Toll-like receptors and cutaneous melanoma (Review)

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Abstract. Innate immune cells recognize highly conserved pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). Previous studies have demonstrated that PRRs also recognize endogenous molecules, termed damage-associated molecular patterns (DAMPs) that are derived from damaged cells. PRRs include Toll-like receptors (TLRs), scavenger receptors, C-type lectin receptors and nucleotide oligomerization domain-like receptors. To date, 10 TLRs have been identified in humans and each receptor responds to a different ligand. The recognition of PAMPS or DAMPs by TLRs leads to the activation of signaling pathways and cellular responses with subsequent pro-inflammatory cytokine release, phagocytosis and antigen presentation. In the human skin, TLRs are expressed by keratinocytes and melanocytes. The main cells from which skin cancers arise. TLRs 1-6 and 9 are expressed in keratinocytes, while TLRs 2-5, 7, 9 and 10 have been identified in melanocytes. It is hypothesized that TLRs may present a target for melanoma therapies. In this review, the involvement of TLRs in the pathogenesis and treatment of melanoma was discussed.

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1. Toll-like receptors and the skin

The skin, which is the largest organ of the human body, represents the interface between the environment and the host. It provides the first line of defense against physical, chemical and biological stressors. The skin is predominantly composed of three cell types: Melanocytes, Langerhans cells and keratinocytes. Keratinocytes are the most common type of skin cell, which serve as a protective physical barrier for the human body and present a fundamental element of the innate immune response (1).

The immune system is classified into two types: innate and adaptive. Innate immunity refers to nonspecific defense mechanisms that are activated immediately following the identification of an antigen in the body. It provides the initial defense against invading pathogens and aids adaptive responses via antigen presentation. By contrast, adaptive immunity provides antigen-specific responses and immunological memory (2).

The innate immune system is composed of numerous cell types, including neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages, dendritic cells, natural killer (NK) cells and γδ T cells. Innate immune cells recognize highly conserved pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). A recent study demonstrated that PRRs also recognize endogenous molecules, termed damage-associated molecular patterns (DAMPs) that are derived from damaged cells (3).

PRRs include Toll-like receptors (TLRs), scavenger receptors, C-type lectin receptors and nucleotide oligomerization domain-like receptors. The recognition of PAMPS or DAMPs by PRRs leads to the activation of signaling pathways and cellular responses with subsequent pro-inflammatory cytokine release, phagocytosis and antigen presentation (4).

The TLR receptor family consists of >10 members in humans and mice, collectively (2,5).

The Toll gene was originally identified as a regulator gene of dorsal-ventral polarity in Drosophila embryos in 1985 (6,7). Subsequent studies revealed that the protein exhibits a key function in Drosophila responses to fungal infections (8). Further studies, based on database searches, identified homologs of Toll in mammals and humans, thus the name ‘TLRs’ was selected (9,10).

TLR1, -2, -4, -5 and 6 are membrane receptors, whereas TLR3, -7, -8 and -9 are intracellular receptors that are localized to the endoplasmic reticulum, endosomes and lysosomes (Table I). TLRs are type I transmembrane proteins composed of 3 domains: An extracellular domain consisting of leucine-rich repeats, a transmembrane domain and an intracellular Toll-interleukin (IL)-1 receptor (TIR) domain (3,11).

The extracellular domain is involved in ligand recognition (PAMPs and DAMPs) and is characterized by the leucine-rich sequence XLXXXLXX, in which X is an amino acid. The transmembrane region determines the
cellular localization of the receptor and exhibits the leucine-rich repeat carboxy-terminal domain. TIR is a conserved protein-protein interaction domain that is required for downstream signaling (1).

Upon recognition of ligands, TLRs dimerize and undergo a conformational change that is required to activate the downstream signaling pathway. Generally, TLRs form homodimers, with the exception of TLR2 and -4, which form heterodimers (3,12).

