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TLR AGONISTS: Are They Good Adjuvants?

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Abstract

Therapeutic immunization leading to cancer regression remains a significant challenge. Successful immunization requires activation of adaptive immunity, including tumor specific CD4 + T cells and CD8+ T cells. Generally speaking, the activation of T cells is compromised in patients with cancer due to immune suppression, loss of tumor antigen expression, and dysfunction of antigen presenting cells (APC). APC such as dendritic cells (DC) are key for the induction of adaptive anti-tumor immune responses. Recently, attention has focused on novel adjuvants that enhance DC function and their ability to prime T cells. Agonists that target toll-like receptors (TLR) are being used clinically either alone or in combination with tumor antigens and showing initial success both in terms of enhancing immune responses and eliciting anti-tumor activity. This review summarizes the application of these adjuvants to treat cancer and the potential for boosting responses in vivo.

Keywords

Toll-like receptors; Cancer vaccines; Dendritic cells; Vaccine adjuvants

Background

Considerable evidence exists showing that the immune system protects the host against progressive growth of primary non-viral cancers and influences the immunogenicity of tumors, a concept known as immune surveillance (1). This has propelled studies to identify effective immune therapeutic approaches to eradicate or reduce outgrowth of human cancers. Immunotherapies fall into two broad categories: those that target antigen presenting cells (APC) by enhancing their ability to stimulate the immune system, and those that target the adaptive immune response i.e. T cells and B cells. In this review we discuss approaches that enhance the activity of APC such dendritic cells (DC). DC are the most potent APC and function to activate innate (e.g. natural killer cells (NK)), and adaptive immune responses

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which are mediated by B cells and T cells (Figure 1). Both innate and adaptive arms of the immune system are key for recognizing and eliminating tumor cells. Since vaccines generally elicit humoral and T cell responses, efforts are being directed towards approaches that elicit tumor-specific, integrated B cell, CD4+ and CD8+ T cell responses in vivo. A challenge in the field of cancer vaccines has been how to elicit these responses and ensure they are not only antigen-specific, but effective, durable and safely administered. In this review we discuss the use of Toll-like receptor agonists, novel compounds that activate DC and other members of the immune system in vivo, and are showing promise in the clinic as vaccine adjuvants in cancer patients.

Toll-like receptors (TLR)

Toll like receptors (TLR) are a family of pattern recognition receptors (PRR) that function as primary sensors of the innate immune system to recognize microbial pathogens. They were initially discovered as factors involved in the embryonic development and resistance of the fly *Drosophila* to bacterial and fungal infection (2,3). TLR recognize distinct structures in microbes, often referred to as “PAMPs” (pathogen associated molecular patterns). Ligand binding to TLR invokes a cascade of intra-cellular signaling pathways that induce the production of factors involved in inflammation and immunity (4,5). PRR also include intracellular proteins e.g. Nod-like receptors, RIG-1 like helicases (RLHs) as well as extracellular receptors, e.g. scavenger receptors and C-type lectin receptors (4,5). TLR, the topic of this review, are typically activated by microbial signals but may also be activated by endogenous ligands (e.g. heat shock proteins, fibronectin and fibrinogen) or synthetic compounds (Table 1). TLR can be expressed on members of the innate and adaptive immune system (DCs, macrophages, granulocytes, T cells, B cells, NK cells and mast cells), as well as by endothelial and epithelial cells (5). More recently, TLR have been found on tumor cells, including melanoma (6). In humans, ten TLR have been identified (Figure 1). These receptors comprise a family of conserved membrane spanning molecules containing an ectodomain of leucine-rich repeats, a transmembrane domain and an intracellular TIR (Toll/IL-1R) domain (7). TLR that are expressed on the surface of cells detect pathogens within the local environment (TLR1,-2,-4,-5,-6). TLR4 recognizes bacterial cell wall component lipopolysaccharide (LPS) through its ectodomain (8), in addition to MPL A (monophosphoryl lipid A). Lipoprotein and lipoteichoic acid are recognized by TLR2 in combination with TLR1 and TLR6, respectively (9). TLR5 recognizes bacterial flagellin (10). In contrast, certain TLR (TLR-3, -7/8, -9) are located within the endoplasmic reticulum (ER) and rapidly recruited to endosomal-lysosomal compartments, where they can detect microbial nucleic acids (dsRNA, ssRNA and ss DNA containing unmethylated CpG motifs, respectively (4,5)).

Ligand binding to TLR induces the recruitment of intracellular adaptors which form signal transduction complexes within the cytoplasm (7). This leads to the activation of signaling pathways including NF-KB and the MAP kinases p38 and JNK, which regulate the expression of genes involved in inflammation (cytokines) and immunity (MHC molecules, adhesion molecules). As different TLR signal through different combinations of adaptors, there is recruitment of dissimilar transcription factors and diverse gene induction. The endosomal receptors TLR7, -8, -9 are activated after ligand engagement and interact with the adaptor MyD88 (myeloid differentiation primary response gene), following which there is association with several signaling complexes ultimately leading to the activation of IRF7, NF- KB and MAP kinases (Figure 2, (7)). The expression of IRF7 facilitates the induction of high levels of type I interferons (IFN). TLR3, also located within endosomes, recognizes dsRNA or its synthetic mimic poly I:C. Unlike other TLR, TLR3 binding induces signal transduction via a MyD88 independent pathway, associating with the TRIF adaptor, signaling through IRF3, and inducing IFNβ production (7). LPS, after engaging TLR4,

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recruits several adaptors (TIRAP, MyD88, TRAM and TRIF) to the TIR intracellular domain. These adaptors subsequently engage both the MyD88 and TRIF- dependent signaling pathways (7).

Basis for using TLR agonists to treat cancer

William Coley, a NY surgeon, was a pioneer in using bacterial components "Coley's toxins" to treat cancer. He documented an association between infection and cancer (11), and subsequently tested extracts of *Streptococcus pyogenes* and *Serratia marcescens* in his patients, thus laying the foundation for using synthetic PAMPs in cancer therapy. PAMPs function by activating many types of APCs through their effects on epithelial and tumor cells. Human DC subsets express distinct TLR, and their response to stimulation is correspondingly differential. When stimulated, the myeloid or "conventional" subset of DC (mDC) which expresses TLR 1–8, upregulates activation markers (e.g. CD80, CD86, MHC class I and II, CCR7), produces pro-inflammatory cytokines and chemokines (e.g. TNF, IL-1, IL-6, MIP-1α, MIP-1β, MIP-3α) and primes antigen-specific CD4+ and CD8+ T cells (Figure 2). Moreover, these DCs acquire an enhanced capacity to take up antigens and present them in an appropriate form to T cells shown in Figure 3 (12). The plasmacytoid subset of DC (pDC) expresses only TLR7 and TLR9. Following activation via TLR7 or -9, pDC produce high levels of type I IFNs and chemokines, prime and boost T cells, and activate NK cells (13,14). Therefore, TLR ligation of these APCs are likely to have consequential effects on stimulating immunity in the host. Given that dying tumor cells may adversely affect DC function (15,16), activating DC with TLR agonists may be critical for priming anti-tumor immunity. TLR are also expressed on other immune related cells (macrophages, NK cells), epithelial cells and even some epithelial cell derived cancers, including breast cancer, squamous cell cancers, and melanoma (17). While the biological function of TLR expression remains to be determined, it has been suggested that TLR ligation may promote tumor progression, through induction of immune suppressive factors, by conferring resistance to apoptosis stimulating, regulatory T cell function or even promoting angiogenesis. Other studies involving TLR3 and TLR9 agonists, however, have shown enhanced production of pro-inflammatory cytokines and even induction of apoptosis (6,18,19). There is increasing evidence that TLR polymorphisms also influence the risk to cancer. Certain TLR4 polymorphisms for example have been associated with an increased risk in prostate cancer (TLR4), gastric cancer (TLR4), and colorectal cancer in certain populations. Follicular lymphoma has been associated with TLR2 polymorphisms, while variants of TLR3 and TLR10 may influence susceptibility to nasopharyngeal carcinoma in Chinese (17). There is evidence to suggest that the efficacy of radiation and chemotherapy in breast cancer requires TLR4 activation via endogenous agonists (high mobility group box-1, HMGB-1) released by dying tumor cells (Table 1). The TLR4 polymorphism (TLR4 Asp299Gly) is associated with worse outcomes in breast cancer patients receiving chemotherapy (20). Altogether, these findings provide a strong rationale for using TLR agonists in the clinic to promote anti-tumor immune responses.

