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Controlling the Origins of Inflammation with a Photo-Active Lipopeptide Immunopotentiator

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Abstract

Inflammatory immune responses are mediated by signaling molecules that are both produced and recognized across highly heterogeneous cell populations. As such, studying inflammation using traditional immunostimulants is complicated by paracrine and autocrine signaling that obscures the origin of a propagating response. To address this challenge, we developed a small molecule probe that can photosensitize immune cells allowing for light-mediated inflammation. We use this probe to control the origin of inflammation using light. Following this motif, inflammation was induced originating from fibroblasts or dendritic cells. We report the contributions of fibroblasts and dendritic cells in initiating inflammation in heterogeneous co-culture which provides insights for the future development of vaccines and treatment of inflammation.

Keywords

photochemistry; immunoassays; inflammation; peptidomimetics; lipids

Inflammation is an important component in vaccine and wound healing therapies, but also plays a role in diseases such as diabetes, heart disease and sepsis. In each inflammatory response, functionally diverse populations of immune cells act in concert to effect a response with nuances such as timing,^[1] location,^[2] and motility patterns^[3] of each cell phenotype all affecting propagation of the resulting signal. Methods to study inflammation initiated from different cell populations disrupt these factors; either isolated cell types are primed (activated) with immunostimulants and washed extensively before introduction into heterogeneous co-culture,^[4] or immunostimulant-exposed cells are separated using physical barriers such as transwell membranes.^[5] These methods have been used to discover logic gates and signaling networks that control immune responses^[6] resulting in the development of increasingly sophisticated immunotherapies.^[7,8] However, removal of critical signaling molecules during post-priming washing steps and disruption of cell-cell contacts often hinder analysis and obscure signals reliant on precise temporal or cell-specific inputs. For instance, cell priming times range from 3–18 h,^[9,10] however, early proinflammatory cytokine transcription begins in as little as 15 min,^[11,12] implying that the initial portion of

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inflammatory signaling is effectively erased during a typical cell priming experiment. A method for *de novo* transduction of specific immune cells in their native environment would therefore be useful as adjacent cells would experience the complete temporal profile of cytokine signaling resulting from stimulation while also maintaining fidelity between cell-cell contacts. A similar problem was faced in the field of neuroscience, where identical receptors on neurons make it difficult to determine activity of subpopulations. Light-guided activation has revolutionized this area, and a variety of optical techniques including optogenetics,^[13] transcription-level photo-caging,^[14] as well as optofluidics^[15] are emerging as tools to study immune cells.

Here we demonstrate a Tagged and Remotely Induced Guided Immune Response (TRIGIR), which we use to tag cells with a photo-active immunopotentiator to effect subsequent Toll-Like Receptor (TLR)^[16] signal transduction and inflammation. Recently, we demonstrated light-mediated activation of immune cells using photo-caged TLR agonists.^[17] Here we disclose a photo-active TLR agonist that can be used to photosensitize immune cells (Figure 1A). We use this probe to address individual cell types in heterogeneous co-culture. Cell populations can be TRIGIRed by exposure to UV light to determine their individual contributions to an inflammatory response. By stimulating cell subsets within a larger cell population, we are able to examine the contribution of each cell subset to an ensemble inflammatory response (*trans*-activation).^[18] Interactions of infiltrating dendritic cells with stromal cells, such as fibroblasts, are implicated in a variety of chronic inflammatory disease states affecting both stimulation and regional memory.^[19] Therefore, we used TRIGIR to investigate the role of *trans*-activation of Bone Marrow Derived Dendritic Cells (BMDCs) and fibroblasts by initiating inflammation from each cell type.

