC *ex vivo* from murine bone marrow (Inaba *et al*., 1992) and human peripheral blood mononuclear cells (Hsu *et al*., 1996), together with an understanding of the factors that activate DC into an appropriate phenotype that promotes Th1, Th2 or Treg cells, has opened up the possibility of using *in vitro*-activated DC for cancer immunotherapy or vaccination. DC immunotherapy has the advantage of activating the DC away from the immunosuppressive influence of the growing tumour. Tumour antigen can be loaded onto DCs in a number of forms, including killed tumour cells, whole tumour supernatants, lysates, purified tumour-specific antigens and synthetic peptides (Banchereau and Palucka, 2005). Maturation of the DC can be induced by stimulation with cytokines such as IL-1β and tumor necrosis factor-α in combination with PGE2 (Dannull *et al*., 2005) or with TLR agonists (Bourquin *et al*., 2006).

The first DC vaccine trials in humans took place over a decade ago, where follicular B-cell lymphoma patients were injected with DCs pulsed *in vitro* with tumour-specific idiotype protein purified from the patient's own tumour (Hsu *et al*., 1996). All four of the patients treated developed antitumour responses, with one patient resolving all traces of disease (Hsu *et al*., 1996). Subsequently, numerous clinical trials have been carried out using DC immunotherapy with varying success (Cranmer *et al*., 2004; Rosenberg *et al*., 2004). Although DC-based immunotherapy has shown some promise in mice, the failures in the clinic may reflect the choice of antigen or maturation stimulus for the DC or the immunosuppressive environment generated by advanced tumours, which may still exert regulatory control on the injected DC or on the T cells induced by the DC. Indeed the tumour antigen preparations, especially killed whole tumour cells or cell lysate, or the immunomodulatory agents used to mature the DC can induce anti-inflammatory as well as pro-inflammatory responses and maturation of the DC. The downstream effect may be to induce regulatory as well as effector T cells and this remains a major obstacle to the development of effective therapeutic vaccines against tumours.

The problem of using whole tumour cells or cell lysates can be circumvented by using peptide antigens; however, the identification of tumour-specific antigen has been a problem. One approach to overcome this problem is to use heat-shock proteins (HSPs) purified from the patient's tumours. These proteins function as chaperones within the cell and so are complexed with a variety of peptides from within the malignant cells. HSP70, HSP90, HSP110, gp96, grp170 and calreticulin have been used for direct immunization against tumours in mice (Srivastava, 2006). We have demonstrated that HSP70 can also be used for maturation as well as antigen pulsing of DC in active immunotherapy against tumours in mice (D Toomey *et al*., unpublished).

Targeting immunosuppressive molecules to promote antitumour immunity

A number of approaches involving the use of blocking antibodies to immunosuppressive cytokines and inhibitors of other immunosuppressive molecules have been used alone or with immunotherapeutics in an attempt to induce tumour-specific immunity by attenuating the immunosuppressive effects of the tumour. Since Cox-2/PGE2 can be produced by both the tumours and DC, it is an important target for reversing immunosuppression. In a Cox2 overexpression mouse model of spontaneous intestinal neoplasia, mice fed with the Cox-2 inhibitor celebrex had a greater than 50% reduction in polyp formation, and in combination with a pox-virus vaccine induced a 95% reduction in polyps (Zeytin *et al*., 2004). In a mouse model of non-small-cell lung cancer model, prophylactic treatment with an irradiated tumour vaccine combined with the non-steroidal anti-inflammatory SC58236 resulted in a reduction in tumour growth in 50% of mice (Baratelli *et al*., 2005).

Another method of reversing the immunosuppressive effects of the tumour involved inhibition of p38 mitogen-activated protein kinase. Mouse DC incubated with tumour culture conditioning medium were found to have decreased IL-12 and defective T-cell activation (Lin *et al*., 2006). Tumour culture conditioning medium appears to induce p38 activation in DC, and inhibition of p38 restored normal levels of IL-12 production and T-cell activation. Similarly, monocyte-derived DCs from myeloma patients were found to be functionally defective, having elevated IL-6, decreased co-stimulatory molecule expression and elevated p38 activation (Lin *et al*., 2006). Treatment with neutralizing anti-IL-6 antibody or p38 inhibitor SB203580 increased cell surface antigen expression and decreased IL-6 production, promoting effective DC function. These studies shave shown that targeting upstream signalling pathways are an effective way of modulating DC phenotype.

Microvesicles released by tumour cells have also recently been shown to modulate DC function (Valenti *et al*., 2006). These membrane-bound bodies are released at a higher rate from neoplastic cells when compared with normal cells. The vesicles or exosomes contain factors from the tumour cells that may directly interfere with the immune response, including ligands, which can induce apoptosis by binding to receptors on the T cells such as Fas and TRAIL. Exosomes purified from the supernatants of melanoma and colorectal carcinoma cell lines were capable of directly inducing apoptosis in Jurkat cells. The vesicles also induced a distinct phenotype in monocytes characterized by retained expression of CD14, reduced expression of co-stimulatory molecules CD80 and CD86 and low expression of HLA-DR. Monocytes with this phenotype isolated from melanoma patients were found to secrete high levels of the immunosuppressive cytokine transforming growth factor-β (TGF-β) (Valenti *et al*., 2006).

