Immune Response Modifiers – Mode of action

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Abbreviations

IFN; interferons

IL-1Rs; interleukin-1 receptor

IRAK; IL-1R-associated kinase

IRF3; interferon-regulatory factor 3

IRM; immune response modifier

LRR; leucine-rich repeat

LC; Langerhans cells

NF-κB; nuclear factor-κB

PBMC; human peripheral blood mononuclear cell

pDC; plasmacytoid dendritic cells

TIR; Toll/IL-1R

TLR; Toll-like receptors

TNF; tumor-necrosis factor

TRAF6; TNF-receptor-associated factor 6

Abstract

The innate immune system governs the inter-connecting pathways of microbial recognition, inflammation, microbial clearance and cell death. A family of evolutionarily conserved receptors, known as the Toll-like receptors (TLRs), is crucial in early host defense against invading pathogens. Upon TLR-stimulation, NF-κB-activation and the IRF3 pathway initiate production of pro-inflammatory cytokines, such as IL-1 and TNF-α, and production of type I interferons (IFN-α and IFN-β), respectively. The innate immunity thereby offers diverse targets for highly selective therapeutics, like small-molecular synthetic compounds that modify innate immune responses. The notion that activation of the innate immune system is a prerequisite for the induction of acquired immunity raised interest in these immune response modifiers as potential therapeutics for viral infections and various tumors. A scenario of dermal events following skin cancer treatment with imiquimod presumably comprise i) an initial low amount of pro-inflammatory cytokine secretion by macrophages and dermal DC's, thereby ii) attracting an increasing number type I IFN producing pDC from the blood; iii) Langerhans cells migrate into draining lymph nodes, leading to an increased presentation of tumor antigen in the draining lymph node, and iv) consequently an increased generation of tumor specific T cells and finally v) an accumulation of tumoricidal effector cells in the treated skin area. The induction of predominately Th1-type cytokine profiles by TLR-agonists like imiquimod might have further benefits by shifting the dominant Th2-type response in atopic diseases like asthma and atopic dermatitis to a more potent Th1 response.

Introduction

The immune system has traditionally been divided into innate and adaptive immunity. Both of these play different roles and functions in defending the organism against the invasion of microorganisms that are constantly present in the environment. Innate immunity could be considered as being the first line of defence against pathogens, such as bacteria or viruses. On the contrary, acquired immune responses are slower processes and are mediated by T and B cells. Both cell types express highly diverse antigen receptors that are generated through DNA rearrangement and are thereby able to respond to a wide range of potential antigens (1). Within the last two decades this highly developed system of antigen detection, which is found only in vertebrates, has been the subject of considerable research.

However, rapidly growing information about the innate immune system, including the identification of cognate ligands of innate immune receptors and the elucidation of their downstream signaling pathways, has become available. The picture that innate immunity is a relatively nonspecific system, with its main roles being to destroy pathogens and to present antigen to the cells involved in acquired immunity, is no longer accurate. The innate immune system must be considered as a system that has a greater degree of specificity than previously thought and that it is highly developed in its ability to discriminate between self and foreign pathogens (2-5). This discrimination relies to a great extent on a family of evolutionarily conserved receptors, known as Toll-like receptors (TLRs), which play a crucial role in early host defense against invading pathogens (5). In addition, activation of the innate immune system leads, via the cytokine secretion from Monocytes/macrophages (interferon-α, interleukin-12, tumor-necrosis factor-α), to the induction of a predominant T helper 1

(Th1)-cell response of the acquired immunity that have been clinically used to treat viral infections and cancerous lesions of the skin (6, 7).

In the past few years, our knowledge about the mode of action of immune response modifiers (IRMs) especially for the TLR7/8 specific ligand imiquimod has greatly increased. In this short review, we will discuss the IRMs, focusing on their receptors, signaling pathways and immunological effects.