The TLR signaling cascade involves the recruitment of the following five adaptor molecules to its TIR: Myeloid differentiation primary-response 88 (MyD88) protein, TIR domain-containing adaptor-inducing interferon (IFN)-β (TRIF), TIR domain containing adaptor protein/MyD88-adaptor-like, TRIF-related adaptor molecule and sterile-α and armadillo motif-containing protein (3,12). Two main TLR signaling pathways have been identified: The MyD88-dependent pathway and the TRIF-dependent pathway, and activation depends on whether MyD88 or TRIF is recruited by the TIR domain (3,12). With the exception of TLR3, which signals through the TRIF, all TLRs recruit MyD88. Both pathways lead to the expression of transcription factors, including nuclear factor-κB (NF-κB), c-Jun N-terminal kinase and mitogen-activated protein kinase, which are required for inflammatory gene transcription. This results in the release of a variety of cytokines and inflammatory markers, such as IL-1, -6 and -8, tumor necrosis factor-α (TNF-α), IFN-α and IFN-β (3,11).

Each of the 10 human TLRs respond to a different ligand. For example, TLR2 is involved in the recognition of lipoproteins and peptidoglycans, TLR4 binds bacterial lipopolysaccharide (LPS) and TLR3, -7 and -8, which are located on endosomes, are involved in the recognition of viral and bacterial nucleic acids. Furthermore, flagellin is recognized by TLR5 and TLR9 recognizes unmethylated CpG motifs in DNA (13,14).

TLRs have been identified in a number of cell types, including dendritic cells, neutrophils, lymphocytes, monocytes and NK cells. TLR7 and -9 are expressed on plasmacytoid dendritic cells (pDCs) and B-lymphocytes, while TLR1-6 and 8 are expressed on myeloid-derived DCs. TLRs 1, 2 and 4-10 are expressed by neutrophils and TLR1 is expressed in NK cells. Monocytes express all TLRs, with the exception of TLR3. B-lymphocytes also express TLR1, while TLR2, -8 and -10 may be present on the membrane of T-lymphocytes (13).

In the human skin, TLRs are expressed by keratinocytes and melanocytes, the main cells from which skin cancers arise. TLR1-6 and -9 are expressed in keratinocytes, while TLRs 2-5, -7, -9 and -10 have been identified in melanocytes (1).

2. TLR-targeted immunotherapies

Immunosuppression allows tumor cells to escape immune-mediated destruction. TLRs are pathogen pattern recognition molecules that identify a variety of pathogens and thus are involved in the regulation of immune responses (15). In addition to exogenous PAMPs, TLRs recognize endogenous ligands, which may alert the innate and adaptive immune systems to the presence of modified tumor cells (1). Therefore, TLR activation of the innate immune response may promote the enhancement of tumor-specific acquired immunity (16).

The involvement of the innate immune response in tumor suppression was first postulated by William B. Coley >100 years ago (17). Coley used heat-killed bacterial cultures of Streptococcus pyogenes and Serratia marcescens (known as Coley’s toxin) to successfully treat patients with inoperable soft tissue sarcoma (17). In the early 1990s, Polly Matzinger hypothesized that tumor antigens are classified as ‘dangerous’ by the immune system in the presence of bacteria that stimulate the immune response (17). Recently, it has been demonstrated that Bacillus Calmette-Guérin induces tumor regression of metastatic melanoma (13). These antitumor effects are associated with TLR activation by LPS and unmethylated bacterial DNA (18).

TLR agonists may present promising drugs for the treatment of malignancies due to their enhancement of the immune response (19). TLR activation induces the release of cytokines involved in cell-mediated immunity and T-regulatory suppression (IL-6 and -12), which shifts the immune response towards Th1 differentiation. This leads to the activation of the type 1 IFN response, which is essential for dendritic cell maturation, antigen cross-presentation and proliferation of NK cells and memory T cells (13).