In developing TRIGIR, we sought to address challenges in measuring an inflammatory immune response initiated in highly heterogeneous immune cell populations. In this environment, signaling by cytokines and prostanoids results in paracrine and autocrine feedback loops that complicate analysis of how distinct subsets of immune cells contribute to inflammation.^[20] Our attention was drawn to TLR2/6, as a critical receptor to initiate inflammation.^[21,22] We elaborated the palmitylated peptide Pam₂CSK₄ (TLR2/6 agonist) because it had three features amenable to TRIGIR. First, structure-activity studies indicated substitution at the *N*-terminus removes immunostimulatory activity.^[23] We hypothesized that attaching the *N*-terminal amine to a photo-labile protecting group would disrupt association of TLR2 with TLR6, an event required for activation.^[24] This hypothesis was supported by the TLR2/6 crystal structure which implies hydrogen bonding of the peptide's *N*-terminus with L318' is the strongest interaction of Pam₂CSK₄ with TLR6.^[25] Second, intercalation of the palmitic acid chains of Pam₂CSK₄ with TLR2 suggested our probe would bind TLR2 without activating the TLR2/6 complex resulting in a tagged population of photosensitized cells (Figure 1B). Lastly, the four lysine residues of Pam₂CSK₄ do not bind TLR2 or 6.^[26] This provided an ideal site to install a 5/6-carboxyfluorescein (FAM) fluorophore to track the TRIGIR probe (Figure 1C).

To develop our TRIGIR probe, we synthesized a photo-caged di-palmitylated cysteine in 8% yield from cystine di-*t*-butyl ester over 5 steps (Scheme 1). The synthesis began with addition of the photo-active compound 2-(2-nitrophenyl)propyl (NPPOC) chloroformate to

di-*tert*-butyl cystine to generate (**1**). This reaction was followed by reduction of the disulfide and addition of excess (10 eq) 1-bromo-2,3-propanediol to afford (**2**) in 42% yield (2 steps).

Subsequent palmitoylation and removal of the *tert*-butyl protecting group provided the terminal amino acid NPPOC-di-palmityl-cysteine (**3**) in 21% yield over 2 steps. Wang resin pre-loaded with *N*- ϵ -(FAM)-lysine was used to build the serine-tetra-lysine (SK₄) sequence before capping with (**3**) over 24 h. Global deprotection and resin cleavage provided the fluorescent, photo-caged sequence NPPOC-C(Pam)₂SK₄K(FAM)-OH (**4**) with the *N*-terminal nitrogen, critical for TLR6 activity, blocked by the photo-labile protecting group (**for complete synthetic details see** Supporting Information). Exposure to long-wave UVA light (Chromato-Vue TL-33, 1.8 A, 115 V, 60 Hz, 365 nm, 10 min) resulted in >75% conversion to the parent Pam₂CSK₄ immunostimulant both in solution and complete cell media formulations ($t_{1/2}$ = 5.1 min). Photolysis was performed *in vitro* with undetectable cytotoxic effects for these irradiation parameters.

Many TLR2⁺ cell types were amenable to tagging with the TRIGIR probe (**4**). TRIGIR parameters were optimized using RAW-Blue macrophages (Invivogen, CA) and Human Embryonic Kidney (HEK) hTLR2⁺ cells. Fibroblasts expressing p65-dsRed fusion protein^[12] were used to observe light-mediated stimulation via confocal microscopy. Lastly, the relationship between the origin of stimulation and resulting inflammatory response was studied in murine 3T3 fibroblasts and primary BMDCs.

Light-dependent inflammation (as measured by the inflammatory transcription factor NF- κ B) was first quantified upon direct addition of the TRIGIR probe to cell culture and was comparable to cells treated with equimolar amounts of the parent Pam₂CSK₄ immunostimulant. Prior to irradiation, cells remained in the resting state across probe concentrations varying by at least 2 orders of magnitude (typically 0.1–100 nM). Upon irradiation (365 nm, 10 min), both RAW-Blue and HEK hTLR2⁺ cell lines exhibited light-mediated increases in NF- κ B (Figure S8 and S9). Similar results were obtained for direct treatment of BMDCs, and light-mediated nuclear translocation of NF- κ B was observed in p65-dsRed fibroblasts (Figure S10). For every cell type tested, the TRIGIR probe demonstrated light-mediated stimulation with only basal levels of stimulation for cells kept in the dark (Figure 1D).