Clinical application and efficacy of TLR agonists

Although TLR agonists have shown great promise in early stage cancers, their anti-tumor activity remains to be established in the adjuvant or metastatic setting. Furthermore, the mechanism(s) of anti-tumor activity has not been fully elucidated and will require further investigation. Agonists either in synthetic form, or as components of bacterial or viral vectors have been evaluated in the clinic. They have been studied either as single agents or in combination with tumor antigens (21). Evidence obtained thus far is consistent with findings in animal models, namely that they can induce potent immunity in humans in

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addition to clinical responses. Below, we summarize pre-clinical and clinical experience, including our own, with various TLR agonists.

Synthetic TLR agonists

TLR3 agonists—TLR3 is expressed on tissue and blood dendritic cells, monocytes, mast cells, NK cells and epithelial cells. Polyribosinic:polyribocytidic acid (Poly I:C), is a synthetic dsRNA complex, which directly activates DC and also triggers NK cells to kill tumor cells (22). In addition to being recognized by endosomal TLR3 (23), it induces high levels of type I interferons and activates several nuclear and cytoplasmic enzyme systems (oligoadenylate synthetase [OAS], the dsRNA dependent protein kinase [PKR], RIG-I Helicase, and MDA5 [melanoma differentiation associated gene]. that are involved in antiviral and antitumor host defenses (5). It has been shown to have broad gene regulatory actions as well. Poly I:C induces prolonged survival of tumor bearing rodents following IP or IV administration, and enhances antigen-specific responses to viral antigens, especially with anti-CD40 (24–27). It appears to exert its therapeutic effects through eliciting antibody responses (28,29), enhancing cross-priming (23), stimulating anti-tumor CD8+ T cells (30–32), and antigen-specific CD4+ T cells (33). Relative to several other toll-like receptor (TLR) agonists, poly IC is the most effective inducer of type I interferon (IFN), which seems to be required for DC maturation and development of CD4+ T cell mediated immunity. Besides TLR3, poly I:C also relies on the intracellular signaling molecule MDA5, to optimize the production of type I IFN (33). It is the TLR3 agonist formulation most extensively tested as a single agent in humans with infectious diseases and cancers including glioblastoma, renal cell cancer, melanoma, leukemia, ovarian cancer, breast cancer (34–41) (A. Salazar personal communication).

Other molecular TLR3 mimics include polyadenosine-polyuridylic acid (poly AU) Ampligen (polyI:polyC (12)U; Hemispherx Biopharma) and Polyinosinic-Polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose (Poly-ICLC, Hiltonol®). The latter is a more stable version of the TLR3 agonist and has been given IV or IM, 2 to 3 times weekly at doses of 10 to 50 μg/kg in patients with gliomas (42). A phase II study in adult patients with recurrent supratentorial anaplastic glioma treated with single agent poly-ICLC, showed no improvement in 6-month progression free survival compared to a historical database. Furthermore no objective radiographic response rates were observed. The authors suggested that poly-ICLC may confer a better outcome in combination with agents such as temozolomide. Administration of poly IC-LC in subjects with melanoma (35) and advanced renal cell cancer (43), was also well-tolerated and while no clinical benefit was observed, it was possible to consistently detect type I IFN in the serum 8 h after a single injection (median titer 199 U/ml), indicating a systemic effect. (35). Ongoing clinical trials are evaluating this agent more extensively in melanoma, prostate, cervical, ovarian, breast, colon and pancreatic cancers, including by intra-tumoral administration. Poly-ICLC has also been used nasally in a recent phase I randomized dose escalation trial in humans (N=50), and was well tolerated at all doses (A. Salazar personal communication). The most common side effects of low-dose Poly-ICLC are temporary discomfort at the injection site and occasional transient malaise, with flu-like symptoms in some patients.

Surprisingly, the agonist is only now being evaluated in humans directly in conjunction with tumor antigens. A phase I/II trial of patients with recurrent malignant glioma receiving intra-lymph-node injections of DCs loaded with HLA-A2 restricted peptides (derived from various glioma-associated antigens such as gp100, and 13Rα2) is currently under review. In addition participants received twice weekly IM injections of 20 ug/kg poly-ICLC. The frequency of CD8+ cells reactive to EphA2- or IL-13Rα2-tetramers was increased post-vaccination in the blood mononuclear cells in 9 of 11 participants evaluated. An interim

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analysis showed an association between positive tetramer response and 6-month progression-free survival, suggesting a possible correlation between antigen-specific responses and clinical response (44). In our own laboratory, we have found that poly I:C of most TLR agonists tested is the most potent activator of human mDC in vitro, and elicits strong anti-tumor immune responses to melanoma antigens (45). These data provide strong support for testing poly I:C together with tumor antigens in humans with cancer.

TLR4 agonists—Bacterial (LPS) and its derivatives are a commonly used vaccine adjuvants. Monophosphoryl lipid A (MPL) a detoxified component of LPS is derived from *Salmonella minnesota*, and contains the lipid A moiety that ligates TLR4 (46). Because it is substantially less toxic than LPS, has a history of safety and potently activates human DC, it has been incorporated into several vaccines. It also produces a different pro-inflammatory profile compared to LPS, including the production of type I IFNs, possibly because it may preferentially activate the TRIF vs. MyD88 signaling pathways (47). MPL has been approved for inclusion in a vaccine for Hepatitis B, Fendrix™ (48) and cervical cancer, Cervarix™ (49,50). Cervarix, a GSK product, is indicated for the prevention of diseases caused by oncogenic human papillomavirus (HPV) types 16 and 18, including cervical cancer, cervical intraepithelial neoplasia (CIN) grade 1 and 2 or worse and adenocarcinoma in situ. Besides MPL, the vaccine contains L1 capsid proteins from HPV and the adjuvant aluminum hydroxide. 100% protection of the vaccine was demonstrated in phase II trials against types 16 and 18 HPV, (51). Over 30,000 women have received this vaccine and it is now approved in both Europe and America. Vaccination offers protection for >6 years, making this vaccine a milestone in the prevention of cancer. A clinical trial to determine whether Cervarix is more effective than Merck's HPV vaccine Gardasil™, which contains L1 capsid proteins and aluminum hydroxyphosphate sulfate, in addition to a yeast protein is under evaluation. MPL is also a component of Glaxo Smith Kline's vaccine formulations for NSCLC and melanoma in combination with TLR9 agonists (see below). Phase IIB studies showed improved median survival time in patients with stage IIIB NSLC devoid of metastases (52).

