Immunostimulatory activity of Toll-like receptor 8 agonists towards human leucocytes: basic mechanisms and translational opportunities

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Abstract

TLR8 (Toll-like receptor 8) is activated by ssRNAs (single-stranded RNAs) and synthetic imidazoquinoline compounds that resemble purines and have immunostimulatory activity. TLR8 agonists are particularly effective at inducing Th1-polarizing responses from human monocytes and myeloid dendritic cells, with the magnitude of response substantially exceeding that induced by agonists of other TLRs. Mechanisms underlying the remarkable efficacy of TLR8 agonists may include: (i) particularly robust activation of intracellular signalling cascades culminating in nuclear translocation of NF-κB (nuclear factor-κB), (ii) activation of BTK (Bruton's tyrosine kinase), and (iii) the ability of some imidazoquinolines to induce TLR-independent effects via antagonism of adenosine receptors. The strong agonist activities of TLR8 agonists also extend to human neonatal leucocytes, which usually display impaired Th1-polarizing responses. Their strong Th1-polarizing properties render TLR8 agonists attractive targets of biopharmaceutical development as agents that may induce protective immune responses in diverse populations, including newborns.

Introduction

TLR (Toll-like receptor) 7, TLR8 and TLR9 represent a subfamily of TLRs expressed in endosomes that mediate recognition of microbial nucleic acids [1]. Throughout this review, the term ‘TLR8 agonists’ is used to encompass also agonists of both TLR7 and TLR8 (i.e. TLR7/8 agonists). Robust activation by TLR8 agonists of Th1-polarizing responses in human neonate and adult monocytes and mDCs [myeloid DCs (dendritic cells)] correlates with stronger TLR8 agonist-induced activation of intracellular signalling cascades, suggesting that the unique aspects of the TLR8 pathway in humans account for such an effective activation. We review the salient features of TLR8 signalling as currently understood, providing insight into the possible mechanisms of action of TLR8 agonists, including possible TLR-independent effects.

Key words: adenosine, adjuvant, imidazoquinoline, immune response modifier, single-stranded RNA (ssRNA), Toll-like receptor 8 (TLR8).

Abbreviations used: APC, antigen-presenting cell; BTK, Bruton's tyrosine kinase; CCR, CC chemokine receptor; DC, dendritic cell; Gag, group-specific antigen; HEK-293 cells, human embryonic kidney cells; xw, inhibitor of nuclear factor-κB; IFN, interferon; E-1, interferon-1; IFI, IFN regulatory factor; LRR, leucine-rich repeat; NAIP, neuronal apoptosis inhibitory protein; NACHT, NOD-like receptor, NACHT, NLRC, NLRP1 (NACHT, leucine-rich repeat, PYD domain-containing proteins); NAIP, nuclear apoptosis inhibitory protein; CIFA, CICA-like immunostimulatory factor; TIR, Toll/IL-1 receptor; TRAF6, TNF-receptor-associated factor 6; Tre, TRE; TNF-receptor-associated factor 6; Treq, T-regulatory cell; XLA, X-linked agammaglobulinaemia.

TLR8 and its agonists

The human TLR7 and TLR8 genes were identified based on homology with other TLRs, and are located next to one another on the X chromosome (Xp22) [2]. Human TLR7 and TLR8 share 42% identity and 60% homology at the amino acid level [2,3]. TLR7/8 orthologues are present in fish, birds, mice and monkeys [4]. TLR8 of Rhesus macaque monkeys closely resembles human TLR8 (96% identity and 98% homology at the amino acid level) (Figure 1). In contrast, murine TLR8 has divergent extracellular LRR (leucine-rich repeat) structure and distinct functional properties, in that murine TLR8 is not activated by agonists of human TLR8 when added in isolation [5]. The TIR [Toll/IL-1 (interleukin-1) receptor] domains of TLR8 orthologues are particularly highly conserved between species [6], suggesting conservation of cytosolic regions of TLR8 that may be essential for interactions with intracellular signalling pathways.