Tregs in cancer

Prior to the rediscovery of suppressor as Treg cells, studies carried out over 30 years ago had shown that suppressor T cells could prevent effective antitumour immune responses (Fujimoto *et al*., 1975). Following the observations that $CD4^+$ T cells expressing the α-chain of the IL-2 receptor (CD25) (Sakaguchi *et al*., 1995) and/or secreting high concentrations of IL-10 (Groux *et al*., 1997) played a pivotal role in the prevention of autoimmune diseases, evidence emerged demonstrating a significant role for Treg cells in suppression of antitumour immune responses. It is generally accepted that there are two main types of Treg cells, $CD4^+CD25^+FoxP3^+$ Treg cells and antigen-specific cytokine-secreting Tr1 ($CD4^+IL\text{-}10^+$) and Th3 ($CD4^+TGF\text{-}\beta^+$) cells. $CD4^+CD25^+FoxP3^+$ Treg cells suppress antitumour effector T cells via a cell-to-cell contact mechanism, which may involve competitive consumption of IL-2 (von Boehmer, 2005), whereas the cytokine-secreting Treg cells suppress via the secretion of immunosuppressive cytokines (IL-10 and TFG-β).

Expansion of Treg cells in cancer

The first report on increased numbers of Treg cells in cancer in 2001 revealed that patients with non-small-cell lung cancer and ovarian cancer had increased numbers of $CD4^+CD25^+$ T cells compared with healthy controls and that these cells were able to suppress T-cell activation *in vitro* (Woo *et al*., 2001). $CD4^+$ $CD25^+$ Treg or $Foxp3^+$ Treg cells have subsequently been shown to be expanded in the peripheral blood and intratumour lymphocytes of many human tumours including breast cancer (Liyanage *et al*., 2002), colorectal cancer (Somasundaram *et al*., 2002), leukaemia (Karube *et al*., 2004), lymphoma (Marshall *et al*., 2004) and melanoma (Viguier *et al*., 2004).

In addition to $CD4^+CD25^+FoxP3^+$ Treg cells and Tr1 cells, several other cell types with suppressive capability are expanded in patients with cancer or have been shown to have a potentially suppressive function against antitumour immunity (Figure 1). IL-10-secreting $CD8^+$ T cells induced by ovarian cancer-derived DCs have been shown to suppress responses of tumour antigen-specific $CD4^+$ T cells (Wei *et al*., 2005). IL-10-secreting $CD8^+$ Treg cells have been shown to infiltrate B16 tumour in mice (Jarnicki *et al*., 2006). $CD3^+$ T cells expressing the NK cell marker NK1.1, termed invariant NKT cells, have been implicated in both the augmentation and suppression of antitumour immune responses (Smyth and Godfrey, 2000; Wilson and Delovitch, 2003). Finally, Peng *et al*. (2007) recently provided evidence that γδ T cells purified from the microenvironment of breast tumours have potent suppressive capability. However, the relative importance of non-$CD4^+$ Treg cells against human tumours *in vivo* has yet to be fully defined.

The question of whether Treg cells found at high frequency in tumours are recruited from the periphery or are activated/matured locally at the tumour site still remains unclear. $CD4^+CD25^+$ Treg cell homing to tumour sites has been shown to occur via CCR4 engagement of CCL22 and CCL2 expressed by tumour cells (Curiel *et al*., 2004; Hirahara *et al*., 2006; Jordan *et al*., 2007). However, there is also growing evidence that both $CD4^+CD25^+$ Treg cells and IL-10- and TGF-β-secreting Treg cells can be expanded or induced locally at the tumour site. Immunosuppressive molecules commonly produced by tumour cells, including IL-10, TGF-β and vascular endothelial growth factor, act on DCs to prevent maturation and to induce a suppressive phenotype (Zou, 2005) capable of inducing IL-10-secreting $CD8^+$ T cells (Wei *et al*., 2005) and IL-10-secreting $CD4^+$ T cells (Chakraborty *et al*., 2004) and stimulating proliferation of $CD4^+CD25^+$ Treg cells (Ghiringhelli *et al*., 2005). Recent studies have also demonstrated that TGF-β in the tumour microenvironment can act directly either on the T cells or DCs to allow the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Tregs (Chen *et al*., 2003; Fantini *et al*., 2004; Liu *et al*., 2007). Thus, the tumour microenvironment is likely to contain Tregs that have been recruited as well as those that are differentiated and/or expanded locally.

The benefit of depletion or inhibition of Treg cells or their products

One of the major challenges of tumour immunotherapy and vaccination is likely to be the development of strategies to overcome the suppressive environment of tumours. Evidence of the importance of the Treg cell and a general regulatory environment of tumours comes from several clinical investigations showing that the frequency of Treg cells in tumours correlates with poor clinical outcome. Several reports have described a significant positive correlation between tumour-infiltrating Treg cell numbers and overall survival in hepatocellular carcinoma and ovarian cancer patients (Curiel *et al*., 2004; Sato *et al*., 2005; Wolf *et al*., 2005; Fu *et al*., 2007). However, there are contradictory reports suggesting that increased expression of Treg markers in certain lymphomas is associated with improved survival (Alvaro *et al*., 2005; Carreras *et al*., 2006), indicating that in certain tumours Treg cells may serve a protective role.