TLR expression is not confined to immune cells; they have been identified in several cell types, including tumor cells and TLR expression is conserved in these cells. Therefore, TLR agonists are considered as extremely promising drugs for cancer immunotherapy due to their immunostimulatory properties and their pro-apoptotic effects on tumor cells (19).

Notably, epidemiological studies have identified an association between chronic infections and cancer-related mortality in 15% of patients, suggesting that TLR-mediated activation of the innate immune response and the NF-κB pathway in particular, may also promote tumor development due to the types of immune cells and cytokines involved. For example, IL-1, -6, -8 and transforming growth factor-β promote angiogenesis and tumor growth (20). Chronic infectious diseases, such as helicobacter pylori and hepatitis B and C, are associated with the development of cancer, which indicates that TLR-mediated inflammation that is associated with bacteria and viruses may promote carcinogenesis (21).

In 1863, Virchow hypothesized that chronic inflammation enhances cell proliferation: Cancer may develop following exposure to certain irritants, which, in addition to the consequent tissue injury and inflammation caused, enhances cell proliferation (22). It has been established that the proliferation of cells alone does not cause cancer, however, it is hypothesized that an environment rich in inflammatory cells, DNA-damage-promoting agents, activated stroma and growth factors promotes and/or potentiates cell proliferation and increases neoplastic risk (17). In malignant tissues, the tumor microenvironment usually contains an excess of inflammatory cells (23). The therapeutic aim for the future is to normalize the host response by reducing the inflammatory network typically observed in neoplastic tissues: Tumor suppression may be achieved by decreasing the high levels of pro-inflammatory cytokines and increasing the levels of anti-inflammatory cytokines (21).

Various TLR agonists have been investigated for skin cancer immunotherapy: Imidazoquinolines (TLR7 and -8 agonists); CpG oligodeoxynucleotides (ODNs) (TLR9 agonists) (13); and
Imiquimod. Imiquimod is a member of the imidazoquinolone family, which also includes resiquimod. These drugs topically stimulate the immune response. Stimulation of TLR7 or TLR8-mediated signaling pathways, following treatment with imiquimod or other imidazoquinolines, leads to the activation of central transcription factors, such as NF-κB. Under normal conditions, heterodimeric NF-κB remains inactive within the cytoplasm while bound to the inhibitory factor, inhibitor of κB (IκB). However, following receptor-mediated stimulation, IκB is phosphorylated via the IκB kinase complex (24). This phosphorylation results in the release, activation and nuclear translocation of NF-κB and the subsequent transcription of numerous genes that transcribe cytokines, chemokines, adhesion molecules and apoptosis-related proteins (21). Furthermore, when imiquimod binds to dendritic cells, macrophages and monocytes, activation results in the release of the following pro-inflammatory mediators: TNF-α, IFN-α, IL-1, -6, -8, -12 and -10 (25). These cytokines drive the immune response toward the T helper (Th-1) profile, which is important for control of viruses and tumors, and inhibits the Th-2 pathway, which is implicated in the response against helminths and allergens (26). Imiquimod also acts as a TLR8 agonist, however, it activates TLR-7 more potently (27). In addition, imiquimod stimulates the maturation of Langerhans cells and their migration to regional lymph nodes, with increased levels of antigen presentation to naïve T cells (25). In a mouse model of subcutaneous melanoma, it was demonstrated that pDCs accumulate in subcutaneous melanoma metastases following treatment with imiquimod (28). Furthermore, plasmacytoid predendritic cells migrate to the skin following the application of imiquimod (29). In addition to the indirect stimulation of lymphocytes and NK cells via the activation of dendritic cells, Stary et al (30) demonstrated that imiquimod-treated DCs acquire direct antitumoral functions in vivo. Imiquimod has been demonstrated to modulate signal transducer and activator of transcription-1 signaling pathways, and this interaction may contribute to the induction of apoptosis in a number of cell types (31). Furthermore, imiquimod leads to increased expression of the death receptor, cluster of differentiation (CD)95 (32). Imiquimod may exhibit indirect pro-apoptotic effects on the respective apoptosis-related proteins via TLR-dependent regulation (32). However, imiquimod exerts an additional direct pro-apoptotic activity against tumor cells via activation of the Fas pathway (27).