MPL is a constituent of the DETOX adjuvant, an oil-droplet complex which also contains purified mycobacterial cell-wall skeleton (CWS) and has been used in combination with melanoma cell lysates (MelacineTM) or irradiated tumor cells (53). Melacine was granted approval in Canada based upon Phase III results demonstrating superior quality of life during active therapy for Stage IV melanoma as compared to a four-drug chemotherapy control, although both therapies achieved similar efficacy results. Furthermore, a meta-analysis of therapies for Stage IV melanoma showed that amongst Melacine recipients, the median survival of 11 months was better than that achieved by other therapies. Moreover, patients who were clinical responders to Melacine had a longer median survival. Melacine was also tested in resected stage II melanoma in a study conducted by the Southwest Oncology Group (SWOG). The primary endpoint was disease-free survival (DFS) in patients who received Melacine or no adjuvant therapy after surgical resection. While Melacine vaccination had no significant benefit in terms of prolongation of disease free survival in the total patient population, 38 percent of patients who expressed two or three of five different HLA genes (HLA-A2, HLA-A28, HLA-B44, HLA-B45, and HLA-C3) showed some benefit from vaccination (54).

Sialyl-Tn (STn) is a carbohydrate associated with the MUC1 mucin on a number of human cancer cells, is associated with more aggressive disease and has been incorporated into cancer vaccines for breast cancer and other epithelial tumors (THERATOPE, Biomera Inc.,). When linked to the neoantigen keyhole limpet haemocyanin (KLH), and given with DETOX, it is safe and may lead to occasional tumor regression in subjects with breast cancer (55). Immune responses in the form of both antibodies or cellular responses have

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been observed in patients with breast, colon and pancreatic cancers using sialyl-Tn (STn) or ras epitopes as antigens and DETOX (55–58) verifying the ability of MPL to stimulate an immune response to tumor associated antigens. MUC1 has also been incorporated into vaccines (BLP25, Stimuvax, Biomira/Merck) targeting non small cell lung cancer (NSCLC). These vaccines are composed of MUC1 peptide incorporated into liposomes containing MPL (52). In a randomized phase IIB trial, sc immunization with Stimuvax improved medium survival time by 17.3 months for patients with stage IIIB NSLC. Phase III studies are in progress.

Finally, results of a placebo controlled randomized phase II study testing GSK's AS-02b vaccine platform, (comprising MPL and the additional adjuvant QS21, a saponin), and the cancer testis antigen, MAGE A3, showed prolonged disease free survival in patients with resected stage Ib-IIIa NSLC (59,60). Ongoing randomized phase III studies are now evaluating MPL in GSK's advanced adjuvant platform AS-15 which also includes TLR9 agonists (see below).

TLR7/8 agonists—Imiquimod (3M)and Resiquimod (R848; 3M) are imidazoquinolines, synthetic immune modulators which target TLR7 and TLR8, TLR that typically recognize viral ssRNA (13,14,61,62). They have the advantage of activating both mDC *and* pDC stimulate innate and adaptive immune responses, while also activating NK cells (63,64). Type I IFN production by pDC facilitates direct priming of CD8+ T cells, as well as cross-priming through promotion of MHC and transporter of antigen peptides (TAP) molecules on DC (65,66). Interestingly, both compounds can activate caspase-1 through intracellular PRRs Nod-like receptors (NLR): cryopyrin/Nalp 3 to induce the production of IL-1β and IL-18 (67) which also facilitate adaptive immune responses. Imiquimod (formulated as 5% cream, Aldara™) is the only approved TLR7 agonist for treatment of genital warts (*C. acuminata*), actinic keratoses, basal cell carcinoma, and lentigo maligna (reviewed in (68)), where it has proven efficacy. It has also been used off-label to treat other HPV-associated lesions, as well as cutaneous melanoma (68). Imiquimod has been useful as adjunctive therapy to treat HIV-infected patients with intra-anal cancer by reducing recurrences of lesions (69). Resiquimod, related to Imiquimod binds to both TLR7 and -8, is a considerably more potent analog, and is in testing stages for treatment of genital HSV. In animal models, when administered together with peptides, proteins, or bacterial vectors and DNA constructs encoding tumor antigens (e.g. melanoma associated antigens, MAA), these agents augment anti-tumor activity (70). DC activity against tumor antigens, including MAA, can also be substantially enhanced *in vivo* if the APC are first activated with TLR7/8 agonists (71–73).

In humans, topical Imiquimod treatment enhanced the immunogenicity of a melanoma peptide vaccine when given with systemic Flt-3 ligand, which mobilizes DC systemically (74). Injection of immature human DCs into imiquimod-pretreated skin lead to DC activation *in situ* and enhanced migratory capacity to draining lymph nodes in cancer patients (75). Recently, we and other investigators demonstrated that imiquimod rapidly recruits significant numbers of human mDC and pDC into topically treated areas (Figure 4; (76)), enhances their survival, induces their trafficking to draining lymph nodes (73,77), and confers human mDC and pDC with cytolytic acivity against tumors in a perforin/granzyme B and TRAIL dependent fashion, respectively (78). Topical application of Imiquimod also caused reversal of T regulatory cell infiltration and suppressive activity in squamous cell cancers of the skin and restored the expression of E-selectin in skin blood vessels (79). In a vaccine trial, our group showed that intradermal injection of the CT antigen NY-ESO-1, as whole protein, into Imiquimod-treated skin of resected melanoma patients, primed new humoral and helper T cell responses and induced local infiltration of T, B, NK and activated mDC and pDC subsets (Figure 4 (21,76)). This study demonstrated, for the first time, the safety and adjuvant activity of Imiquimod when administered simultaneously with protein

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antigen. It also confirmed the agonist's mDC and pDC activating potential in vivo. Of note, no indoleamine 2'3'-dioxygenase (IDO) was detected *in vivo*. We have shown that IDO is triggered *in vitro* by ligation of TLR7/TLR9 on pDC. IDO metabolizes tryptophan to kynenurenine, which is responsible for the induction of T regulatory cells (14).

We are currently undertaking a randomized controlled study evaluating the immunogenicity of topical Resiquimod in combination with the cancer testis antigen NY-ESO-1 protein delivered SC in Montanide ISA 51. Given the proven efficacy of Imiquimod in treating cutaneous pre-malignant and malignant lesions, it is likely that these agents, in the absence of potent systemically administered formulations (80,81) will gain greater use in the treatment of additional cutaneous pre-malignant and malignant conditions e.g. cervical intraepithelial neoplasia.