Evidence of a role for Treg cells influencing the course of human cancers is supported by experimental studies in mice demonstrating the benefits of Treg cell depletion on tumour progression. $CD4^+CD25^+$ Treg cells have been targeted for elimination using depleting anti-CD25 antibodies. Evidence from mouse models has demonstrated that depletion of $CD25^+$ T cells can result in the development of tumour-specific effector T cells, which cause tumour regression (Onizuka *et al*., 1999; Shimizu *et al*., 1999; Golgher *et al*., 2002; Jarnicki *et al*., 2006). When combined with vaccination and local tumour radiation, CD25 depletion led to the elimination of established tumours (Kudo-Saito *et al*., 2005). The promising results from animal models led to the assessment of two compounds, human anti-Tac (anti-CD25) (Kreitman, 2004) and ONTAK (IL-2 receptor-binding domain of IL-2 fused to diphtheria toxin) (Foss, 2000), in clinical trials for the treatment of human cancer; however, such approaches have had limited success to date (Frankel *et al*., 2002; Kreitman, 2004; Zou, 2006). A major concern with this approach is a lack of specificity; activated $CD4^+$ and $CD8^+$ cytotoxic T cells also express CD25 and thus depletion of cells expressing this molecule may prove more damaging than beneficial. In addition, global depletion of Treg cells may leave patients vulnerable to autoimmune disease.

The co-stimulatory molecule CTLA-4 is a key negative regulator of T-cell responses and is expressed at high levels on Treg cells. It has been shown in several animal models that CTLA-4 signalling can restrict antitumour immune responses (Leach *et al*., 1996; Kwon *et al*., 1997; Espenschied *et al*., 2003). Antibodies against CTLA-4 are currently being tested for their ability to treat human tumours. Evidence of tumour regression with prolonged time to progression has been seen in patients with melanoma treated with anti-CTLA-4 (Phan *et al*., 2003; Ribas *et al*., 2005) and durable responses have been observed in patients with several other tumour types (Hodi *et al*., 2003; Attia *et al*., 2005; Weber, 2007). However, treatment of tumours with anti-CTLA-4 antibodies is associated with the induction of a wide range of grade 3 and 4 autoimmune manifestations,

potentially limiting its usefulness in the clinic (Phan *et al*., 2003; Attia *et al*., 2005; Weber, 2007).

In addition to CD25 depletion and CTLA4 blockade, other approaches, including anti-GITR antibody therapy (Ko *et al*., 2005), antibody blockade of PD-1 (Iwai *et al*., 2002; Curiel *et al*., 2003; Hirano *et al*., 2005) and neutralization of immunosuppressive cytokines, such as IL-10, TGF-β, IL-13 and vascular endothelial growth factor (Lizee *et al*., 2006), have been assessed for their ability to reverse Treg cell-mediated suppression of antitumour responses. However, it is likely that such non-targeted approaches will share some or all of the problems associated with CD25 depletion and anti-CTLA-4 treatment. All of the above approaches highlight the need for a holistic approach to tumour immunotherapy that works to combine the prevention of regulatory cells while augmenting/inducing strong antitumour immunity. Preferably, such approaches would work upstream of the induction of regulation so as to prevent, rather than fight, the effects of tumour-infiltrating Treg cells.

TLR ligands as immunotherapeutics or adjuvants for cancer vaccines

The activation of innate immune cells is fundamental to the development of effective adaptive immune responses. Innate immune cells not only act as antigen-presenting cells for T cells, but also provide signals necessary for T-cell activation. In addition, cytokines secreted by innate immune cells help to direct the induction of distinct T-cell subtypes (Janeway and Medzhitov, 2002). Innate immune cells express pathogen recognition receptors, which allow them to sense and respond to PAMPs, conserved structures expressed by pathogens. Pathogen recognition receptors include TLRs, which are membrane glycoproteins with a cytoplasmic Toll/IL-1 receptor homologue signalling domain and an external PAMP recognition domain with leucine-rich repeats (Creagh and O'Neill, 2006). Binding of ligands for TLRs, such as lipopolysaccharide (LPS) to TLR4, flagellin to TLR5 or CpG to TLR9, activates intracellular signalling pathways through NF-κB, mitogen-activated protein kinases and interferon regulatory factors 3, leading to the production of pro-inflammatory cytokines and type I interferons (IFN), which in turn activate co-stimulatory molecule expression, necessary for the antigen-presenting cell function of DC and macrophages. Therefore, TLR agonists are potent activators of innate and adaptive immune responses and when coadministered with a foreign antigen can act as adjuvants to promote immune responses specific for the foreign antigen. This property has been exploited in the development of infectious disease vaccines, where they are effective as adjuvants in promoting protective T-cell and antibody responses against pathogenic antigens (Krieg, 2006; Kanzler *et al*., 2007). However, they can also help to break tolerance and allow the induction of immune responses to self–antigen, and have therefore also been exploited as tumour immunotherapeutics and as adjuvants for cancer vaccines.