Small, low-molecular-mass (< 400 Da) synthetic imidazoquinoline compounds bearing structural resemblance to purines were the first TLR7 and TLR8 agonists identified [7] (Figure 2). Whereas imiquimod (molecular mass 240 Da) activates human cells via TLR7 only, R-848 (also known as resiquimod, molecular mass 314 Da) is a more polar and water-soluble congener that activates both TLR7 and TLR8 (TLR7/8 agonist) and is ~100-fold more potent than imiquimod [8,9]. In addition to its TLR8 agonist properties, the ability of R-848 as a weak base to accumulate more efficiently in late endosomes and lysosomes may also contribute to its strong bioactivity [10]. Subsequently,
Figure 1 | Close similarity of human and R. macaque TLR8 proteins
LRR and TIR domains are indicated. Note the close similarity of human and R. macaque TLR8 structures (96% identity and 98% similarity at the amino acid level) and the divergent structure of murine LRR repeats. This Figure was created using SMART software tool (http://smart.embl-heidelberg.de/). R. macaque predicted TLR8 protein sequence was taken from the NCBI (National Center for Biotechnology Information) website (accession number XP_001095602).

Figure 2 | Imidazoquinoline compounds bear structural similarity to adenosine and guanosine
Imidazoquinolines are small (molecular mass < 400 Da) synthetic purine (guanosine) congeners that activate human cells via TLR7 (imiquimod, gardiquimod and loxoribine), TLR7/8 (R-848, CL097) or TLR8 only (3M-002). Imidazoquinolines resemble adenosine, an endogenous purine metabolite with immunomodulatory properties. A recent study suggests that, in addition to TLR-mediated effects, imidazoquinolines also activate cells in a TLR-independent fashion via antagonism of adenosine [41].

Other novel synthetic imidazoquinoline congeners have been identified that specifically activate TLR7 or TLR8 alone, as determined by studies using TLR-transfected HEK-293 cells (human embryonic kidney cells) [11]. Viral ssRNAs (single-stranded RNAs), such as those of influenza virus, are natural agonists for TLR7 and TLR8. In particular, guanosine- and uridine-rich ssRNAs, such as that found in the U5 region of HIV and in the influenza virus, are recognized by TLR7 in mice but via TLR8 in humans [12,13]. Murine TLR8, which possesses a divergent extracellular LRR region (Figure 1), is not activated by agonists of human TLR8 added in isolation, but can be activated by a combination of TLR8-directed imidazoquinolines and polyT oligodeoxynucleotides to induce TLR8-dependent TNFα (tumour necrosis factor α) production [5].

The ability of TLR agonists to activate leucocyte subsets is heavily influenced by their TLR expression. Accordingly, TLR7 agonists directly activate purified pDCs (plasmacytoid DCs), a DC population that expresses large amounts of TLR7 and produces antiviral type 1 IFNs (interferons) and, to a lesser degree, monocytes. In contrast, TLR8 agonists directly activate not only monocytes, but also mDCs and moDCs (monocyte-derived DCs), corresponding to the robust TLR8 expression of these cells. These expression patterns are likely to contribute to the efficacy of TLR8 agonists in inducing Th1-polarizing cytokines such as TNFα and IL-12 [11].
The recognition of ssRNA and guanine analogues in mouse DCs is abrogated in the presence of chloroquine (an inhibitor of endosomal acidification), suggesting that TLR7 and TLR8 are located within endosomal or lysosomal compartments [13]. Initiation of TLR8 signalling following ssRNA and imidazoquinoline stimulation is dependent on a short cysteine-rich insert sequence within LRR8 [10], predicted to form a loop projecting from the solenoidal ectodomain of LRR8. An aspartic acid residue within LRR17 is also required for TLR8 signalling [10]. It is as yet unknown whether TLR8 agonists directly bind to TLR8 or whether intermediate molecules are involved in their recognition, by analogy to MD-2 (myeloid differentiation 2)/TLR4 [15].