TLR9 agonists—We and others have shown that synthetic oligonucleotides containing unmethylated CpG dinucleotide (CpG-ODN) bind TLR9 on pDC, leading to their activation and type I IFN production (13,14). Ligation of TLR9 on B cells induces their activation and proliferation (82). CpG-ODNs have been classified into three families: D-, K or C-type ODNs. These differ based on their backbones (phosphodiester or phosphorothioate), location and number of CpG dinucleotides, and palindromic sequences. In animal models, these constructs have anti-tumor effects when given either as monotherapy, or together with vaccines or other treatments (82–85). In humans, the responses to monotherapy, whether used to treat HCV (86) or cancers (non small cell lung cancer, cutaneous T-cell lymphoma, renal cell cancer, non-Hodgkin's lymphoma, chronic lymphocytic leukemia) regardless of delivery route (i.v., s.c, or intratumorally) or if given with adjunct chemotherapy have been generally low (68,82,87,88). Early phase 1 trials have shown that CPG ODN are well-tolerated at levels that can stimulate immune activation: NK cell activation, inflammatory cytokine production and reduction of regulatory T cell number in draining lymph nodes (89). These findings suggest that CpG should be used with additional agents to achieve maximal effects. Indeed, Romero et al., (90,91) showed that the addition of CpG to a Melan A/MART-1 HLA A2-restricted peptide and the "water in oil" adjuvant Montanide, dramatically increased the number of antigen-reactive cells elicited (upto 1.15% of circulating CD8+ T cells). Moreover, enhanced tumor-reactive CD8+ T-cell responses were also observed after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51 in patients with NY-ESO-1 expressing tumors and responses were associated with survival (92).

In a similar manner, we tested the immunogenicity of CpG plus NY-ESO-1 antigen emulsified in Montanide in patients with resected melanoma. Specific and strong integrated $CD4^+$ T cell and antibody responses were elicited in most vaccinated patients, along with $CD8^+$ T cell responses in approximately half the patients (93). These findings clearly established that protein antigen, presented on the right platform can elicit class I restricted responses. Given that peptide/Montanide combinations are significantly immunogenic (90,91), it will be important to dissect the precise contribution of Montanide vs. CpG agonist in additional trials.

Recently, GSK reported on immune responses in subjects with metastatic melanoma in a phase II randomized trial receiving their vaccine AS02B comprising MPL, MAGE A3 antigen, QS21 in oil/water emulsion vs. AS15, comprising CpG in addition to MPL, MAGEA3 antigen, QS21 and liposome formulation. The addition of CpG to the vaccine formulation significantly enhanced the induction of antigen-specific CD4+ T cell and antibody responses. A difference between arms in time to treatment failure was also documented (94). Phase III trials of MAGE A3/AS15 are in progress in patients with resected stage 1b-IIIa NSCLC and resected, high risk melanoma.

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TLR9 agonists have also been used in combination with chemotherapy, radiotherapy or monoclonal antibodies targeting CD20 molecules on B cells (Rituximab) with evidence of clinical activity. However, Phase III studies exploring the combination of PF-3512676 (a Pfizer product formerly known as CpG7909) with paclitaxel/carboplatin or gemcitabine/cisplatin vs. chemotherapy alone as first-line treatment of patients with advanced NSLC indicated no improvement in progression free survival or overall survival with the addition of the CpG-ODN (86,89)

Overall TLR9 agonists appear to be generally well tolerated with side effects including flu-like symptoms local injection site reactions and in some cases hematological side effects. Evidence is accumulating that they have potential as components of immunotherapies that are administered in the adjuvant setting, and are likely to work best when combined with other immunomodulators or treatments.

Pathogens expressing TLR agonists

Several inactivated or attenuated pathogens are components of vaccines against infectious agents (Table 1). Many are now also being tested as vectors for cancer vaccines. *Mycobacterium bovis*, (Bacillus-Calmette Guérin, BCG), is the only vaccine currently available for tuberculosis and is also approved to treat superficial bladder cancer and bladder cancer in situ. BCG cell wall skeleton and peptidoglycan activate TLR 2 and TLR4 signaling (95), and induce local inflammation in addition to tumor-specific immunity (96,97). Addition of BCG to vaccination with NY-ESO-1 protein has also led to induction of antibody and CD4+ T cells in humans (98). Yellow fever vaccine stimulates innate and adaptive immune responses through its ability to activate DC via TLR2, -7, -8 and -9 (99), and is being actively investigated as a vaccine adjuvant. Pox vectors, such as vaccinia or canarypox are being evaluated as vaccine vectors in melanoma and ovarian cancer amongst other tumors. Pox vectors expressing the cancer testis antigen NY-ESO-1 have proven immunogenicity (100) and current modifications involving the inclusion of co-stimulatory molecules may enhance their function further. Fowlpox vectors (ALVAC) have recently been used as a component of a preventive vaccine for HIV infection and may have a modest beneficial effect, (101). Pox vectors (e.g. Modified Vaccinia Ankara, MVA) can signal APC via TLR2 (102–104). Bacterial vectors recombinant for cancer-testis antigens such as *Salmonella typhimurium* may provide efficient stimulation by engaging multiple TLRs (105). Finally, adenovirus vectors expressing various tumor associated antigens including telomerase and cancer-testis antigens are in evaluation in the clinic. Adenovirus is reported to activate DC via TLR9 (106,107). Besides BCG, it remains to be seen whether other pathogens expressing TLR agonists will prove to be efficacious in treating either early or advanced stages of cancer.

Prospects for TLR agonists

We predict that synthetic TLR agonists will be most efficacious when used in optimal combinations together with antigen(s) and combined with other modalities including other vaccines, adjuvants and immune modulators. Activation of TLR9 with CpG ODNs for example, increases the immunogenicity of peptide-, DNA-, tumor cell- or DC-based vaccines (108). Fusion of antigen to the TLR agonist presents yet another attractive approach to enhance the immune response to poorly immunogenic antigens (109–111)

Route of injection is also likely to influence outcome. Intra-tumoral administration of TLR agonists may directly activate locally infiltrating DC, directly promote tumor cell apoptosis or sensitize tumor cells to cytotoxic agents. Intratumoral injection is safe when delivered in combination with rituximab (anti-CD20), an antibody which targets B cells (112,113), and the effects of local injection of poly I:C into cutaneous melanoma is currently under

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evaluation. Although clinical efficacy has not yet been shown, with the advent of more potent analogs it seems likely that intratumoral injection approaches in combination with other interventions will yield clinical responses of targeted lesions. Imiquimod, for example, is used off label to treat small in transit melanomas

Therapeutic interventions such as chemotherapy and radiation induce cell death leading to the release of tumor antigens and endogenous cellular factors (e.g. heat shock proteins, HMGB-1) that activate TLRs on DC, in addition to triggering various intracellular signaling pathways through the release of ATP (e.g. NLR members of the inflammasome, (114). Tumor antigens released as a consequence of cell death can be acquired by DC and crosspresented to T cells (20,115). In animal models, lymphodepletion (through radiation or chemotherapy) activates TLR4, an essential step in promoting the effectiveness of adoptively transferred T cells in preclinical models (116). Preclinical models also indicate that combining chemotherapy or radiation with systemic administration of synthetic TLR agonists, or vaccines which incorporate synthetic TLR agonists, are synergistic and enhance stimulation of anti-tumor immunity as well as tumor regression.