An important immunomodulatory property of TLR agonists is their capacity to enhance IL-12 production from DC and other innate immune cells and consequently their ability to promote Th1-type responses (Hemmi *et al*., 2000; Agrawal *et al*., 2003), which play a key protective role in immunity to tumours. DCs activated with TLR agonists, especially TLR8 agonists, stimulate IL-12-producing human myeloid DCs, which activate CD8 CTL against tumours (Xu *et al*., 2006). This along with their other pro-inflammatory properties has put TLR agonists to the forefront of potential immunotherapeutic agents against cancer.

TLR ligands as tumour immunotherapeutics

Mycobacteria bovis Bacillus Calmette–Guerin has been used for over 30 years as a immunotherapy against bladder cancer (Brandau and Suttmann, 2007). It has recently been shown that Bacillus Calmette–Guerin cell wall skeleton can act as an immunomodulator through PAMPs activation of DC via TLR2 and TLR4 (Uehori *et al*., 2003). More recently, synthetic ligands for TLR7, TLR4 and TLR9 have been through preclinical evaluation and clinical trials against cancer (Krieg, 2006; Kanzler *et al*., 2007). Furthermore, the TLR7 target imiquimod (Aldara), developed by 3M Pharamaceuticals, has been licensed for use as a topical preparation against basal cell carcinoma (Beutner *et al*., 1999). CpG and other TLR9 agonists are in phase I, II or III trial for non-small-cell lung carcinoma, non-Hodgkin's lymphoma, renal cell carcinoma and colorectal cancer (Krieg, 2006). The TLR4 agonist OK-432 from *Streptococcus pyogenus* or a purified lipoteichoic acid-related derivative, OK-PSA, have been evaluated in uterine cervical cancer and non-small-cell lung cancer (Watanabe and Iwa, 1987; Kikkawa *et al*., 1993). While many of the trials reported enhancement of antitumour immune responses, there was no definitive mechanism of action and in many patients there was no discernable clinical response (Krieg, 2007). However, successful topical treatment of basal cell carcinoma with imiquimod, a synthetic TLR7 agonist, was associated with infiltration of myloid and plasmacytoid DCs in the peritumoral infiltrate and it appears that these cells may mediate direct antitumour cytotoxicity (Stary *et al*., 2007).

Studies in mice have demonstrated that TLR agonists have the potential to break tolerance to self-antigens by inhibiting the function of Treg cells (Pasare and Medzhitov, 2003; Yang *et al*., 2004; Peng *et al*., 2005). While prophylactic administration of CpG or CpG with irradiated tumour cells did not prevent establishment of C26 tumours, peritumoral administration of CpG alone or with irradiated tumour cells was effective at treating established tumours (Heckelsmiller *et al*., 2002b). The protection was dependent on injection of CpG into the tumour site and was mediated by induction of tumour-specific $CD8^+$ T cells. Furthermore, DC pulsed with tumour antigen and CpG at a distant site to the tumour combined with peritumoral injection of CpG resulted in regression of large murine tumours resistant to chemotherapy (Heckelsmiller *et al*., 2002a). It has also been demonstrated that injection of DCs pulsed with antigen (irradiated tumour cells) and

CpG is effective at treating established C26 tumours in mice (Bourquin *et al*., 2006). Therapeutic administration of DC pulsed with a human papillomavirus-16-derived peptide mixed with CpG-ODN resulted in eradication of HPV-16-expressing tumours in mice (Zwaveling *et al*., 2002). Synergy between different TLR agonists in activation of innate immune cells and induction of Th1 cells is now well documented (Napolitani *et al*., 2005). The TLR9 ligand CpG has been shown to synergize with the TLR5 ligand flagellin to promote antitumour immunity (Sfondrini *et al*., 2006). Furthermore, the synergy between CpG and the TLR3 ligand dsRNA in promoting antitumour immunity has been linked with synergistic activation of IL-6 and IL-12 production (Whitmore *et al*., 2004).

TLR ligands as adjuvants in cancer vaccines

In addition to their use as direct tumour immunotherapeutics where the aim is to generate immune responses against antigens derived from the tumour *in vivo*, TLR agonists have also been used as adjuvants for co-administration with tumour antigens, either peptides, recombinant proteins or killed tumour cells. Immunization of melanoma patients with the melanoma antigen Melan-A/MART-1 formulated in incomplete Freund's adjuvant with CpG-ODN induced strong antigen-specific $CD8^+$ T-cell responses (Speiser *et al*., 2005). Prophylactic immunization of Tg mice expressing the Her-2/*neu* gene product with a synthetic peptide corresponding to a CTL epitope from Her-2/neu in combination with CpG-ODNs was effective at preventing tumour growth in mice (Nava-Parada *et al*., 2007). Therapeutic immunization delayed tumour growth but mice eventually developed large tumours and had to be euthanized. Furthermore, therapeutic administration of CpG combined with depletion of $CD25^+$ Treg cells did not alter the course of tumour growth. However, depletion of $CD25^+$ cells prior to therapeutic immunization with the peptide and CpG resulted in a significant augmentation of tumour-specific CTL responses and a reduction in tumour growth (Nava-Parada *et al*., 2007). Peritumoral injection of CpG and polyI:C enhanced the therapeutic efficacy of recombinant adenovirus expressing human tyrosinase-related protein 2 against established B16 melanoma in mice (Tormo *et al*., 2006). In a transgenic mouse model where the mice expressed SV40 T Ag (Tag) and developed spontaneous tumours, prophylactic but not therapeutic immunization with Tag with CpG-ODN prevented tumour growth (Garbi *et al*., 2004). Despite the activation of innate immunity, it appears that the therapeutic vaccine failed to generate CTL or the effector T cells failed to infiltrate the established tumour and this was overcome by co-injection of pre-activated Tag-specific $CD4^+$ and $CD8^+$ T cells (Garbi *et al*., 2004).