TLR superfamily: structure, function and their ligands

TLRs comprise a family of cell-surface and endosomally expressed receptors that recognize conserved products unique to microorganisms, such as lipopolysaccharide, bacterial DNA or double-stranded RNA. The discovery of the TLR family began with the identification of Toll, a receptor that is expressed by insects (8). Subsequent studies revealed that toll plays an essential role in the insect innate immune response against fungal infections (9). So far, 13 TLRs, the mammalian homologues of the Drosophila protein Toll, have been identified, although only TLR1 to TLR10 are expressed in humans.

TLRs are transmembrane glycoproteins with a considerable homology in their cytoplasmic region, while the extracellular region of the TLRs differs markedly. All TLRs and interleukin-1 receptor (IL-1Rs) family members have a conserved region of ~200 amino acids in their cytoplasmatic tails, which is known as Toll/IL-1R (TIR) domain (Figure 1). This domain contains three conserved regions that are crucial for assembly of downstream signaling complexes, as it mediates the interaction with adaptor protein MyD88 (10). The extracellular domain of TLRs contains 19-25 tandem copies of leucine-rich repeat (LRR) motifs and differs therefore markedly

from IL-1Rs, which contain three immunoglobulin-like domains. The LRR domain is thought to be involved directly in ligand recognition. The TLRs that are involved in the recognition of microbial products TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 – are displayed on the cell surface. By contrast, TLR7, TLR8 and TLR9, all of which are involved in the recognition of nucleic-acid-like structures, are localized intracellularly (11). TLR9 for example is expressed in the endoplasmatic reticulum, and has been shown to be recruited to endosomal/lysosomal compartments after stimulation with CpG-containing DNA (12). The main ligands recognized by different TLRs are summarized in Figure 1. There are four broad types of ligands: naturally occurring molecules that are constituents of microorganisms, synthetic analogues of naturally occurring substances, small-molecule fully synthetic compounds, and endogenous components that are released from host cells during sterile inflammation. Sterile inflammation is thought to be driven by ligands derived from damaged cells, which are usually not present in the extracellular environment: for example, heat-shock proteins, β-defensins and oxidized lipids. Nevertheless, most of the known ligands are derived from microbial sources, and they include polymeric molecules such as peptidoglycans (which bind TLR2), bacterial LPS (TLR4), bacterial and viral DNA (TLR 9) and double stranded RNA (TLR3). Due to their pharmacodynamic and pharmacokinetic properties, large polymeric molecules are not ideal drug candidates. Nonetheless, CpG olideoxynucleotides (ODNs) modeled on bacterial DNA sequences are good examples of synthetic analogues of naturally occurring ligands in clinical use. The antiviral and antitumoral imidazoquinoline compounds, imiquimod (R-837) and resiquimod (R-848) were the first fully synthetic low molecular-mass molecules, which have been described as ligands for TLRs (TLR7 and TLR8).

TLR/IL-1R-superfamily signaling cascade

Upon ligand binding, TLRs/IL-1Rs form either homo- or heterodimeric receptor complexes, and undergo the conformational change required for recruitment of downstream signaling molecules. TLRs activate signaling pathways that are similar to those induced by IL-1 because of the presence of the TIR domain. In activated receptor complexes the TIR domain interacts with the adaptor protein MyD88 and the IL-1R-associated kinase (IRAK), which leads to activation of the adaptor molecule tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) (Figure 2) (13). Subsequently, TRAF6 mediates the activation of the inhibitor of NF-κB (IκB) kinase (IKK). NF-κB dimers are usually sequestered in the cytoplasm in an inactive form by molecules of the inhibitor of NF-κB (IκB) family (14). After activation by upstream signals, IKK phosphorylates IkBs, leading to their polyubiquitylation and proteasome-mediated degradation, thereby initiating liberation of nuclear factor-κB (NF-κB) from the IκB. The NF-kB family of transcription factors, p65(REL-A), REL-B, cytoplasmatic (c) REL, p50 and p52 - which function as homo- and heterodimers - then translocate into the nucleus and activate NF-κB-dependent gene transcription (15).