Certain adjuvants selectively activate other cellular non-TLR sensors a prominent example being aluminum hydroxide, an adjuvant that is the component of many FDA approved vaccines, (117). Alum formulations induce the secretion of IL-1β and IL-18 in vitro, and in vivo (IL-1β) and recruit and activate monocytes and granulocytes (118). Their role as inducers of the inflammasome has come under question but it is clear that they exert pro-inflammatory effects (117). Sharp *et al.* found that poly(lactic-*co*-glycolic acid) (PLGA) and polystyrene microparticles activated the NLRP3 pathway of the inflammasome (119), and TLR agonists (e.g. LPS) in conjunction with these experimental adjuvants or approved adjuvants such as aluminum hydroxide may be more effective when given in combination, as they mimic viruses and other pathogens which can target multiple pathways (118,120). Moreover, as TLR agonists may sensitize tumor cells to cytotoxic agents, their future lies in combination with other therapies including cancer vaccines and monoclonal antibodies. Interestingly, GSK's Cervarix vaccine contains MPL and aluminum hydroxide while its AS15 vaccine platform contains MPL, CpG ODN and QS21 (QuilA, a saponin extracted from the bark of the *Quillaria saponaria* tree) which triggers IL-1 release in an inflammasome-dependent way (121). New immune modulators such as anti-CTLA-4 and anti-PD-1 which block regulatory molecules on T cells and improve their anti-tumor function, may also improve the immunogenicity of TLR agonists.

Preclinical models indicate that combinations of TLR agonists are superior to individual use in vivo (122,123). TLR4 agonists act synergistically with TLR7, TLR8 or TLR9 agonists in the induction of a selected set of genes, including the Th1 polarizing cytokines IL-12 and IL-23, thereby conferring potent Th1 polarizing activity to human DC. Ligands for 3 TLRs (TLR2/6, TLR3, and TLR9) increased protective efficacy in mice towards a viral protein by enhancing the avidity of antigen-specific T cells, when compared with using ligands for any 2 of these TLRs (122). On the other hand, ligation of TLR2 and the PRR dectin-1 with zymosan, induces DC to produce IL-10 but not IL-12 or IL-6, and *in vivo* administration of zymosan suppresses antigen-specific responses response in a IL-10, and TGFβ dependent manner (124). Studies are needed to carefully evaluate stimulatory vs. inhibitory combinations of TLR agonists. In humans trials are investigating a combinatorial approach which include GSK's AS15 platform which includes agonists that activate both TLR4 and TLR9, thereby targeting both mDC and pDC subsets and allowing for optimal activation of both. The next decade is likely to bring several new synthetic agonists into the clinic such as acylated monosacharides that are structurally related to lipid-A, flagellin and novel TLR7 and TLR8 agonists. By taking advantage of the divergent signaling pathways used by various TLR to enhance DC activation, it should be possible to improve the overall immune

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response. An important goal will be to ascertain which combination of TLR agonists induces desirable anti-tumor immune responses (Th1, cytolytic T cells, NK cell activation) overcomes tolerance and reverses the immunosuppressive effects of T regulatory cells.

Challenges for the future

For TLR agonists to achieve recognition in the clinic it will be critical to undertake side-by-side comparisons against the same antigen using selected immune monitoring assays that measure the quantity and quality of responses (e.g. avidity, memory cell generation, durability). Through a program of the Cancer Vaccine Collaborative, a joint program of the Cancer Research Institute and the Ludwig Institute for Cancer Research, a coordinated global network of clinical trial sites has been conducting a series of parallel early-stage clinical trials to identify the optimal composition of successful therapeutic cancer vaccines. The antigens selected for these trials were primarily cancer/testis antigens, such as NY-ESO-1 and MAGE-A3, as well as melanoma differentiation antigens, such as Melan-A/MART-1. As discussed above, various forms of these antigens (peptides, protein, long peptides) have been mixed or co-administered with a series of Toll-like receptor ligands: CpG, Imiquimod, Resiquimod, PolyIC-LC, OK-432, Monophosphoryl lipid A (MPL), ISCOMatrix®, BCG. Additionally, these antigens have also been formulated as recombinant viruses (Vaccinia, Folwpox) or DNA endowed with natural CpG signals. Efforts such as these will yield important new information regarding successful vaccine platforms. Despite this progress, these studies highlight an endemic problem in the field of cancer vaccines: the lack of commercial availability of most of the TLR ligands discussed in this review. Efforts to systematically test these reagents, let alone to combine them, are thwarted by proprietary issues that should hopefully become less pronounced as these reagents prove their value in the clinic and become readily available.

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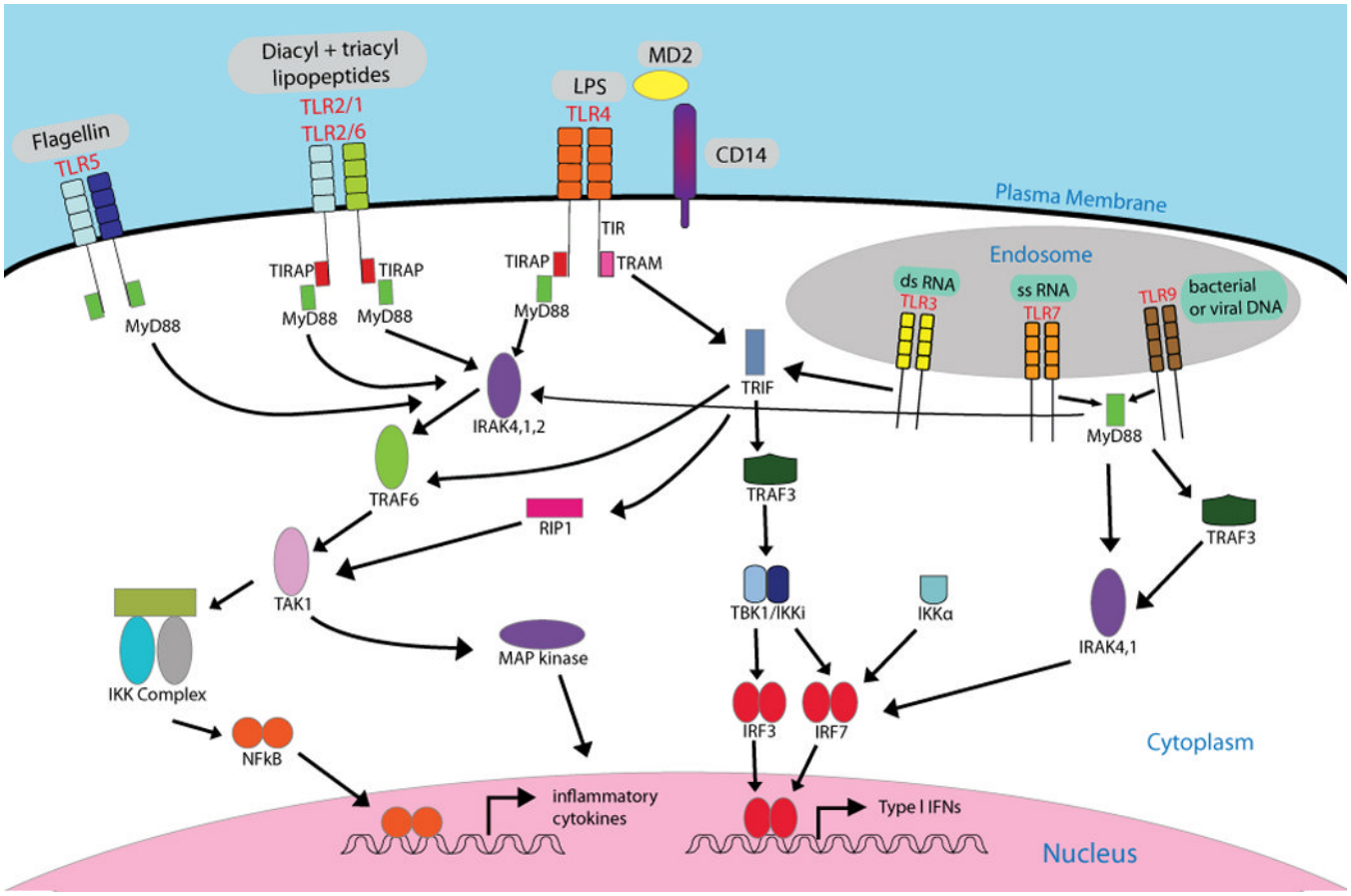