TLR agonists can activate as well as inhibit Treg cells

It is now generally accepted that Treg cells have a major role in constraining antitumour immunity (Figure 2). The most convincing evidence comes from *in vivo* experiments in mice where depletion or inhibition of Treg cells enhanced antitumour immunity and slowed tumour progression (Onizuka *et al*., 1999; Shimizu *et al*., 1999; Golgher *et al*., 2002; Jarnicki *et al*., 2006). Depletion, inhibition or preventing the induction of Treg cells also has considerable potential for augmenting immunotherapeutic and vaccine approaches against cancer. Indeed, there is already some direct and indirect evidence of the beneficial effects of Treg cell depletion during vaccination either against tumours or infectious disease antigens. Early studies had shown that cyclophosphamide treatment could enhance the therapeutic effect of T-cell immunotherapy by eliminating tumour-induced suppressor T cells (North, 1982). More recently, it has been demonstrated that depletion of $CD25^+$ T cells in metastatic renal cell carcinoma patients using recombinant IL-2 diphtheria toxin conjugate DAB_{389}IL-2 (ONTAK) enhanced tumour-specific CTL responses induced by intradermal injection of DC transfected *in vitro* with tumour RNA and matured with IL-1β, tumor necrosis factor-α and PGE2 (Dannull *et al*., 2005).

Studies in mouse models that have inhibited or depleted natural Treg cells with antibodies against CD25, GITR or CTLA4 have also provided convincing evidence that Treg cells can constrain the induction of effector T-cell responses by vaccination. Depletion of $CD25^+$ T cell prior to vaccination of mice with a herpes simplex virus 1 peptide combined with CpG enhanced virus-specific CTL responses and enhanced protection against viral challenge (Toka *et al*., 2004). Depletion of $CD25^+$ cells 7 or 35 days prior to administration of mature DC pulsed with a model antigen enhanced the induction of antigen-specific T cells *in vivo* (Oldenhove *et al*., 2003). The combination of $CD25^+$ T-cell depletion and CTLA4 blockade enhanced the therapeutic efficacy of granulocyte–macrophage colony–stimulating factor-producing tumour cell vaccine against B16 melanoma in mice (Sutmuller *et al*., 2001). However, this study also demonstrated that CD25 depletion can remove effector T cells and can decrease the efficacy of prophylactic vaccination. Furthermore, vaccination of mice harbouring a B-cell lymphoma expressing influenza virus haemagglutinin (HA) with recombinant vaccinia virus-HA expanded Treg and these cells suppressed effector T cells and IFN-γ production (Zhou *et al*., 2006).

The use of TLR agonists as vaccine adjuvants and tumour immunotherapeutics is based on the belief that these cells selectively promote the differentiation of IFN-γ-secreting Th1 subtype of $CD4^+$ T cells. However, studies on the basic biology of TLR agonist activation of cells of the innate and adaptive immune systems suggest that TLR agonists may have the potential to inhibit as well as enhance effector T-cell responses that mediate antitumour immunity (Table 1). Nevertheless, with the exception of TLR2 agonists, many ligands for TLRs have been shown to promote the induction of Th1 cells against model antigens (Hemmi *et al*., 2000; Agrawal *et al*., 2003). The demonstration that TLR agonists can precipitate autoimmune diseases in mouse models (Obermeier *et al*., 2002) (Ichikawa *et al*., 2002; Ronaghy *et al*., 2002) has also been used as evidence that TLR