Although MyD88-dependent signaling is essential for recognition of a broad range of microbial components, studies with MyD88-deficient cells have revealed the existence of MyD88-dependent and –independent pathways leading to NF-kB activation upon LPS initiated signaling (16). It became clear, at least for TLR4 dependent signaling, that the MyD88-dependent pathway involves the early phase of NF-kB activation, leading to the production of inflammatory cytokines. In contrast, MyD88-independent pathways activate the interferon-regulatory factor 3 (IRF3) and involve the late phase of NF-κB activation, together leading to the production of type I interferons (IFN-α and IFN-β) and the expression of IFN-inducible genes (Figure 2).

Interestingly, it has been shown that the MyD88-independent pathway is in addition responsible for LPS-mediated maturation of dendritic cells (DCs) (17). The identification of MyD88-independent pathways of TLR4 signaling resulted in the discovery of several adaptors, like TIRAP (TIR-domain-containing adaptor), TRIF (TIR-domain-containing adaptor protein inducing IFN-β) and TRAM (TRIF-related adaptor molecule), all of which have TIR domains as a unique feature. All of these TIR containing adaptors, including MyD88 itself, are believed to play crucial roles in TLR-signaling pathways, because they provide additional specificity to the response generated by signaling through each TLR.

Finally, as excessive production of inflammatory cytokines upon TLR stimulation may induce serious systemic disorders such as endotoxic shock or exacerbation of atopic diseases such as asthma and atopic dermatitis, it is not surprising that organisms have developed mechanisms for modulating their TLR-mediated responses. Indeed, TLR-signaling pathways have been shown to be negatively regulated by several molecules, such as IRAK-M, SOCS1 (suppressor of cytokine signaling1) and SIGIRR (single immunoglobulin IL-1R-related molecule)(18). Consequently, SOCS1-deficient mice, for example, are more sensitive to LPS shock and lethality than their wild-type littermates (19). Interestingly, SOCS1 belong to the family of SOCS proteins, which are induced by cytokines and are known to be negative regulators of cytokine-signaling pathways (20), and have not been implicated as regulators of TLR-signaling pathways until recently (21). Although SOCS1 has been shown to associate with IRAK1 and may thereby inhibit its activity, the precise mechanism by which SOCS1 inhibits TLR signaling remains unclear.

Immunomodulatory effects of imidazoquinolines, fully synthetic TLR ligands

Imiquimod and its analog resiquimod (R-848) mainly act through indirect (i.e. immune activating mechanisms) rather than exerting anti-viral or anti-tumor effects. However, a number of studies have demonstrated their efficacy in the treatment of infectious and neoplastic diseases including primary and recurrent genital herpes simplex virus and cytomegalovirus infection in guinea pigs (22-25). Imiquimod has been demonstrated to have anti-tumoral properties in a number of murine models, including MC-26 colon carcinoma, Lewis lung carcinoma and FCB bladder tumor (26). Initial studies have also been undertaken in human subjects for the treatment of various types of cancer (27-30). Much of the biologic activity of these compounds can be attributed to the induction of cytokines, especially interferon-α (IFNα) but including also tumor necrosis factor-α (TNFα) (31, 32).

Early in vitro studies have already shown the importance of cells from the immune system in the production of these cytokines. Human peripheral blood mononuclear cell (PBMC) cultures produce IFNα, tumor necrosis factor (TNF), IL-1, IL-6, IL-8 and several other cytokines in response to the imidazoquinolines (33-38). Imiquimod and R-848 have also been shown to increase the secretion of chemokines such as MIP (macrophage inflammatory protein)-1α, MIP-1β, and MCP (monocyte chemotactic protein)-1 (39).