Figure 1. Expression of Toll-like receptors on innate immune cells

TLR 1,-2,-4, -5 and -6 are expressed in the plasma membrane where TLR2 associates with either TLR1 or TLR6. TLR3, -7, -8 and -9 traffic from the endoplasmic reticulum to the endosome where they encounter their ligands. MYD88 (myeloid differentiation primary response protein 88) and TRIF (TIR domain-containing adaptor protein inducing IFN) are signalling adaptors that link Toll-like receptors (TLRs) are to downstream kinases that define a given signalling pathway. All TLRs use MyD88, except for TLR3 which uses TRIF. The sorting adaptor TIRAP (TIR domain-containing adaptor protein) is used by TLR1, TLR2, TLR4 and TLR6 and links the TIR domain to MyD88, whereas TRIF is recruited by both TLR4 and TLR3. An additional adaptor TRAM, links the TIR domain of TLR4 with TRIF. TLRs which use the MyD88 dependent pathway recruit the IRAK family of proteins and TRAF6 resulting in the activation of TAK1. This in turn leads to the activation of NFKB and the MAPK pathway and results in the induction of pro-inflammatory cyokines and upregulation of phenotypic markers of activation (CD80, CD86). TLR4 (which relies on additional accessory molecules MD2 and CD14) and TLR3 both trigger the TRIF-dependent pathway, which also leads to activation of inflammatory cytokines via NFKB and MAP Kinase. In addition, TRIF recruits TRAF, leading to the activation of TBK1/IKKi, IRF3 and IRF7 and transcription of type I IFN. MyD88 also associates with the IRAK family of proteins. A complex of proteins (TRAF3, IRAK1 and Ikkα) subsequently activates IRF7. Examples of ligands binding the TLRs are shown. (Adapted from Kumar et al.,(125)).

Abbreviations: LPS, lipopolysaccharide; PtdIns(4,5)P2, phosphatidylinositol-4,5-bisphospate; TRAF3, TNFR-associated factor 3.

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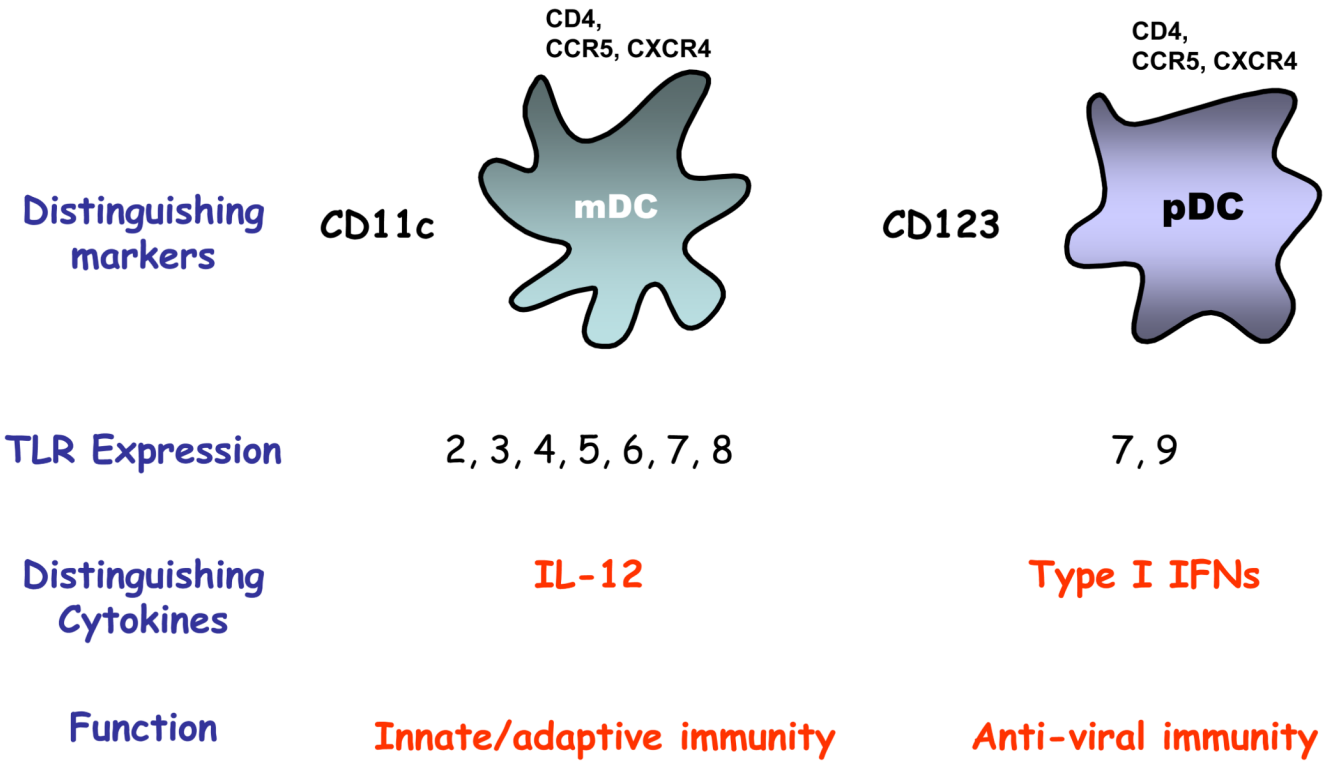

Figure 2. Major DC subsets in blood

There are two subsets in blood, the myeloid DC (mDC) or the plasmacytoid DC (pDC). They are distinguished by surface marker expression, TLR expression, cytokine production and primary functional roles. It is now appreciated that pDC can also participate in the induction of adaptive immune responses although their precise roles need to be determined. While pDC do not synthesize IL-12, mDC can produce type I IFN via TLR3 ligation.