To generate photosensitized cells, we used TRIGIR to tag each cell type individually (**See supporting information for complete TRIGIR procedure**). The TRIGIR probe was observed to tag cells in a dose-dependent manner over 1–3 h with strong preference for TLR2⁺ (>95% tagging) compared to TLR2⁻ (<10% tagging) cells (Figure S11 and S12). Next, cells were washed until irradiated wash buffer did not stimulate RAW-Blue cells (Figure S13). To test activation, tagged cells were irradiated to effect transduction of TLR2/6 as measured by light-mediated TNF production (BMDCs) or NF- κ B transcription (HEK hTLR2⁺, RAW-Blue, p65-dsRed fibroblasts) (Figure S14 and S15).

Once tagged, the TRIGIR probe persisted on cells for the duration of all experiments (Figure S16), and was found to distribute on cell surfaces and in endosomes, consistent with reported TLR2 trafficking.^[27] As we intended to use the TRIGIR probe in heterogeneous

co-cultures containing tagged and untagged cells, it was important to know the dynamics of intercellular exchange with the TRIGIR probe. Therefore, an intercellular exchange experiment was performed at durations used for activation and quantification of inflammation using TRIGIR (2 and 24 h). Tagged BMDCs or tagged fibroblasts were incubated with untagged BMDCs (stained for CD86). Intercellular transfer of the TRIGIR probe was quantified by measuring the percentage of cells double positive for both TRIGIR and CD86 tags (untagged CD86⁺ BMDCs that acquire the TRIGIR probe from TRIGIR-tagged cells in co-culture). At 2 h, minimal (<1%) exchange was observed (Figure S17). As activation and subsequent production of TNF occur within 1–2 h, we determined this level of exchange acceptable for co-culture experiments. After 24 h, 16% of fibroblasts exchanged the TRIGIR probe with BMDCs; BMDC-BMDC co-cultures were more promiscuous resulting in 39% exchange.

Next, we used TRIGIR to study the effect of initiating inflammation from different cell types in co-culture (Figure 2A). In inflammation, it is particularly challenging to determine cytokines, such as TNF, that trigger positive feedback loops. We selected BMDCs and fibroblasts because the proinflammatory paracrine and autocrine signaling circuits make origins of inflammation difficult to establish for this pairing. As such, the exact contribution of different signaling molecules in fibroblast-immune cell signaling, such as TNF or prostanoids remains ill-defined,^[28,29] although impaired signaling is associated with a variety of disease states.^[30,31] At a minimum, *trans*-activation of fibroblasts can occur from BMDCs through TNF, while BMDCs can be stimulated by fibroblast prostanoids such as prostaglandin E₂.^[32]

We measured TNF obtained from mixed populations of BMDCs and fibroblasts relative to isolated populations while using TRIGIR to vary TLR signal transduction, and therefore, the origin of inflammation (Figure 2B). This approach allowed quantification of TNF production due to direct interaction of BMDCs with immunostimulant relative to *trans*-activation of BMDCs by fibroblasts or intercellular signaling between BMDCs. We considered each cell type (fibroblasts and BMDCs) along with each cell type tagged with equimolar amounts of TRIGIR probe (tagged-fibroblasts and tagged-BMDCs). Our experiments were matched with previously established direct-stimulation protocols, whereby each cell type was stimulated in isolation (with native Pam₂CSK₄ or irradiated TRIGIR probe), washed, and then added to co-culture. Bulk inflammation in co-cultures was quantified as serum TNF after 20 h for both TRIGIRed and directly stimulated cultures.

The TRIGIR technique produced marked differences depending on the initiating cell type, indicative of the differences each cell type plays in the origin of an inflammatory response. These differences were not as pronounced or not discernible using standard direct-stimulation techniques. In direct-stimulation experiments, all cell combinations involving BMDCs produced TNF, with stimulated BMDCs alone producing the most (21 pg/mL). Slightly lower levels of TNF were observed for direct stimulation of fibroblasts with unstimulated BMDCs or direct stimulation of BMDCs with unstimulated fibroblasts (Figure 2C).