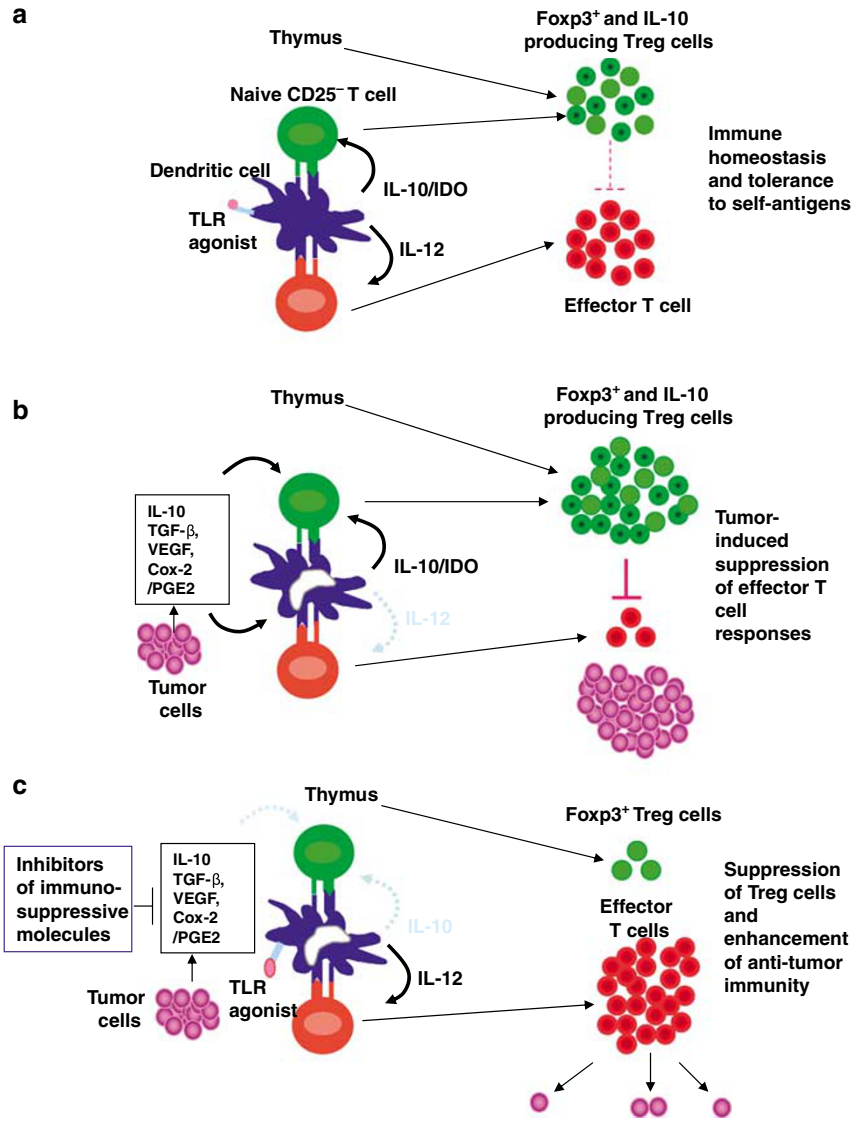

Figure 2 Regulatory T (Treg) cell control of antitumour immunity and their manipulation in the development of tumour immunotherapeutics. (**a**) Immune homeostasis in normal individuals is in part maintained by $CD4^+CD25^+Foxp3^+$ Treg cells that emerge from the thymus and exert regulatory control on effector T-cell responses to self-antigens. The introduction of TLR agonists during infection or sterile inflammation promotes interleukin (IL)-10 and IL-12 production by dentrite cells (DCs) (and other innate immune cells), and as a consequence activation of IL-10-secreting Treg cells as well as effector Th1 cells. (**b**) During the development of cancer, tumour cells express a range of immunosuppressive molecules, including IL-10, transforming growth factor-β (TGF-β), indoleamine 2-3 dioxygenase (IDO), vascular endothelial growth factor (VEGF), cyclooxygenase-2 (Cox-2)/prostaglandin E2 (PGE2), or induce anti-inflammatory molecule production by DCs. These help to expand the population of IL-10-secreting Treg cells and convert $CD4^+CD25^-$ T cells to $CD4^+CD25^+Foxp3^+$ Treg cells, which inhibit the induction and function of effector T cells allowing tumour progression. (**c**) Administration of TLR agonists alone or with tumour antigens can help to break tolerance to the tumour antigen, but can stimulate the production of anti-inflammatory molecules that promote further Treg cell expansion. However, inhibition of immunosuppressive molecules induced by the tumour during TLR immunotherapy should allow selective expansion of effector T cells and the development of antitumour immunity.

agonists can promote Th1 cells specific for self-antigens. However, recent evidence has suggested that these autoimmune diseases are mediated not by Th1 cells but by IL-17-secreting T cells (Langrish *et al*., 2005) and that Th1 cells as well as classical Treg cells, which are also induced against self-antigens during the development of autoimmunity in mouse models, may play a regulatory role (Boccaccio *et al*., 1999; Korn *et al*., 2007). TLR9 agonists have also been shown to protect mice from colitis through induction of IFN-α production (Katakura *et al*., 2005). Furthermore, a synthetic lipopeptide agonist of TLR2 has been shown to stimulate IFN-γ and IL-10 production, which had a regulatory role against Th2 type responses (Akdis *et al*., 2003).

It has been reported that TLR agonists can break tolerance to self-antigens by directly or indirectly inhibiting

