JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS
Open Access on 07/16/2015

Stimulation of Innate Immune Cells by Light-Activated TLR7/8 Agonists

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Supporting Information

ABSTRACT: The innate immune response is controlled, in part, by the synergistic interaction of multiple Toll-like receptors (TLRs). This multi-receptor cooperation is responsible for the potent activity of many vaccines, but few tools have been developed to understand the spatio-temporal elements of TLR synergies. In this Communication, we present photo-controlled agonists of TLR7/8. By strategically protecting the active agonist moiety based on an agonist-bound crystal structure, TLR activity is suppressed and then regained upon exposure to light. We confirmed NF-κB production upon light exposure in a model macrophage cell line. Primary cell activity was confirmed by examining cytokine and cell surface marker production in bone-marrow-derived dendritic cells. Finally, we used light to activate dendritic cell sub-populations within a larger population.

The innate immune response plays a large role in determining self from non-self.[1] It is guided, in part, by the interaction of chemical agonists with Toll-like receptors (TLRs).[2] TLRs recognize distinct chemical species leading to the activation of the innate and adaptive immune system, influencing everything from vaccines to atherosclerosis.[3] The spatial and temporal responses of these receptors are critical for understanding their activity,[4] yet few techniques exist to control the spatial activity of TLR agonists. Here we show a photo-caging method[5] to control the activity of TLRs using light. Understanding the signaling of TLRs using photo-controlled agonists can aid understanding of how the innate immune system determines non-self, potentially leading to better vaccine design and understanding of inflammatory responses.

TLR7/8 are TLRs for which a small-molecule agonist and its binding interaction have been defined. The imidazoquinoline and thiazoquinoline families, of which the potent anti-tumor drug Imiquimod is a member, are widely used TLR7/8 agonists.[6] Here we report a photo-controlled agonist of TLR7/8 (Figure 1). We designed this photo-controlled agonist on the basis of the recently published crystal structure of TLR8.[7] We present results on the photo-activation of two molecules, a TLR7 agonist, Imiquimod (R837),[8] and a TLR7 and TLR8 agonist, Resiquimod (R848).[9] Activating both of these compounds with UV light led to stimulation of NF-κB[10] in model cell lines and primary cells. In addition, we selected antigen presenting cells (APCs) for activation within a population of cells using light.

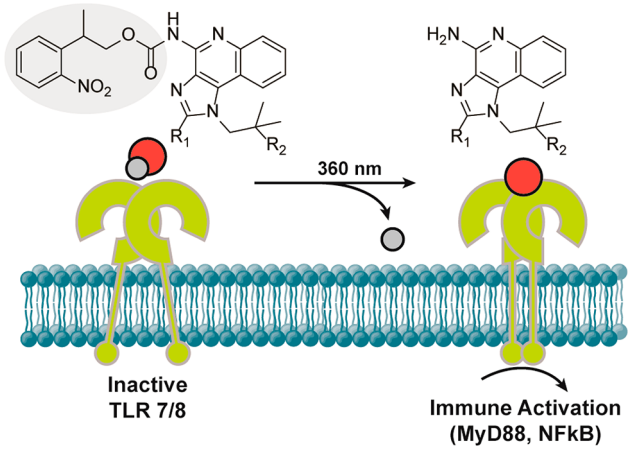

Figure 1. TLR7/8 activation and subsequent MyD88 signaling cascade following deprotection of photocaged small-molecule agonist.

In creating photo-caged agonists, we sought to control the signaling of TLRs. In the crystal structure as reported by Tanji and co-workers,[7] TLR 8 exists as a homo-dimeric pair. The C-terminus of each monomer is separated by 53 Å. Once the agonist is bound, the C termini are brought together to 30 Å initiating downstream signaling. In addition to the alanine scanning studies of Tanji, previous structure−activity relationship studies[11] showed the critical role of the C4 amine in the imidazoquinoline molecules and its binding to D543 and V573.

On the basis of the crystal structure of TLR 8, we proposed that the C4 amine of the imidazoquinolines was critical for activity (Figure 2). We predicted that caging the amine would block activity by inhibiting those same interactions. To protect the C4 amine, we created a carbamate of 2-(2-nitrophenyl)-propyloxycarbonyl (NPPOC), a well-studied photo-protecting group,[12] on both Resiquimod (Resiq) and Imiquimod (Imiq). The agonist was reacted with NPPOC-Cl in dioxane, heated to 50 °C, and then purified to yield the protected agonist (SI).