Several types of immune and non-immune cells have been identified to be responsive to triggering by imidazoquinolines:

Keratinocytes: As the skin is well recognized as an important source of cytokines with several cells being able to secrete cytokines , i.e. keratinocytes, dermal fibroblasts, melanocytes and Langerhans cells, it was not surprising that normal

human keratinocytes produce increased levels of IL-6 and IL-8 mRNA upon stimulation with imiquimod (40). However, more recent data showing the lack of TLR-7 or 8 in human keratinocytes as well as the inability to produce IL-8 protein in response to R-848 (41) call these results into question. Nevertheless, topical administration of imiquimod (imiquimod cream 5%) to the flanks of hairless mice and rats leads to increases in local concentration of IFNα and TNFα mRNA and protein levels within hours (31). These studies could also demonstrate that imiquimod, when applied locally as cream, leads to the local production of cytokines without generating a systemic response (31). Despite the potential ability of non-immune cells of the skin to react towards imiquimod stimulation (40), classical immune cells are considered the primary responsive cell population in the skin upon topical application of imidazoquinolines.

Plasmacytoid dendritic cells: Systemically, Plasmacytoid dendritic cells (pDC), cells of B-lineage plasma cell morphology but lacking all antibody producing capacity (42-44) have been found as the major producers of IFNα (45). They express TLR7 (43, 46), react directly towards stimulation with imidazoquinolines by rapidly producing large amounts of IFNα and other pro-inflammatory cytokines and maturing into $CD8^+$ DC (47-50). Thereby the production of IFNα as well as IL-12 and TNFα in response to R-848 is dependent on TLR7 itself (39, 49, 51) as well as the central adaptor molecule of TLRs, MyD88 (51) in the mouse. While being neglected as dermal cells for a long time, it now becomes clear, that pDC are resident in normal mouse (52) and human skin (53), albeit at very low numbers. However, a topical treatment of skin with imiquimod induces massive recruitment of, supposedly, peripheral pDC which is associated with regression of natural (53) or experimental tumors (52). In addition, pDC are the only producers of IFNα in the skin upon

imiquimod exposure (53). It is interesting to note, that a small number of human patients do neither show an inflammatory response towards imiquimod nor an increased pDC recruitment, the biological explanation of which is currently unclear (53).

Langerhans cells: Recent results suggest that the mechanism by which topical imiquimod enhances immune function in the skin is to a great extent due to effects on Langerhans cells. In vitro it has been shown that imiquimod and R-848 induce functional maturation of human Epidermal Langerhans Cells, proffering TLR agonists as adjuvants in future vaccine strategies to induce protective antiviral and antitumoral responses (54). Moreover, topically applied imiquimod increases Langerhans cell migration from the skin to the regional draining lymph node in mice (55). The effect of this on Langerhans cells leads in turn to enhanced Langerhans cell antigen presentation exclusively upon hapten sensitization during the induction of contact hypersensitivity (CHS) (55). Interestingly, also systemic dosing of R-848 sharply increases CHS responses in mice, pointing also to the ability of imidazoquinolines to induce a general dendritic cell activation in vivo (56). These results explain the notion that in the treatment of diseases where enhanced Langerhans cell antigen presentation appears to be desirable, like in cutaneous neoplasms, topical application of imiquimod has been proven to be beneficial (57-59). In addition, the powerful adjuvanticity of TLR7 ligands (56) is now increasingly being recognized in vaccination studies (60).

Other dendritic cells: Human monocyte derived DC respond to stimulation with R-848 by producing bioactive IL-12, IL-6, TNFα, IFNα and several chemokines, upregulating maturation markers as well as with enhanced antigen presentation (61,

62). The combination of R848 with the triggering of TLR3 or TlR4 induced a synergistic activation of both human and murine DC, which could even be superagonized by the addition of IFNγ or CD40-L (63). Also in vivo a combination of imidazoquinolines and agonistic anti CD40 treatment during vaccination of whole animals lead to a sharply enhanced generation of antigen specific, lytically active CD8+ CTL (64). Thus, in general, DC treated with imidazoquinolines, either alone or in combination with other pro-inflammatory agonists shift the acquired immune system towards a Th1-dominated immune response (63, 65).