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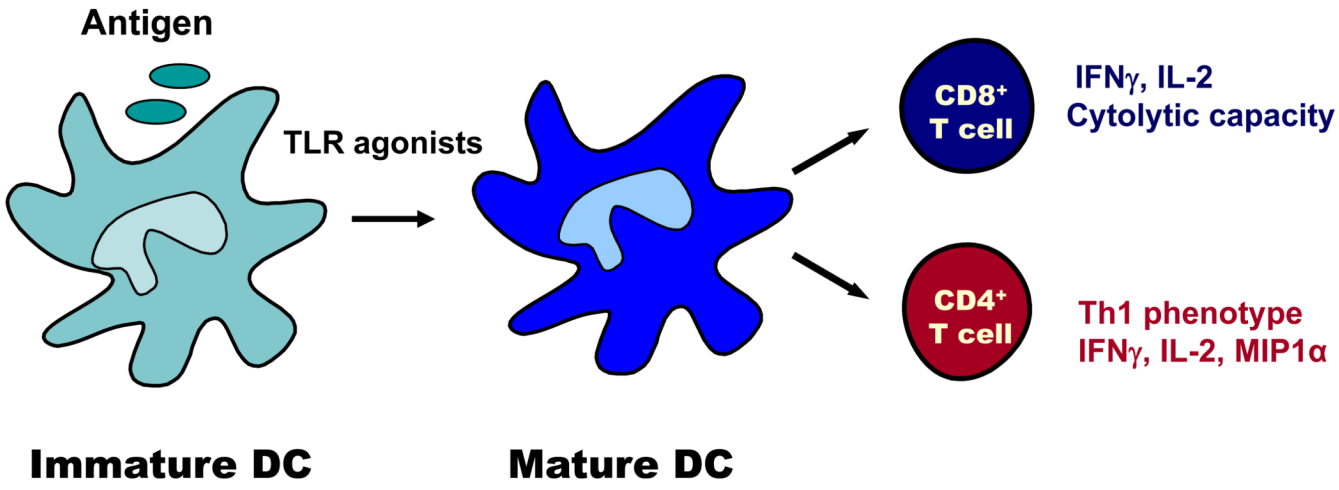

Figure 3a

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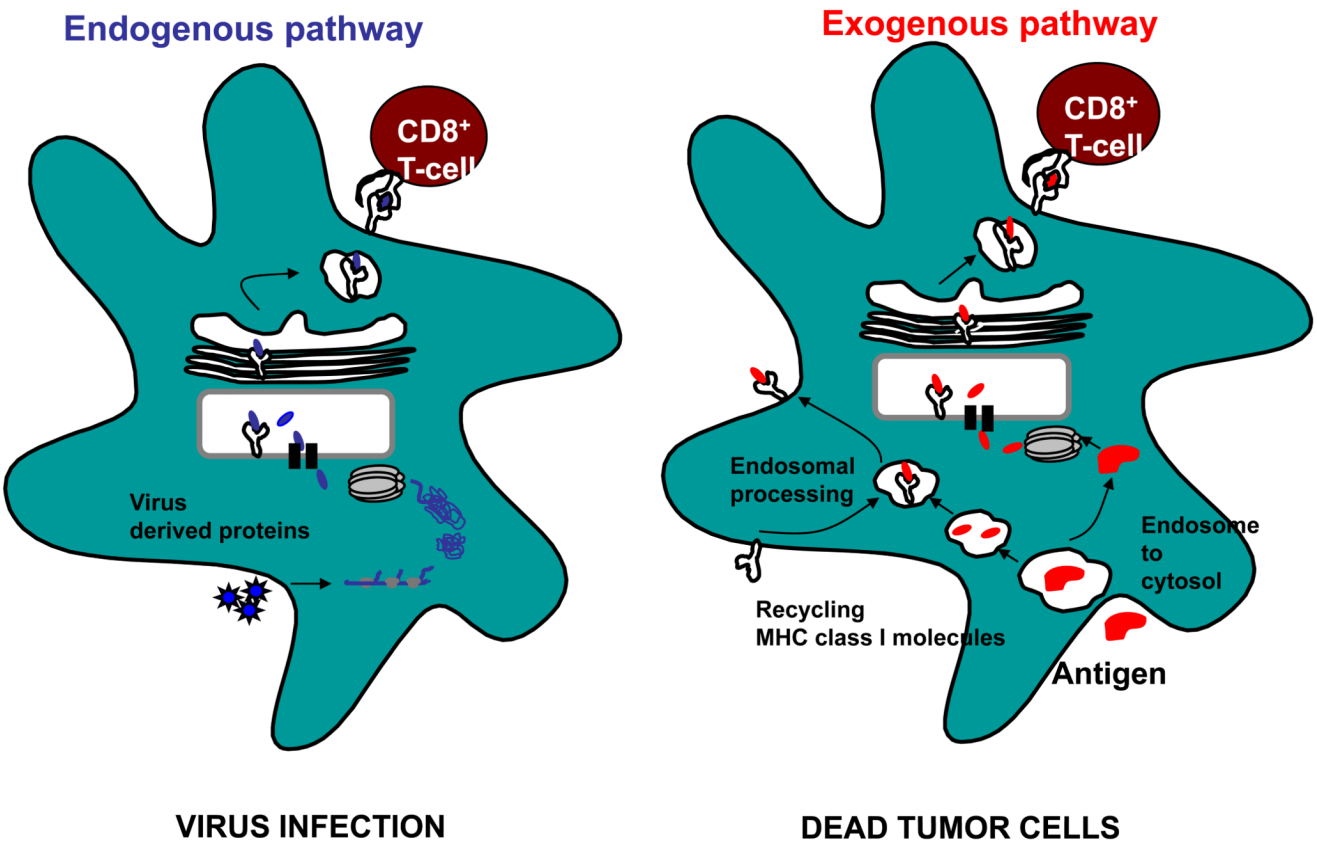

Figure 3b

Figure 3. DC undergo activation following ligation of TLR and prime CD4+ and CD8+ T cells

A. DC are most efficient at acquiring antigen when they are in their immature state through mechanisms that include phangocytosis, endocytosis and receptor mediated uptake. After encountering TLR ligands, they undergo maturation and upregulate HLA molecules (which present peptide antigens to T cell receptors on T cells) as well as co-stimulatory molecules such as CD80 and CD86, which interact with CD28 on T cells. DC also produce cytokines (IL-12, type I IFN) that aid in priming of CD4+ helper cells and cytolytic T cells. **B.** DC utilize endogenous and exogenous pathways to process and present antigens to CD8+ T cells. In the endogenous pathway, exemplified by virus infection or transduction of cells with RNA or DNA encoding antigens, antigen is processed in the cytoplasm by the proteosome and then transported into the ER where further processing can take place and peptides access newly synthesized HLA class I molecules. The peptide-HLA complex is then transported to the cell surface where it can interact with the T cell receptor. In the exogenous pathway, dying virus- infected cells or tumor cells (e.g. following chemotherapy or irradiation) are phagocytosed by DC and crosspresented to T cells. Dying tumor cells also release factors that activate DC via TLRs or components of the inflammasome. Antigens from these cells may access the cytoplasm and intersect with the conventional endogenous pathway of antigen processing. Alternatively, they may be processed within the endosomes themselves and acquired by recycling class I molecules which return to the cell surface. The exogenous pathway explains how antigens from dead cells can be acquired and presented to T cells.