**Effects of TLR8 agonists on human leukocytes**

**Monocytes**

When directly compared with agonists of other TLRs each tested at optimal concentrations, TLR8 agonists including R-848 (TLR7/8), the imidazoquinoline congeners 3M-003 (TLR7/8), 3M-002 (TLR8), as well as lipid complexed ssRNA and poly U sequences (TLR8), all induced robust production of TNFα by both human neonate and adult monocytes [9]. This TNFα response was greater than that by agonists of TLRs 1–7 (alone) [8,9]. Typically, agonists of TLR2/6 (bacterial lipopptide) and TLR7 (imiquimod) induce less than 10,000 pg/ml of TNFα in human whole blood. In marked contrast, TLR8 agonists induce up to 50,000–80,000 pg/ml of TNFα [9].

The strong TNFα-inducing activity of TLR8 agonists on human monocytes significantly correlates with the greater magnitude of TLR8 agonist-induced p38 MAPK (mitogen-activated protein kinase) phosphorylation and more profound/prolonged depletion (degradation) of IkBa [inhibitor of NF-κB (nuclear factor κB) α] in both neonatal and adult monocytes [9].

**APCs (antigen-presenting cells)**

TLR8 agonists induce marked surface expression of the co-stimulatory molecule CD40 on human mDCs tested in whole blood, again exceeding effects by agonists of other TLRs [9]. In addition, TLR8 (3M-002, ssRNA) and TLR7/8 (3M-003, R-848) agonists effectively induce robust up-regulation of IL-12 production in both neonate and adult monocytes [9]. This cytokine is of particular importance due to its ability to induce proliferation of CD4+ T-cells towards a Th1 phenotype.

Of note, however, is the observation that agonists that activate TLR8 only do not induce production of IFN-α, a cytokine that contributes to adaptive immune responses [16,17]. However, TLR7/8 agonists strongly induce production of TNFα and IL-12, as well as up-regulation of the co-stimulatory molecule CD40 from human monocytes/macrophages [8,9] while also inducing robust IFN-α production via pDCs [18]. Naïve or resting B-cells express low levels of human TLR8, which subsequently rises to moderate expression levels following B-cell activation [19]. The functional consequences of TLR8 expression on B-cells still remain to be determined.

**Tregs (T-regulatory cells)**

Human Tregs, classified as either naturally occurring (CD4+CD25+Foxp3+) or induced (CD4+CD25hi) Tregs, down-regulate a broad array of immune responses, maintain self-tolerance and are particularly abundant and potent in newborns [20–24]. Activated Tregs induce non-specific suppression of both CD4 and CD8+ T-cells via cell–cell contact and via production of cytokines such as IL-10 and TGF-β (transforming growth factor-β). Of note, Tregs express relatively high amounts of TLR8 mRNA, and both synthetic and natural agonists of human TLR8 can reverse Treg function via a TLR8–MyD88 (myeloid differentiation factor 88)–IRAK4 (IL-1-receptor-associated kinase 4) signalling pathway [25]. Thus TLR8 apparently plays unique roles in the biology of human Treg cells, contributing to the overall ability of these agents to strongly induce innate immunity and enhance adaptive immunity (Figure 3). In addition, TLR-mediated activation of accessory cells can also indirectly regulate Treg function [26]. Activation of TLRs on APCs induces production of IL-6, a cytokine that renders CD4+ cells refractory to Treg suppression in vitro [27] and in vivo, representing an important means by which TLR agonists can indirectly modulate Treg function [28].

**The TLR8 signalling pathway**

Studies using TLR-transfected HEK-293 cells demonstrated that agonist-induced stimulation of TLR8 induces MyD88-dependent activation of the classical TLR signalling pathway, culminating in the activation of NF-κB [7]. In addition, TLR8 can also utilize MEKK3 (MAPK/extracellular-signal-regulated kinase 3) to mediate NF-κB and JNK (c-Jun N-terminal kinase) activation, as demonstrated using transfected HEK-293 cells [30]. TLR8 agonists induce the production of various inflammatory cytokines (IL-6, IL-12, TNFα and IFN-γ), up-regulation of co-stimulatory molecules (CD40, CD80 and CD86), MHC molecules and CCRs (CC chemokine receptors) (CCR7) [7,31,32]. BTK (Bruno's tyrosine kinase) has been shown to interact directly with the intracellular domain of TLR8 and plays an important role in downstream signalling events following TLR8 activation [33–35]. For example, cells derived from patients with XLA (X-linked agammaglobulinaemia) demonstrate selective deficiency in TLR8-induced production of TNFα and IL-6 [34,37]. Furthermore, DCs derived from healthy adult controls treated in vitro with the BTK-selective inhibitor LFM-A13 also demonstrated selective impairment of TLR8-induced TNFα and IL-6, similarly to XLA cells.