We first examined the kinetics of uncaging of both derivatives. Light-mediated production of the original agonists with the half-life was determined through UV absorbance and LC-MS. Initially, the maximum absorbances of NPPOC-Imiq and NPPOC-Resiq were 335 and 320 nm, respectively. During 1 h of continued UV exposure (4 W, 360 nm), these peaks decrease, indicating deprotection (Figures S1 and S2). Production of imidazoquinoline agonist following UV exposure

Received: December 3, 2013
Published: July 16, 2014

dx.doi.org/10.1021/ja412314j | J. Am. Chem. Soc. 2014, 136, 10823−10825

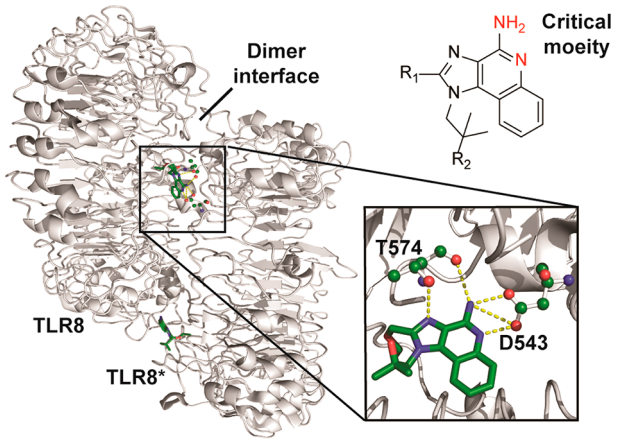

Figure 2. Crystal structure of TLR8 dimer and key binding sites of agonist (Resiquimod) upon activation. D543 and T573 knockout studies showed a decrease in NF-κB activity, demonstrating the importance of hydrogen bonding in activation.

was also confirmed by LC-MS where aliquots of the NPPOC-agonist in DMSO were analyzed via LC-MS from 0 to 60 min. After 1 min of UV exposure, 30−40% of each agonist was produced reaching up to 88% after 60 min (Figure 3). These results confirmed that Resiquimod and Imiquimod are recovered after brief exposures to UV light.

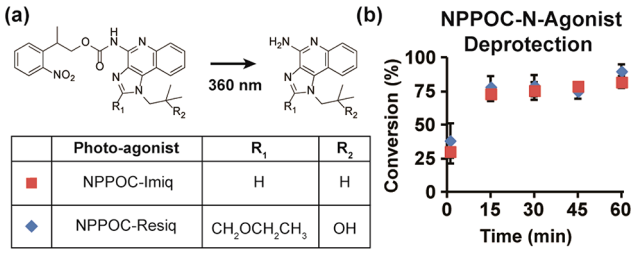

	Photo-agonist	R_1	R_2
■	NPPOC-Imiq	H	H
◆	NPPOC-Resiq	$CH_2OCH_2CH_3$	OH

Figure 3. (a) Deprotection of NPPOC-Imiq and NPPOC-Resiq with 360 nm light. (b) Percent conversion of protected agonists to Imiquimod (red squares) and Resiquimod (blue diamonds) measured by LC-MS. After 20 min of irradiation, the deprotection reaches 80% conversion. The first data points represent t = 1 min.

Next, we confirmed that photo-deprotection translated into activation of TLRs and a cellular response. We determined the effect of caging on TLR activity and innate immune stimulation using a reporter cell-line, RAW 264.7 (RAW-Blue).[13] The protected agonists were added to RAW-Blue cells, and half of the samples from each set were irradiated for 20 min. Without irradiation, samples with protected agonist yielded no NF-κB activity. Upon irradiation, NF-κB activity increased, as the protecting group is removed from the C4 amine and the TLR can dimerize to initiate signaling. Activation with uncaged agonists was comparable to the activation with either Imiquimod or Resiquimod (Figure 4a,b). [Note: Higher levels of NF-κB at 2 μM for Resiquimod are thought to be specific to the agonist. The discrepancy between the concentrations may be due to incomplete deprotection of NPPOC-Resiq leading to lower RAW-Blue stimulation.] As Imiquimod is a weaker agonist,[14] only Resiquimod and NPPOC-Resiq were used in subsequent experiments.

NPPOC-Resiq was tested with bone marrow-derived dendritic cells (BMDCs). The photo-activation of BMDCs was confirmed via flow cytometry by measuring secretion of

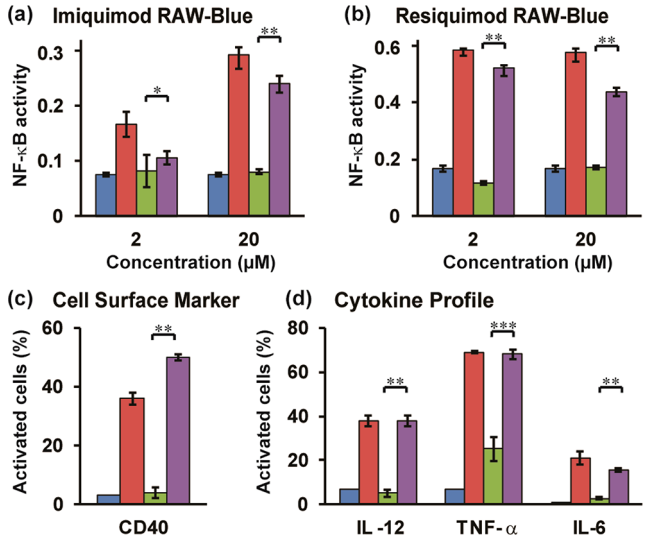

Figure 4. (a) RAW-Blue activation via NF-κB stimulation after 24 h incubation at 37 °C of Imiquimod (red), NPPOC-Imiq (green), *in situ* deprotected NPPOC-Imiq (purple), and resting (blue). (b) RAW-Blue activation via NF-κB stimulation after 24 h incubation at 37 °C of Resiquimod (red), NPPOC-Resiq (green), *in situ* deprotected NPPOC-Resiq (purple), and resting (blue). (c) BMDC activation via cell surface marker expression when incubated with agonists for 18 h at 37 °C. (d) BMDC intracellular cytokine production when incubated with agonists for 8 h at 37 °C. For flow cytometry experiments, Resting (blue), Resiquimod (red), NPPOC-Resiq (green), *in situ* deprotected NPPOC-Resiq (purple). Each result is from three independent experiments, where $*p < 0.047$, $**p < 0.0001$, and $***p < 0.0002$.