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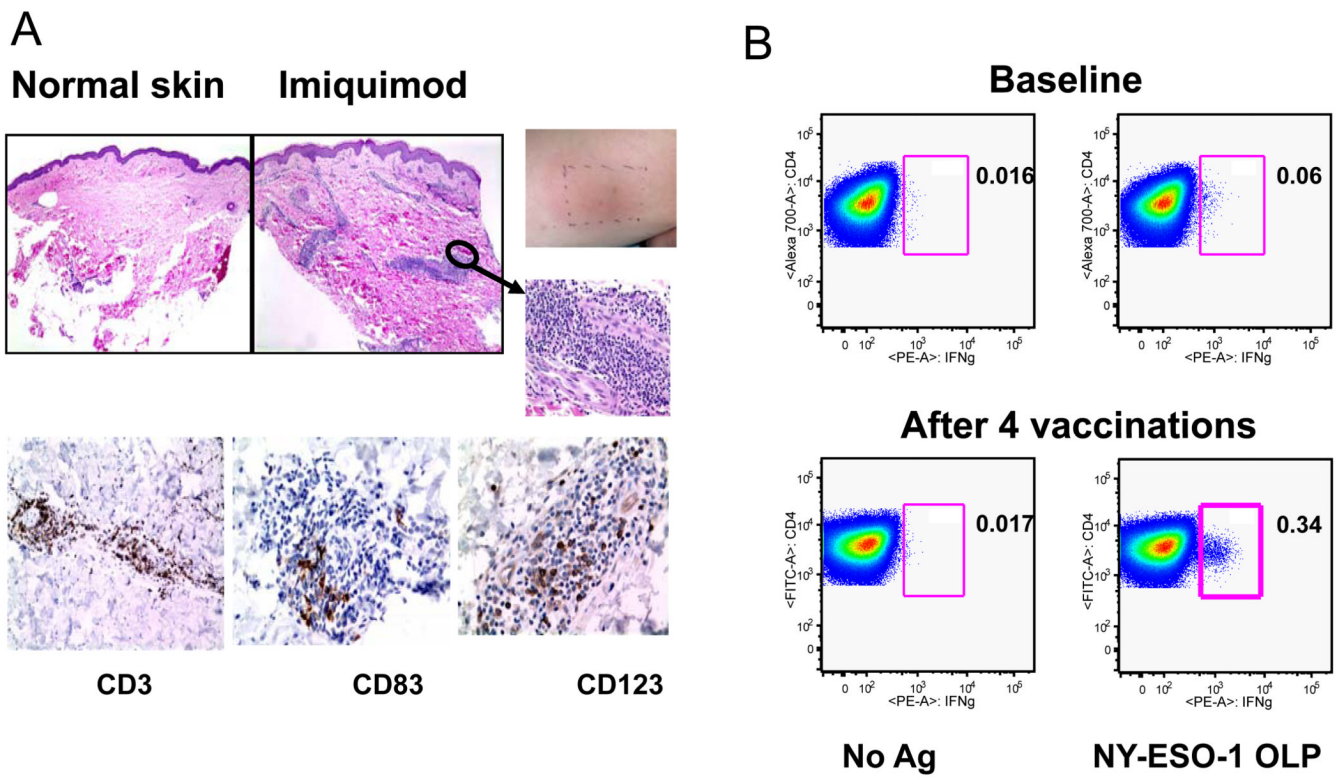

Figure 4. Imiquimod induces local inflammation and NY-ESO-1 specific CD4+ T cell responses

A. Representative H and E stained sections of control skin and Imiquimod treated skin (left panels). Right upper panels show inflammation at the Imiquimod treated of one patient. Representative immunohistochemistry sections for three tested markers are shown (CD3: T cells; CD83: mature DC; CD123: plasmacytoid DC). **B.** Quantification of IFNγ-secreting NY-ESO-1-specfic CD4+ T cells. Representative before and after vaccine samples for one patient are shown. Following a one week in vitro stimulation with pooled NY-ESO-1 overlapping peptides, cells were re-stimulated and stained for intracellular IFNγ. CD4 staining is shown on the y axis and IFNγ staining is shown on the x-axis.(76). Copyright 2008. The American Association of Immunologists, Inc.

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Table 1

Natural and synthetic ligands of Toll-like receptors

Receptor	Pathogen Associated ligands (PAMPs)	Endogenous ligands	Synthetic ligands
TLR 1/2	Triacylated lipopeptides (Bacteria and Mycobacteria)*	Not known	Pam3Cys *
TLR 2	Peptidoglycan (gram positive bacteria); Bacterial lipoprotein; Lipoteichoic acid; LPS (*Porphyromonas gingivalis*, *Leptospira interrogans*); GPI-anchor proteins (*Trypanosoma cruzi*); Neisserial porins, Hemagglutinin (*MV*); phospholipomannan (*Candida*); LAM (*Mycobacteria*)	Not known	CFA MALP2** Pam2Cys** FSL-1** Hib-OMPC
TLR 3	ssRNA virus (*WNV*), dsRNA virus (*RSV*, *MCMV*)	Not known	Poly I:C; poly A:U
TLR 4	LPS (Gram-negative bacteria); F-protein (*RSV*); Mannan (*Candida*); Glycoinositolphospholipids (*Trypanosoma*); Envelope proteins (RSV and MMTV)	Hsp60, Hsp70, fibronectin domain A surfactant protein A, hyaluronan; HMGB-1	AGP MPL A RC-529 MDF2β CFA
TLR 5	Flagellin (Flagellated bacteria)	Not known	Flagellin
TLR 2/6	Phenol-soluble modulin (*Staphylococcus epidermidis*) Diacylated lipopeptides (*Mycoplasma*); LTA (*Streptococcus*); Zymosan (*Saccharomyces*)	Not known	MALP-2** Pam2Cys** FSL-1**
TLR 7	Viral ssRNA (Influenza, VSV, HIV, HCV)	Human RNA	Guanosine analogs; imidazoquinolines (e.g. Imiquimod, Aldara ® R848, Resiquimod®); Loxoribine
TLR 8	ssRNA from RNA virus	Human RNA	Imidazoquinolines; Loxoribine; ssPolyU 3M-012
TLR 9	dsDNA viruses (HSV, MCMV); Hemozoin (Plasmodium); Unmethylated CpG DNA (bacteria and viruses)	Human DNA/chromatin, LL37-DNA	CpG-oligonucleotides
TLR10	Not known	Not known	Not known

*Ligands recognized by TLR1 and TLR2

**Ligands recognized by TLR2 and TLR6

Adapted from: (68,108,125)