Imiquimod has been approved for the treatment of condylomata acuminata, superficial basal cell carcinomas (BCCs) and actinic keratosis, however, a number of studies have indicated that it may also be an efficacious treatment for lentigo maligna (LM) and metastatic melanoma (26).
generally identified on the face or neck of middle-aged or elderly patients (32). The gold standard treatment for LM is conventional surgery using a 5-10 mm margin. However, the localization of the disease, which often arises on the face, makes surgical removal difficult and patients may require extensive plastic repair.

In 2000, Ahmed and Berth-Jones reported the first therapeutic use of imiquimod (5%) in an elderly patient with a large LM on the scalp that refused surgery. Following 7 months of intermittent topical imiquimod application (due to localized reactions), the patient exhibited complete clinical and histological remission and no evidence of recurrence was identified during 9 months of follow up (33).

Additional case reports have demonstrated similar results with regard to LM lesions. Particularly noteworthy is the case of a patient with recurrent LM initially treated using a CO₂ laser, who underwent treatment with imiquimod (5%) once or twice a day for 3 months. Following treatment in this patient, biopsy revealed no residual LM (34). Other studies have reported similar outcomes (35); Naylor et al (36) demonstrated a clinical and histological resolution rate of 93% in 28 cases of LM 4 weeks after a 12-week treatment regimen. Additionally, 80% of patients exhibited no evidence of relapse after a year of follow up (36). Similar results were also obtained by Craythorne and Lawrence (37), who demonstrated that in 6/8 LM patients treated with imiquimod, the tumor resolved clinically with no evidence of recurrence after a mean follow up period of 34.2 months. A brisk inflammatory reaction was a prerequisite of therapeutic response (37).

Despite the positive evidence regarding the treatment of LM with imiquimod, at present surgery is considered the best approach for LM treatment.

A recent literature review (38) postulated that for the treatment of LM surgical intervention remains the most widely used and recommended available treatment, however, no randomized controlled trials have demonstrated that surgery is the best therapeutic modality for LM. The use of non-surgical interventions, such as imiquimod as a monotherapy, may be effective and may be considered in selected cases whereby surgical procedures are contraindicated.

The same hypothesis was supported by Kallini et al (39), which considered topical imiquimod as a second line therapy for LM, with surgery as the primary therapeutic option. LM usually occurs in elderly patients, often with concomitant conditions that make surgery difficult to perform; in these conditions imiquimod may represent an alternative treatment choice (40).

Disagreements between certain authors may be due to the absence of shared guidelines for the treatment of LM with imiquimod. In previous studies, dosage (3 times daily or weekly) and treatment duration (2 weeks-7 months) has varied and reported follow-up periods were short, with a median follow up time of <24 months, as reported by Erickson et al in 2010 (41).

A small number of cases that exhibited progression from LM to LMM during treatment with imiquimod have been reported (42,43). We postulate that imiquimod acts by increasing the production of TNF-α, which stimulates the production of metalloproteinase 9, a factor that contributes to the invasive capacity of melanoma, thereby inducing recurrence. However, the treatment of LM that already exhibits an unknown invasive component presents a problem: The application of imiquimod in these cases represents a significant risk for tumor progression (43).

Data regarding imiquimod use for amelanotic lesions is limited. A recent study demonstrated the histologically-confirmed resolution of an amelanotic LM treated with imiquimod 7 times a week for 8 weeks (44). By contrast, another study reported the accidental use of imiquimod for an achromic superficial spreading melanoma due to incorrect diagnosis, which resulted in a poor response to topical treatment and an increase in lesion size (45).