Table 1 Direct and indirect effect of TLR agonists on Treg cell function

TLR	*TLR agonist*	*Cell type acting on*	*Effect on regulatory cell*	*Influence on effector immune response*	*Reference*
TLR2	PGN	Mu $CD4^+CD25^+$	Promotes survival and IL-10 production	Reduces resistance to *Candida albicans*	Netea *et al*. (2004)
TLR2	Pam_3Cys	Mu $CD4^+CD25^+$	Induces proliferation and temporary loss of suppressive function	Enhances effector responses to *C. albicans* infection *in vivo*	Sutmuller *et al*. (2006)
TLR2	Pam_3Cys-SK4	Mu $CD4^+CD25^+$	Enhances proliferation and transient suppression of Foxp3 expression	Renders effectors resistant to suppression	Liu *et al*. (2006)
TLR2	Lipo-peptide	Mu T cells	Enhances IL-10 and IFN-γ production	Inhibits Th2 responses	Akdis *et al*. (2003)
TLR2 TLR3 TLR4 TLR9	LTA polyI:C LPS CpG	Mu DC	Induces IP-10 production by regulatory DCs	Recruits and inhibits Th1 proliferation	Qian *et al*. (2007)
TLR4	LPS	Mu $CD4^+CD25^+$	Enhances proliferation and suppressive function	Controls $CD4^+$ T cell-dependent wasting disease	Caramalho *et al*. (2003)
TLR4	LPS	Mu $CD4^+CD25^+$	Induces IL-10, IFN-γ and IDO in the presence of DCs		Fallarino *et al*. (2003)
TLR4	LPS	Mu DC	Promotes IL-10-secreting Treg cells	Control infection-induced immunopathology	Higgins *et al*. (2003)
TLR4	LPS	?	Induces IL-10-secreting Treg cells	Inhibits $CD8^+$ T-cell responses	den Haan *et al*. (2007)
TLR4	LPS	Mu DC	Induces IL-6 and blocks suppressive function of $CD4^+CD25^+$ T cells	Enhances $CD4^+CD25^-$ proliferation	Pasare and Medzhitov (2003)
TLR4	LPS	Mu DC	Reverses Treg cell anergy	Enhances $CD4^+CD25^-$ T-cell proliferation	Kubo *et al*. (2004)
TLR4 TLR8	LPS CpG	Mu DC	Blocks $CD4^+CD25^+$ T-cell tolerance	Enhances CD8+ T-cell responses to tumours	Yang *et al*. (2004)
TLR5	Flagellin	Hu $CD4^+CD25^+$	Enhances Foxp3 expression and suppressive function	Supresses $CD4^+CD25^-$ T-cell proliferation	Crellin *et al*. (2005)
TLR5 TLR7/8	Flagellin R484	Hu $CD4^+CD25^+$	Enhances IL-10 and IFN-γ production		Caron *et al*. (2005)
TLR9	CpG	Mu $CD4^+CD25^+$	Blocks suppressive function	Enhances naive T-cell proliferation	Peng *et al*. (2005)
TLR9	CpG	Mu DC	Induces IDO-dependent T regulatory responses	Suppresses T-cell proliferation in MLR	Mellor *et al*. (2005)
TLR9	CpG	Mu DC	Enhances DC IL-10 and Treg induction	Constrains antitumour immunity	Jarnicki (in review)

Abbreviations: DC, dendrite cell; Hu, human; IDO, indoleamine 2-3 dioxygenase; MLR, mixed lymphocyte culture; Mu, murine; IFN, interferon; IL, interleukin; LTA, lipoteichoic acid; LPS, lipopolysaccharide; PGN, peptidoglycan; TLR, Toll-like receptor; Treg, regulatory T cells.

Treg cell responses (Pasare and Medzhitov, 2003; Yang *et al*., 2004; Peng *et al*., 2005). TLR-induced IL-6 production by DC blocked the suppressive function of $CD4^+CD25^+$ Treg cells (Pasare and Medzhitov, 2003). More recently, it has been demonstrated that immature DCs are required for the suppressive function of $CD4^+CD25^+$ Treg cells and that TLR activation of IL-1 and IL-6 production by DCs is required to reverse Treg cell anergy (Kubo *et al*., 2004). Thus, it appears that TLR ligands can indirectly enhance the suppressive function of Treg by promoting inflammatory cytokine production by DCs. TLR agonists can also act directly on T cells. Synthetic and natural ligands for TLR8 can reverse the suppressive function of human $CD25^+$ Treg cells. Transfer of Treg cells enhanced tumour growth in mice; this was reversed when the Treg cells were stimulated with a TLR8 ligand (Peng *et al*., 2005). Recombinant vaccinia virus expressing influenza virus HA can protect HA-transgenic mice against tumour challenge by induction of antigen-specific $CD8^+$ CTL, but HA-pulsed DC could only induce protection when $CD25^+$ Treg cells were depleted or following four daily administration of LPS or an unrelated virus (Yang *et al*., 2004).

In addition to the reports that TLR agonists can inhibit Treg cell function, there is also evidence that TLR agonists can directly or indirectly, through activation of innate cells, induce differentiation, proliferation or activation of Treg cells (Table 1). Murine $CD25^+$ Treg cells express TLR4, 5, 7 and 8, and stimulation with the TLR4 agonist LPS induced proliferation and enhanced the suppressive function of $CD25^+$ Treg cells (Caramalho *et al*., 2003). In addition, the TLR2 ligand Pam_3Cys can induce proliferation of murine $CD4^+CD25^+$ T cells and temporary loss of Treg suppressive activity, which is restored after the removal of the TLR agonist (Sutmuller *et al*., 2006). Studies with TLR-defective mice have demonstrated that TLR signalling does play a role in the induction or expansion of Treg cells. TLR4-defective mice have reduced inducible Treg cells as a result of attenuated IL-10 production by DCs and as a consequence have enhanced immunopathology in the lungs during *Bordetella pertussis* infection (Higgins *et al*., 2003). Furthermore, TLR2-defective mice have reduced numbers of $CD25^+$ Treg cells and IL-10 production and enhanced resistance to infection with *Candida albicans* (Netea *et al*., 2004). It has also been reported that a TLR2 agonist can enhance proliferation of

$CD4^+CD25^+$ Tregs as well as $CD4^+CD25^-$ effectors, but also rendered effectors resistant to suppression by Treg cells and transiently suppressed Foxp3 expression (Liu *et al*., 2006). Human $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells have been shown to express TLR5, and stimulation of $CD4^+CD25^+$ T cells with flagellin and anti-CD3 enhances expression of Foxp3 and their suppressive function (Crellin *et al*., 2005). It has also been demonstrated that flagellin and the TLR7/8 ligand R-848 directly interact with purified $CD4^+$ T cells and enhance IFN-γ and IL-10 production independent of antigen-presenting cells (Caron *et al*., 2005).