T cells: In humans, imidazoquinolines can induce IFNγ, IL-8 and IL-10 in T cells either directly or in combination with TCR triggering (66), while murine T cells appear to be non-responsive, at least with regard to upregulation of adhesion molecules (56). Interestingly, however, it has recently been described, that systemic dosing of R848 leads to a rapid (1h) almost complete deletion of peripheral leukocytes, especially CD4 and CD8 T lymphocytes in mice. This phenomenon was dependent on MyD88 and left animals in a transient state of immune incompetence, where they were not able to mount otherwise prominent CHS responses. The most likely explanation was an increase of adhesion molecules in blood vessel endothelial cells leading to increased rolling of leukocytes (56). These results are in line with observations of other TLR ligands such as CpG to induce rapid leukopenia (67) and underscore the notion, that the systemic use of TLR ligands in humans has to be tightly controlled for possible detrimental side effects.

B cells: The stimulation of TLRs expressed on B-cells can lead to antigen-specific proliferation of B lymphocytes and the expression of differentiation markers such as major histocompatibility class II and B7.2 and B cell proliferation (68, 69). Moreover,

human memory B cells produce antibodies in response to CpG stimulation independently of antigen-specific cognate T- cell help (70). As B cells express TLR7 in mice (56) and humans (71), it is not surprising, that these cells can also be directly activated by imidazoquinolines (68, 72). Thereby, the compounds appear to mimic CD40 triggering, which is known as a potent B cell stimulus (72, 73). After a single in vivo dose of R-848 huge, almost confluent B cell follicles develop in the spleen of mice (Gunzer, unpublished observations). However, a more complex interplay between pro-inflammatory cytokines produced by other cells such as plasmacytoid DC might modulate the response of B cells in vivo (74).

Interestingly, R-848 has been shown to be a potent modulator of in vitro IgE production in normal but also in allergic donors (75, 76). In detail, R-848 appears to be a strong inhibitor of IgE production from PBMC. This inhibition of IgE production occurs not only in anti-CD40 and IL-4 stimulated cells, but also in PBMC from allergic donors suffering from rhinitis or atopic eczema (76). These results and the above-discussed TLR-mediated TH1-shift further support the potential use of imiquimod and R-848 in allergic diseases.

Microglia: Finally, as many viral infections have severe neurological involvements (77), which, e.g. in the case of influenza are often responsible for fatal outcome (78), the test of drugs for their ability to manipulate the CNS immune system is an important task. The recent finding, that R848 is able to activate microglia (79), the primary phagocytic cells of the CNS therefore is important and requires further study as to the mechanisms involved as well as the efficiency of the process for neuroprotection during viral infection.

In summary, imidazoquinolines, especially imiquimod and R848 have been shown to exert a plethora of effects on the immune system, either during local or systemic application. It needs to be tested in good animal models and in clinical trials, which of these effects can be used therapeutically for applications beyond the original purpose of the treatment of virus induced genital warts. Recent data also point out, that imiquimod, but not R848, can directly induce apoptosis in melanoma (80) as well as basal or squamous cell carcinomas and immortalized keratinocyte lines (81). Although not directly tested, this seems to be completely independent of TLR signaling and works via release of mitochondrial cytochrome C and induction of several caspases (81). Interestingly, in vivo, the inflammatory infiltrate of basal cell carcinomas is not apoptotic upon imiquimod treatment, while tumor cells show increased numbers of apoptotic cells (81).

Histological and Immunohistochemical findings upon imiquimod treatment:
Biopsies of inflammed tumor areas under imiquimod therapy are characterized either by a dense lichenoid inflammatory infiltrate in the papillary dermis or a diffus interstitial inflammatory infiltrate in the reticular dermis that obscures the neoplasm (Figure 3a) (82). The infiltrate is composed of lymphocytes, and, more variably, a few macrophages, plasma cells, or eosinophils (83). Tumor cells show signs of degeneration and necrosis. Thereby, in-situ carcinoma, such as solar keratosis and Bowen’s disease imitate the histologic pattern of interface- dermatitis that must be differentiated from inflammatory dermatoses (Figure 3b). Immunostainings for keratin frequently highlights the formation of colloidal bodies among the inflammatory cells. Deposits of amyloid can be occasionally found in the dermal connective tissue upon special stainings (Figure 3c) (Metze, unpublished observation). The histological changes as induced by imiquimod are identical to those of spontaneous tumor

regression as seen in solar and reticulate seborrheic keratosis (Lichen planus-like keratosis), keratoakanthoma, superficial basal cell carcinoma, porokeratosis, melanocytic nevi and melanoma (halo-phenomenon) (Metze, unpublished observation).