cytokines; IL-6, IL-12, and TNF-α and the increase in the expression of a costimulatory molecule, CD40. As observed in Figure 4c,d, NPPOC-Resiq without irradiation shows no activation compared to the Resiquimod control. After deprotection, the cytokine production and cell surface marker expression of the NPPOC-Resiq treated cells are comparable to the Resiquimod control. [Note: During the experiment, a signal decrease after UV exposure was observed; therefore, results were compared to UV-exposed Resiquimod.]

The photo-activation of BMDCs was also observed using confocal microscopy. Dendritic cells, when stimulated, initiate macropinocytosis within 15 min.[15] Cells treated with Resiquimod dendronize and engulf FITC-labeled dextran (Figure 5a). To confirm that agonist protection inhibits activity, cells were treated with NPPOC-Resiq, without (Figure 5b) and with (Figure 5c) irradiation. Without irradiation, BMDCs remain spherical with basal levels of endocytosis. Following 2 min of UV exposure using a Hg lamp (120 W), the cells become activated, comparable to cells treated with unmodified Resiquimod.

While activation of BMDCs showed spatial selectivity, upon activation, BMDCs rapidly migrated outside the activation area making quantification difficult. Therefore, to test spatial constraints, we instead used the less mobile DC 2.4s and a circular photo-mask of 3 mm diameter to activate a portion of the cells in a 14 mm diameter well (SI). After 1 min of photo-activation, we visualized intracellular expression levels of IL-12 (Figure 4). Comparing the irradiated circular region relative to the periphery, revealed that only irradiated cells exhibited higher levels of IL-12, while the periphery cells were not activated (Figure 5e,f). From this, we conclude that activation

dx.doi.org/10.1021/ja412314j | J. Am. Chem. Soc. 2014, 136, 10823−10825

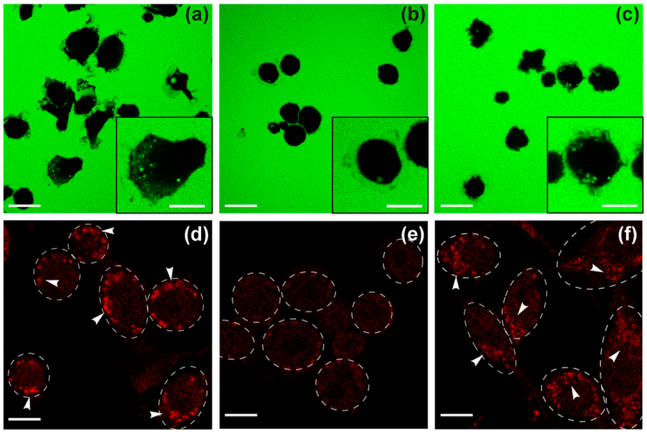

Figure 5. Confocal images of BMDCs in FITC-labeled dextran 10 min after treatment with (a) Resiquimod, (b) NPPOC-Resiq, and (c) NPPOC-Resiq and UV exposure. Scale bar is equal to 20 and 10 μm for inlaid images. (d–f) Visualization of spatially activated DC 2.4 via IL-12 staining. Individual cells are highlighted by dashed lines. (d) DC 2.4's treated with Resiquimod. Selections of a single cell culture containing DC 2.4 treated with NPPOC-Resiq, (e) at edge of culture with no activation and (f) at center of culture with activated DCs exposed to UV light. Scale bar is equal to 10 μm. See Supporting Information for videos of BMDC activation and experimental setup of spatial control of DC 2.4 activation.

of specific cellular populations is possible using a photo-caging approach.

In this work, we have successfully developed a technique to control immune cell activation through photolysis of a caging group. We found that the designed, protected agonist removes TLR activity and selectively stimulates activity when exposed to UV light. This has been tested on both a model cell line, as well as primary dendritic cells.

In the future, we plan to develop a series of caged agonists for different TLRs with selective deprotection at different wavelengths using two-photon excitation. These molecules can be used in conjunction to probe TLR signaling in a spatio-temporal manner.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, characterization, tables, figures, and videos of BMDC activation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Guan and Weiss laboratories for instrumentation use. We also thank the Cahalan and Kwon laboratories for advice on cell culture protocols. J.K.T. acknowledges the NSF (DGE-0808392) for a fellowship. We acknowledge UC-Irvine, a Hellman Faculty Fellowship, and an NIH New Innovator Award (DP2-AI112194) for funding.

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