Conversely, TRIGERed cell cultures displayed significantly higher TNF levels with clear differences among experiments. Tagged-BMDCs alone produced TNF in appreciable amounts (48 pg/mL), and addition of untagged-fibroblasts to tagged-BMDCs resulted in attenuated TNF (30 pg/mL). Fibroblasts do not secrete TNF, however, tagged-fibroblasts mixed with untagged-BMDCs produced the most TNF (56 pg/mL). This result indicates that TNF production is sensitive to the origin of inflammation; BMDCs are more sensitive to *trans*-activation from TRIGIRed fibroblasts than to direct activation via TRIGIRing TLR2/6. While these results do not conflict with the paradigm of BMDC-mediated immune responses, it is interesting that transduction of TLR2/6 on fibroblasts appears to be a dominant factor for TNF production in BMDC-fibroblast interactions. This finding could imply that targeting fibroblasts rather than BMDCs might be a better approach to treating inflammation or that designing adjuvants that target both DCs and fibroblasts could improve responses.

In conclusion, we demonstrated the synthesis and utility of a light-controlled immunostimulant probe for the immune cell receptor complex TLR2/6. This probe can be used to Tag and Remotely Induce a Guided Immune Response (TRIGIR) in TLR2⁺ cells. TRIGIR can be used to direct inflammation initiated from Bone Marrow-Derived Dendritic Cells (BMDCs) and fibroblasts. By tagging isolated populations of each cell type and TRIGIRing them in co-culture, we determined that the ensemble proinflammatory immune response generated from fibroblasts and BMDCs is amplified by activation of TLR2/6 on fibroblasts to a greater extent than direct transduction of TLR2/6 on BMDCs for TRIGIRed cell populations. We expect that this class of photosensitizing immunostimulant and the TRIGIR technique will provide a valuable tool in understanding the spatial, temporal and intercellular contributions to inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design of the TRIGIR probe and light activation of tagged cells. **(A)** The TRIGIR probe can associate with TLR2 without subsequent TLR2/6 dimerization and signal transduction. Upon irradiation, the photo-labile protecting group is released, activating the TLR2/6 complex resulting in inflammatory signaling. **(B)** The crystal structure of TLR2/6 bound to Pam₂CSK₄. Interactions with the *N*-terminus of the peptide with L318' on TLR6 and intercalation of palmitoyl chains into the TLR2 binding pocket are highlighted. The probe associates to TLR2 through interactions with the palmitoyl chains and the photo-labile protecting group prevents dimerization by preventing binding to TLR6 through L318' **(C)** The TRIGIR probe tags both fibroblasts (Fibs) and Bone Marrow Derived Dendritic Cells (BMDCs) in a dose-dependent manner as observed by the fluorescent tag attached to the probe. **(D)** Light-mediated immune cell stimulation was observed for a variety of cell types treated with the TRIGIR probe including BMDCs, fibroblasts, and macrophages. Errors report standard deviations for experiments performed in triplicate.

Figure 2.

The TRIGIR technique compared to direct stimulation provides a method to study *trans*-activation as initiated by tagged cells (A) TRIGIR preserves early signaling that is removed in experiments involving direct stimulation. (B) Bright field (left) and fluorescent (right, blue DAPI and green TRIGIR probe) images for mixtures of BMDCs and Fibroblasts (Fibs). * denotes cells tagged with the TRIGIR probe. Scale bar is 20 μ m. (C) TNF-ELISA data indicating the differences in the *trans*-activation of BMDCs and Fibs using TRIGIR. These changes in TNF production were not observed using direct stimulation techniques. Error bars are standard deviations for experiments repeated 6 times each (* $p < 0.025$, ** $p < 0.01$, *** $p < 0.001$).

Scheme 1.

a) NPPOC-Cl, TEA, DCM, 0 °C, 12 h, **b**) 1. DTT, TEA, chloroform, rt, 3 h, 2. 3-bromo-1,2-propanediol (10 eq), TEA, DMF, 80 °C, 15 min, **c**) 1. Palmitoyl chloride, TEA, DCM, 0 °C, 12 h 2. TFA, TIPS, DCM, rt, 3 h **d**) 1. (**3**, 3 eq) DIC, DMAP, rt, 24 h 2. TFA, DCM, TIPS, rt, 6 h