Tumor remission after successful imiquimod therapy is characterized by stereotypic histologic changes that are identical to those of spontaneous tumor regression, namely, a hyperplastic or atrophic well differentiated epidermis devoid of atypic cells and fibroplasia of the dermal tissue. Residuals of flat epidermal neoplasms or melanomas are a flattened epidermis and a papillary dermis that is thickened by an increased number of fibroblasts, collagen fibers (fibroplasia), mucin, ectatic small blood vessels with loss of elastic fibers, melanophages, and a patchy lymphocytic infiltrate (83, 84). Primary, invasive neoplasms and cutaneous tumor metastases result in circumscribed fibrosis or sclerosis of the reticular dermis with a variably dense infiltrate composed of lymphocytes, and, to a variable extent, neutrophils, plasmacells, erythrocytes, macrophages, and, in the case of pigmented tumors, melanophages (Metze, unpublished observation).

Immunohistochemical characterization of the inflammatory cells under imiquimod treatment exhibits many CD4+ lymphocytes, CD68+ macrophages, a few CD25+ and CD8+cells, and scattered lymphocytes with TIA-1 expression. CD1+ Langerhans cells or CD56+ lymphocytes are almost absent (57, 82, 83, 85) (Figure 3d and e). Interestingly, this immunoprofile thus not differ substantially from that found in spontaneous tumor regression (Metze, unpublished observation).

Dermal events following imiquimod treatment – a hypothetical scenario

With the above mentioned results from numerous studies on the immunomodulatory events of imidazochinolines in combination with the steadily increasing number of experimental and clinical studies using imiquimod for dermal disorders, we will try to construct a scenario of events, which might be going on in the skin upon imiquimod encounter:

(1) A given skin area around a developing tumor is not inflamed and immunologically silent for a long time (86, 87). When detected by the dermatologist and treated with topical imiquimod, a measurable reaction is developing slowly, as can be detected by the appearance of erythema and local pain only in a matter of several days. This is most likely due to the relatively low number of cells, which are able to directly respond to imiquimod locally, this being mostly dermal DC (Figure 4a) (53). This slow response also sharply contrasts to the rapid response to imidazoquinolines given systemically (56).

(2) The initial low amount of pro-inflammatory chemo- and cytokines being produced in the treated skin will attract increasing numbers of circulating pDC from the blood. Since these cells are extremely potent producers of IFNα and directly react towards imiquimod, the pDC influx will lead to an increasingly steep increase of IFNα in the skin which has been calculated to reach as much as 1.000 IU per 2-3 cm^2 of skin. Despite inducing massive gene expression, the so-called IFNα signature, in situ (29, 53), another very important response is the mobilization of Langerhans cells (LC) to leave the epidermis and migrate into draining lymph nodes, which is maximal at day 3 in mice (52) (Figure 4a and b).

3) The increased appearance of LC from an area, where tumor cells reside, might lead to an increased presentation of tumor antigen in the draining lymph nodes and consequently an increased generation of tumor specific T cells. This has not been experimentally tested in a time resolved manner so far. In addition, imiquimod is also able to powerfully activate B cells, which then might also present antigen to T cells. A hallmark of this effect is the massive enlargement of spleens in mice treated with imidazoquinolines topically (52) or systemically (Figure 4c) (56).

(4) The increased availability of tumor specific T cells in the peripheral blood together with the increased rolling of lymphocytes on TLR7 triggered endothelia (Figure 4d and e) (56) in the vicinity of the treated skin area will lead to a preferential infiltration and accumulation of tumoricidal effector cells (in case of BCC this will be mostly cells of the CD4 subtype (88)) in the treated skin area and ideally leads to an immunogenic destruction of the neoplasm, which can be detetcted by shrinkage or disappearance of the nodule (Figure 4a) (25, 27, 52). In addition, prolonged topical application of imiquimod might also directly lead to killing of melanoma cells, which increases the destruction of the tumor (Figure 4a) (81, 89).