In addition to the aforementioned pathways, TLR7/8 agonists activate, via TLR7, production of type I IFNs (IFN-α and IFN-β) [12,31] by a MyD88–TRAF6 (TNF-factor-receptor-associated factor 6)–IRF7 (IFN regulatory factor-7) complex that induces phosphorylation, dimerization and
TLR8 agonists activate human APCs and reverse human Treg function

TLR8 agonists strongly activate human APCs via TLR8-dependent and, possibly, via a TLR8-independent mechanism involving antagonism of adenosine, an endogenous purine metabolite that acts via seven-transmembrane adenosine receptors to induce the second messenger cAMP. Exposure of human APCs to TLR8 agonists induces robust phosphorylation of p38 MAPK and profound/prolonged disappearance of IκBα, resulting in robust induction of protective Th1-type immune responses, including production of IL-12 and up-regulation of the co-stimulatory molecule CD40. TLR8 agonists also reverse suppression mediated by human Treg cells, via both direct action on Treg as well as by induction of APC production of IL-6, a cytokine that renders T responder cells refractory to Treg-mediated inhibition.

nuclear translocation of IRF-7 and consequent activation of the IFN-α promoter [38]. Human pDCs constitutively express IRF-7 and efficiently produce IFN-α after TLR7 stimulation. IRF-5 has also been implicated in TLR7 and TLR8 signalling after R-848 stimulation, inducing both IFN-α and IFN-β in a human monocytic cell line [39].

In addition to TLR-mediated effects, the imidazoquinoline compounds R-848 (TLR7/8) and imiquimod (TLR7) activate caspase 1 via NALP3 (cryopyrin/NACHT [NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from Podospora anserina) and TP1 (telomerase-associated protein 1)]- LRR- and pyrin-domain-containing protein 3), leading to IL-1β and IL-18 production [43]. These results reveal yet another TLR-independent mechanism by which the imidazoquinoline compounds can activate immune responses in human cells.

TLR8 agonists uniquely activate the neonatal immune system

Impaired functional responses of neonatal APCs to a wide variety of stimuli represent a major impediment to inducing protective immunity in human newborns, a population...
at high risk of bacterial and viral infection [44,45]. It is increasingly recognized that although expression of Th1-type immunity in response to a variety of microbial stimuli is markedly impaired at birth, there is relative preservation of neonatal Th2-immune responses [46,47]. Such a Th2 polarization bias against the Th1-type immune responses required for effective cell-mediated immunity and for effective immune responses to many vaccines. Efforts to prevent neonatal infections and to prevent newborns and infants from serving as a major reservoir for infection of older individuals [48] are therefore limited by incomplete understanding of the neonatal immune response, and in particular mechanisms by which the neonatal adaptive immune system may be instructed to provide Th1-type responses.

The discovery and characterization of the TLR system have facilitated a fresh approach to characterizing the human neonatal immune system. Although full-term human newborns express normal basal amounts of TLRs at birth, activation of neonatal blood monocytes with agonists of TLRs 1–7 results in impaired TNFα production relative to adult peripheral blood monocytes [8]. This impairment is due, at least in part, to the distinct functional expression of the adenosine system at birth [49]. Neonatal blood plasma contains high concentrations of adenosine, an endogenous purine metabolite that induces intracellular accumulation in mononuclear cells of cAMP, a second messenger that inhibits production of Th1-polarizing cytokines, while preserving production of cytokines with Th2-polarizing and anti-inflammatory properties [47]. In marked contrast with the general pattern of impaired stimulus- (including TLR agonist) induced Th1-polarizing neonatal responses, TLR8 agonists, including TLR7/8 agonists, induce robust immune responses by neonatal APC, comparable with those of healthy adult controls [8,9].