**Imiquimod use in metastatic melanoma.** The immune system is essential for the restriction of melanocyte proliferation. This may explain why the eruptive melanocytic nevi phenomenon is observed in organ transplant recipients with clear immunosuppressive conditions, as the immune system of these patients is no longer able to inhibit melanocyte proliferation (46). Furthermore, restoration of complete immune responsiveness leads to regression of melanocytic nevi (47).

These findings indicate an association between melanocytic proliferation and the immune system. However, the mechanism by which immunosuppression induces melanoma remains unclear. Notably, the incidence of melanoma in organ transplant recipients is only 2-3 folds higher than that in the general population (47).

In halo nevi and atopic dermatitis, melanocyte proliferation is inhibited due to the pro-inflammatory response (48,49). On the basis of this finding, it has been postulated that imiquimod application, as a result of the pro-inflammatory responses exhibited, may stimulate immune recognition of atypical melanocytes leading to complete or partial elimination of melanocytes within atypical nevi (50). However, in a study by Somani et al (50) no resolution of atypical nevi was observed after twelve weeks of imiquimod treatment, suggesting that melanocytic neoplasms, such as dysplastic nevi, were resistant to imiquimod therapy (50).

By contrast, several studies have demonstrated that topical administration of imiquimod induces regression in melanoma lesions (51-54). In 2000, Steinmann et al (54) suggested that topical treatment of cutaneous melanoma metastasis with imiquimod may stimulate melanoma-specific cytotoxic T cells as a consequence of the cross-presentation of melanoma antigens by dendritic cells. Following this, Bong et al (51) investigated the efficacy of imiquimod in the treatment of cutaneous metastasis of melanoma in 3 patients with >15 cutaneous in-transit metastases with unilateral localization in the leg. Imiquimod (5%) was applied topically under occlusion twice a day for 21-28 weeks. Two patients exhibited >90% regression, however, the third patient only responded following the administration of intralesional IL-2 for 2 weeks (51).

Wolf et al (52) observed complete clinical and histopathological remission of melanoma skin metastases in 2 patients following 4 and 8 months treatment with imiquimod (5% cream) on cutaneous lesions, respectively, whereby imiquimod was applied with a 1-cm surrounding margin 3 times a week.

However, poor drug penetration following topical application may limit imiquimod efficacy (55). Turza et al (56)
reported that after treatment with imiquimod, a number of dermal melanomas showed clinical regression, but exhibited histopathologically-proven persistence of subcutaneous disease. This suggests that subcutaneous melanomas are resistant to imiquimod as a monotherapy (56,57). In addition, melanomas with high constitutive B cell lymphoma 2 expression appear to be imiquimod-resistant (58).

Therefore, at present imiquimod is not suitable for use as a first line therapy for metastases of cutaneous melanoma (55).

Resiquimod. Resiquimod is a TLR7/8 agonist that is chemically similar to imiquimod. The two drugs are synthetic low molecular weight imidazoquinolinamines. These drugs are immune response modifiers, which exhibit antiviral and antitumor activity by enhancing the production of cytokine and antigen-specific antibodies leading to a shift in immunity towards a Th1 response (59,60). Resiquimod is 10-fold more potent than imiquimod in the stimulation of the Th1 response (61). Furthermore, by contrast to imiquimod, resiquimod may be orally administered (60). Resiquimod may be considered as a potential cancer vaccine adjuvant due to its ability to increase antigen presentation via the direct activation of dendritic cells (DCs), determine local activation of immune cells and enhance the production of pro-inflammatory cytokines and the subsequent transcription of NF-κB and type I IFN (62). A previous study revealed that combined treatment with NY-ESO-1 antigens (a frequently expressed tumor-specific antigen that stimulate humoral and cellular immune responses in cancer patients), Montanide (an immune adjuvant that ensures the slow release of antigens and the recruitment of antigen-presenting cells to the injection site) and topical resiquimod results in high immunogenicity in melanoma patients. This combination increased the number of antibodies and CD4+ T cells, however, no consistent CD8+ T-cell response was identified (62). Conversely, another study demonstrated that topical resiquimod exhibits potent cytotoxic T lymphocyte responses to parenteral antigens in mice (63).