This scenario is partly supported by experimental data and the currently available clinical results are very promising. However, a number of issues have still to be clarified and the hope is high, that future studies making use of the now available methods such as intravital imaging and new reporter mice will help to elucidate the full complement of events following the impact of imidazoquinolines in the skin and the whole body.

Figure legends

Figure 1: **TLR structure, function and their ligands.** All TLRs and interleukin-1 receptor (IL-1Rs) family members have a conserved Toll/IL-1R (TIR) domain in their cytoplasmatic tails crucial for assembly of downstream signaling complexes. The extracellular domain of TLRs contain 19-25 tandem copies of leucine-rich repeat (LRR) motifs involved in ligand recognition, while IL-1Rs contain three immunoglobulin-like domains. The TLRs that are involved in the recognition of microbial products are displayed on the cell surface, while TLR7, TLR8 and TLR9, all of which are involved in the recognition of nucleic-acid-like structures, are localized intracellularly. MyD88 is an essential TIR domain-containing adaptor (TLR 5, 7, 8, 9 and 10), for the induction of inflammatory cytokines via TLR. TIRAP is a second adaptor that specifically mediate the MyD88-dependent pathway via TLR2 and TLR4. The TIR domain-containing adaptor TRIF mediates the MyD88-independent TLR4 and TLR3 signaling pathway.

Figure 2: **TLR signaling**. Upon ligand binding TLRs form either homo- or heterodimeric receptor complexes and the TIR domain interacts with the adaptor protein MyD88 and the IL-1R-associated kinase (IRAK), which leads to activation of the adaptor molecule tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6). Subsequently, TRAF6 mediates the activation of the inhibitor of NF-κB (IκB) kinase (IKK), thereby initiating liberation of nuclear factor-κB (NF-κB), which then translocate into the nucleus and activate NF-κB-dependent inflammatory cytokine gene transcription. In contrast, MyD88-independent pathways activate the interferon-regulatory factor 3 (IRF3) and involves the late phase of NF-κB activation, together leading to the production of type I interferons (IFN-α).

Figure 3: **Histology and Immunohistochemistry.** Histopathologic findings of a nodular BCC after 2 weeks of 5 % imiquimod cream (3×/week); a dense, diffuse interstitial, inflammatory infiltrate is present in the dermis, mainly composed of lymphocytes (hematoxylin-eosin, original magnification ×50) (a). Solar keratosis after successful treatment (3×/week imiquimod for 6 weeks): thickened papillary dermis with fibrosis, ectatic blood vessels and signs of lichenoid interface-dermatitis (hematoxylin-eosin, original magnification ×200) (b). Deposits of amyloid in the papillary dermis are visualized by Congo red staining (Bowen's disease, 6 weeks of treatment) (original magnification ×200) (c). Immunohistolologic investigation shows predominance of CD4+ lymphocytes and a few CD8+ lymphocytes (d and e) (immunoperoxidase, original magnification ×50, ×100, respectively).

Figure 4: **Cellular interactions following topical treatment with imiquimod.** The TLR7/8 specific ligand imiquimod promotes an initial low amount of pro-inflammatory cytokine secretion by macrophages and dermal DC's, thereby attracting an increasing number type I IFN producing pDC from the blood (a and e). In response to proinflammatory cytokines Langerhans cells (LC) leave the epidermis and migrate into draining lymph nodes, leading to an increased presentation of tumor antigen in the draining lymph node, and consequently an increased generation of tumor specific T cells (a, b, and c). B cells are activated directly to form antibody secreting plasma cells. The increased availability of tumor specific T cells in the peripheral blood together with the increased rolling of lymphocytes on TLR7 triggered endothelia in the vicinity of the treated skin area will lead to a preferential infiltration and accumulation of tumoricidal effector cells in the treated skin area (d and e).

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