Similar to most other TLR agonists, imiquimod (TLR7) fails to effectively induce TNFα production in newborns. In marked contrast, the compound R-848 (TLR7/8) fully activates robust neonatal TNFα comparable with that of adult cells [8], suggesting that it is the TLR8 agonist activity of R-848 that renders it an effective inducer of neonatal synthesis of the Th1-polarizing cytokine TNFα. Accordingly, studies of human neonatal APCs cultured in vitro have demonstrated that TLR8 agonists possess unique efficacy to induce the Th1-polarizing cytokine TNFα at both the mRNA and protein level [8,9]. TLR8 agonists, including R-848 (TLR7/8), the imidazoquinoline congeners 3M-003 (TLR7/8) and 3M-002 (TLR8), as well as ssRNAs (TLR8) induced robust production of the Th1-polarizing cytokines TNFα and IL-12 from neonatal (and adult) APCs that substantially exceeds responses induced by TLR2, 4 or 7 (alone) agonists [9]. TLR8 agonists also effectively induced up-regulation of the co-stimulatory molecule CD40 on neonatal and adult mDCs. The strong activity of TLR8 agonists in both neonatal and adult correlates with their induction of p38 MAPK phosphorylation and degradation of IκBα in both neonatal and adult monocytes. TLR8 agonists are thus uniquely efficacious in activating co-stimulatory responses in neonatal APCs, suggesting that these agents are promising candidate adjuvants for enhancing immune responses in newborns.

Of note, human neonatal cord blood has high adenosine concentrations, and human neonatal mononuclear cells are especially sensitive to adenosine-induced cAMP accumulation and inhibition of TLR-mediated TNF production [49]. The extent to which antagonism of adenosine receptors may contribute to the strong immunostimulatory activity of certain imidazoquinoline compounds towards leucocytes of both neonates and adults remains to be defined.

Translational development of TLR8 agonists

Lessons from the clinical development of TLR7 agonists

Imiquimod (brand name Aldara™) was initially developed by 3M Pharmaceuticals as an antiviral agent and was approved by the U.S. FDA (Food and Drug Administration) in 1997 for the treatment of external genital and peri-anal warts caused by certain strains of HPV (human papillomavirus) [7]. Well after imiquimod was in clinical use, the molecular mechanism of Aldara™ was found to be via a TLR7/MyD88-dependent pathway [7,52]. pDCs are a major cellular target of imiquimod, that induce up-regulation of the lymphoid homing receptor CCR-7 and enhance co-stimulatory molecule expression and IFN-α production [32]. Imiquimod has also been studied as a potential treatment for several primary skin cancers and cutaneous tumours including, basal cell carcinomas, keratoacanthomas, actinic keratoses and Bowen’s disease [54]. Accordingly, humans treated with Aldara™ for cutaneous cancers have increased local pDCs with the phenotype CD123+CD83+BDCA-2+ and increased IFN-α production [55]. In general, the antiviral and antitumour properties of imiquimod are attributed to the following series of cellular and molecular events: (i) activation of TLR7 on local pDCs, resident macrophages, mDCs, monocytes and moDCs inducing the NF-κB signalling pathway and pro-inflammatory cytokine production; (ii) chemoattraction of additional pDCs; (iii) migration of Langerhans cells into the lymph nodes and IL-12 production by NK (natural killer) cells; and (iv) increased antigen presentation from DCs leading to T-cell activation.

The TLR7/8 agonist resiquimod (R-848) has been assessed in a pilot study and a randomized clinical trial in humans for genital herpes [56,57]. These data demonstrated that topical application of resiquimod reduced reactivation of herpes simplex virus infection, as determined by reduced viral shedding 60 days after drug administration [56,57]. Although resiquimod treatment effectively reduced reactivation, resiquimod-treated patients demonstrated an increase in the severity of local signs, compared with that for vehicle-treated patients [56]. Clinical development will therefore have to include monitoring of the inflammatory response induced by the imidazoquinoline compounds to ensure that their therapeutic index is not exceeded.
Animal models for clinical development of TLR8 agonists