Clinical studies have demonstrated the safety and the efficacy of topical application of resiquimod and its analogs in activating the local immune response (64-67). However, resiquimod injection may induce systemic cytokine release and thus, must only be formulated to cause local immune activation, preventing systemic effects (68). Parenteral resiquimod has been associated with transient peripheral blood leukopenia and lymphopenia due to general endothelial cell activation with consequent transiently reduced availability of peripheral-blood leukocytes (69).

CpG ODNs (TLR9 agonists). CpG are short single-strand DNA cytosine and guanine-rich sequences or ODNs (13,18). CpGs may be classified into 3 types according to their effect on immune cells: CpG-A, a stimulator of NK cells due to its marked IFN-α-producing effect on pDCs; CpG-B, a moderate IFN-α inducer that enhances antigen-specific immune responses; and CpG-C, which combines the properties of CpG-A and CpG-B (13).

CpGs are highly potent immune activators that trigger TLR9 and activate pDCs. Activated pDCs subsequently, release IFN-α, which augments T and NK cell responses and activates conventional myeloid DCs (mDCs) (70).

PF-3512676 is a synthetic CpG-B sequence and TLR9 agonist that has been studied in a variety of tumor types, including renal cell carcinoma, glioblastoma, non-Hodgkin's lymphoma, melanoma and mycosis fungoides (13).

A phase II trial of CpG administered by subcutaneous injections demonstrated a response in 10% of melanoma patients, in addition to evidence of immune system activation (71). A phase I study of intralesional treatment with PF-3512676 in BCC and melanoma patients demonstrated local tumor regression and immune cell activation (72).

Intradermal CpG injections surrounding primary melanoma excisions have been demonstrated to activate pDC and mDCs, reduce the number of regulatory T cells in the draining lymph node and increase the number of melanoma-specific CD8+ T cells, as well as NK cell responses (70,73).

These data suggest potential for investigation of CpG intratumoral injections to induce immunomodulatory responses in melanoma patients.

Poly I:C. Poly I:C and its more stable derivative poly I:C-poly-L-lysine (Poly ICLC) are synthetic double-stranded RNA sequences that induce IFN production. Their biological effects are mediated by two major double-stranded RNA receptors: TLR3 in the endosome and melanoma differentiation associated gene 5 (MDA5) in the cytosol. After binding poly I:C or poly ICLC, TLR3 and MDA5 initiate downstream signaling that leads to the activation of transcription factors, including IFN regulatory factor (IRF)-3, IRF-7 and NF-κB, resulting in the increased production of type I IFNs and pro-inflammatory cytokines (74).

Previously, poly I:C and poly ICLC were evaluated as single agents in metastatic melanoma, anaplastic glioma and renal cell carcinoma, however, no antitumor efficacy was observed (13). However, recently, they have been used as adjuvants in cancer vaccines. A clinical study demonstrated that the co-administration of poly-ICLC with dendritic cell vaccines decreased the recurrence of malignant glioma (75). These data indicate that poly I:C and poly ICLC may potentially be used as immunological adjuvants to enhance the efficiency of therapeutic cancer vaccines (74).

3. Conclusion

Development of successful immunotherapies against melanoma has been hindered due to the complex interactions that occur between melanoma and the immune system. In particular, TLRs are expressed by a number of distinct cell types and thus may trigger different responses depending on the cell and the environment. The diverse cell- and stimulus-specific patterns of TLR expression and the distinct actions of TLR agonists indicate the requirement for a more complete understanding of their function in melanoma therapies. The application of TLR agonists presents a novel immunotherapeutic approach for the treatment of melanoma.

References