While most investigations in mice have shown a positive effect of TLR ligands as adjuvants or immunotherapeutics on tumour progression, the successes in the clinic have so far been limited. Furthermore, there is indirect evidence from the mouse models that TLR agonists can promote potentially suppressive as well as beneficial effector responses. Peritumoral administration of the TLR5 agonist flagellin 8–10 days post challenge did not affect the growth of the weakly immunogenic murine breast carcinoma D2F2, but significantly reduced the growth of the same tumour expressing the human HER-2 oncoprotein. The protective effect of the flagellin was associated with enhanced IFN-γ production and reduced frequency of $CD4^+CD25^+$ Tregs in blood and spleen. However, when the TLR5 agonist was administered at the time of tumour challenge, it accelerated the growth of the antigenic tumour and this was associated with reduced IFN-γ production and enhanced $CD4^+CD25^+$ Tregs (Sfondrini *et al*., 2006). TLR ligands can induce immunosuppressive molecules that promote the expansion of Treg cells. CPG-ODN induces IFN-α-dependent expression of IDO in $CD19^+$ DCs, which mediate potent IDO-dependent T-cell suppression *in vivo* (Mellor *et al*., 2005). Furthermore, CpG-ODN-induced IDO in the lungs inhibits Th2-driven experimental asthma (Hayashi *et al*., 2004). IDO limits the initial-rate limiting step of tryptophan catabolism, and activation of $CD4^+CD25^+$ T cells with the TLR4 ligand LPS induces tryptophan catabolism in DCs (Fallarino *et al*., 2003). Therefore, TLR-activated Treg cells appear to be capable of enhancing the tolerogenic effect of DCs. Furthermore, stimulation of regulatory DCs with TLR2, TLR3, TLR4 or TLR9 agonists induces IP-10 production, which recruits Th1 cells, but inhibits proliferation of the recruited cells (Qian *et al*., 2007). CpG also induces Cox-2 and PGE2 expression in murine macrophages and spleen cells, and inhibition of Cox-2 enhanced serum levels of IFN-γ in response to injection with CpG (Chen *et al*., 2001).

Microbial molecules that promote IL-10 production from DC and other innate immune cells have been shown to promote the expansion of inducible Treg cells (McGuirk and Mills, 2002; Lavelle *et al*., 2003; Boyd *et al*., 2005). However, TLR agonists, in addition to inducing IL-12 production, can also stimulate IL-10 production from DC and thereby promote the induction of Tr1-type Treg cells as well as Th1 cells (AG Jarnicki *et al*., in review). Furthermore, it has recently been reported that LPS can promote IL-10-secreting Treg cells that inhibit $CD8^+$ CTL responses (den Haan *et al*., 2007). Inhibition of IL-10 with an anti-IL-10 receptor antibody combined with CpG activation was shown to reverse the tolerogenic state of tumour-infiltrating DC and augment their therapeutic efficacy in mouse tumour model (Vicari *et al*., 2002). We have recently demonstrated that selective modulation of TLR signalling in DC, such that production of IL-10 and other immunosuppressive molecules is inhibited and IL-12 is augmented, considerably enhances the therapeutic efficacy of antigen-pulsed DC against B16 tumours in mice (AG Jarnicki *et al*., in review; D Toomey *et al*., unpublished).

Conclusions

Studies in animal models have underlined the potential of TLR agonist as tumour immunotherapeutics and adjuvants for therapeutic vaccines. However, apart from the TLR7 agonist aldara, which is in human use for superficial basal cell carcinoma, the results from the clinic have not been that impressive and one company has recently halted clinical programmes with a TLR9 agonist in cancer because interim data from phase 3 trials showed that it failed to improve on the efficacy achieved with chemotherapy (Schmidt, 2007). While most publications have highlighted the pro-inflammatory and Th1-promoting activity of TLR agonists, many investigators have ignored the anti-inflammatory and regulatory arm, which is also induced by pathogen-derived ligands for TLRs as a means of controlling excessive inflammation and exerting feedback control to terminate immune responses. Approaches for selective manipulation of the innate immune responses induced by TLR agonists have potential to promote effector over regulatory T-cell responses and to enhance their efficacy against tumours (Figure 2). Although this could have a risk of inducing excessive inflammation, if used in the vaccine setting where it is administered infrequently with a tumour antigen, it has considerable potential for selectively promoting tumour-specific Th1 and CTL responses and thereby limiting tumour progression without causing excessive inflammation or systemic autoimmunity.

Abbreviations

Cox2, cyclooxygenase-2; CTL, cytotoxic T-lymphocytes; DC, dendritic cell; HSP, heat-shock protein; IDO, indoleamine 2-3 dioxygenase; PAMP, pathogen-associated molecular pattern; PGE2, prostaglandin E2; TLR, Toll-like receptor; Treg cell, regulatory T cell.

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