R. macaque is a primate species closely related to humans and serves as a useful model in translational research [58]. To date, ten TLR/TIR orthologues have been identified within the R. macaque genome, with an overall mean amino acid identity of 96.7% to their corresponding human TLR/TIR sequences, compared with 87.4% to mouse TLR/TIR sequences [6]. The most highly conserved TLR/TIR is TLR8, which demonstrates 98.6% amino acid identity to human TLR8. Moreover, TLR8 in R. macaque and humans is highly conserved in terms of its predicted distribution pattern of extracellular LRRs, compared with mouse TLR8 when analysed using the SMART (simple modular architecture research tool) protein domain prediction software tool (Figure 1). Agonists of human TLR8 (e.g. 3M-002) have also been demonstrated to activate R. macaque PBMCs (peripheral blood mononuclear cells) in vitro, inducing IL-12p40/p70 production [59]. Therefore R. macaque is likely to be a highly relevant translational medicine model with predictive value for study of the activity of TLR8 agonists as novel immunostimulatory compounds in humans.

TLR7/8 agonists are efficacious as vaccine adjuvants in an R. macaque model. The TLR7/8 agonist 3M-012 conjugated to the HIV Gag (group-specific antigen) protein improved the magnitude of Th1 and CD8+ responses in adult R. macaques [59]. Immunization with the HIV Gag protein in combination with a TLR8 agonist (3M-002) alone demonstrated less Th1 responses compared with the conjugated TLR7/8 agonist. This additional effect seen with a combined TLR7/8 agonist compared with a pure TLR8 agonist may be due to the ability of the combined agonist to induce pDCs (that express TLR7 but not TLR8) to produce IFNα, a cytokine that enhances adaptive immune responses, in part via inducing Th1 differentiation of CD4+ T-cells [7,32].

Given the potential adjuvant capability of TLR agonists, we are currently characterizing TLR-mediated cytokine production in leukocytes derived from neonate, infant and adult R. macaques. Preliminary results indicate that the relative advantage of TLR8 agonists is also maintained in this species (V.J. Philbin and O. Levy, unpublished work).

Future prospects for translational development of TLR8 agonists

TLR8 agonists will undoubtedly be evaluated for a variety of indications as stand alone immunostimulatory compounds that increase resistance to infection/malignancy and as vaccine adjuvants. Our laboratory has been particularly focused on the preservation of human neonatal responses to TLR8 agonists as it raises the possibility that these agents might serve to enhance neonatal host defence. Human newborns suffer a high frequency of microbial infection, resulting in millions of excess neonatal and infant deaths per year (WHO (World Health Organization) [60]). In this context, neonatal immunization is desirable because: (i) newborns are susceptible to multiple bacterial and viral pathogens, (ii) strategies involving immunization of the mother pose substanstial logistical and medicolegal challenges, and (iii) birth represents a likely point of contact of the newborn with health care providers and therefore early life immunization is associated with a substantially higher rate of vaccination coverage than immunization given at later time points [61,62]. There is therefore a major unmet medical need for improved means of vaccinating newborns at birth, including novel neonatal vaccine adjuvants. Thus the evaluation of the clinical potential of TLR8 agonists in newborns is of great interest and potential global public health importance.

Conclusion

TLR8 agonists are particularly efficacious in inducing robust Th1-polarizing immune responses in human leucocytes, including those derived from newborns. This activity may reflect unique aspects of the TLR8 pathway, including involvement of BTK and robust p38 phosphorylation and profound/prolonged IκB degradation. In addition, imidazoquinoline agonists may also activate cells via TLR-independent mechanisms, including antagonism of adenosine, an endogenous purine metabolite with immunosuppressive properties, as well as engagement of the NACHT/inflammasome pathway. Future mechanistic studies will undoubtedly shed more light on the TLR8 signalling pathways, as well as the use of physiologically relevant animal models to examine the potential role of the TLR8 agonists as vaccine adjuvants and immune response modifiers. The ability of TLR8 agonists to induce strong Th1-polarizing responses suggests that they may be useful for a broad array of indications, including enhancement of host defence in immunocompromised populations. Although safety concerns will need to be carefully addressed with respect to avoiding overexuberant immune responses, the identification of TLR8 agonists as potential vaccine adjuvants [9] capable of enhancing protective immune responses at birth and throughout infancy has a clear potential to reduce infectious disease-induced morbidity and mortality during the first years of life.

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References
