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Controlling the Origins of Inflammation with a Photoactive Lipopeptide Immunopotentiator**

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Abstract: *Inflammatory immune responses are mediated by signaling molecules that are both produced by and recognized across highly heterogeneous cell populations. As such, the study of inflammation using traditional immunostimulants is complicated by paracrine and autocrine signaling, which obscures the origin of a propagating response. To address this challenge, we developed a small-molecule probe that can photosensitize immune cells, thus allowing light-mediated inflammation. This probe was used to control the origin of inflammation using light. Following this motif, inflammation was initiated from fibroblasts or dendritic cells. The contributions of fibroblasts and dendritic cells in initiating inflammation in heterogeneous co-culture are reported, thus providing insights into the future development of vaccines and treatment of inflammation.*

Inflammation is not only an important factor in vaccine and wound-healing therapies, but also plays a role in diseases such as diabetes, heart disease, and sepsis. When inflammation occurs, functionally diverse populations of immune cells act in concert to effect a response depending on timing,[1] location,[2] and motility patterns[3] of each cell phenotype, which all affect propagation of the resulting signal. Methods to study inflammation initiated by different cell populations disrupt these factors; either isolated cells are primed (activated) with immunostimulants and washed extensively before their introduction into 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] thus implying that the initial portion of 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 that results 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 the activity of subpopulations. Light-guided activation has revolutionized this area, and a variety of optical techniques, including optogenetics,[13] transcription-level photocaging,[14] and optofluidics,[15] are emerging as methods to study immune cells.

Here we demonstrate a method to tag and remotely induce a guided immune response (TRIGIR), which we used to tag cells with a photoactive immunopotentiator to effect subsequent toll-like receptor (TLR)[16] signal transduction and inflammation. Recently, we demonstrated light-mediated activation of immune cells using photocaged TLR agonists.[17] The photoactive immunopotentiator we disclose here is a modified TLR agonist that can be used to photosensitize immune cells (Figure 1 A). We used this probe to address individual cell types in co-culture. Using TRIGIR the individual contributions of cell populations to an inflammatory response were determined. By stimulating cell subsets within a larger cell population, we were able to examine the contribution of each cell subset to the 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 populations of immune cells. In this environment, signaling, for example by cytokines and prostanoids, results in paracrine and autocrine feedback loops that complicate the analysis of how distinct subsets of immune cells contribute to inflammation.[20] Our attention was drawn to the complex of TLR2 with TLR6 (TLR2/6), which is a critical receptor complex to initiate inflammation.[21,22] We elaborated the palmitylated peptide Pam_2CSK_4 (TLR2/6 agonist) because it had three features amenable to TRIGIR. First, structure–activity studies indicated that substitution at the N-terminus removes immunostimulatory activity.[23] We hypothesized that attaching the *N*-terminal amine to a photolabile protecting group would disrupt association of TLR2 with TLR6, an event required for

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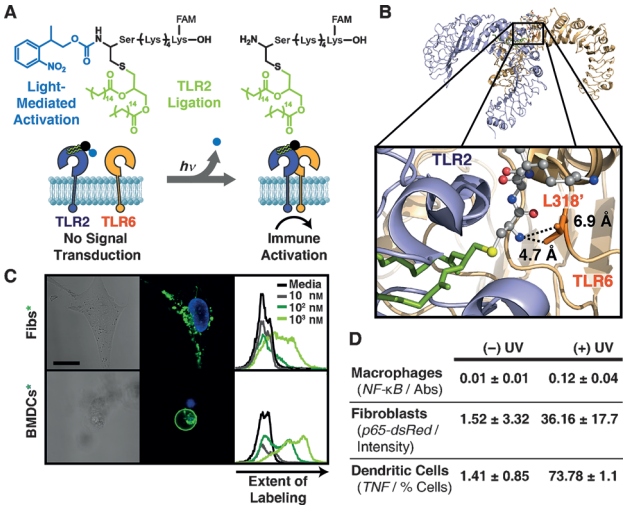
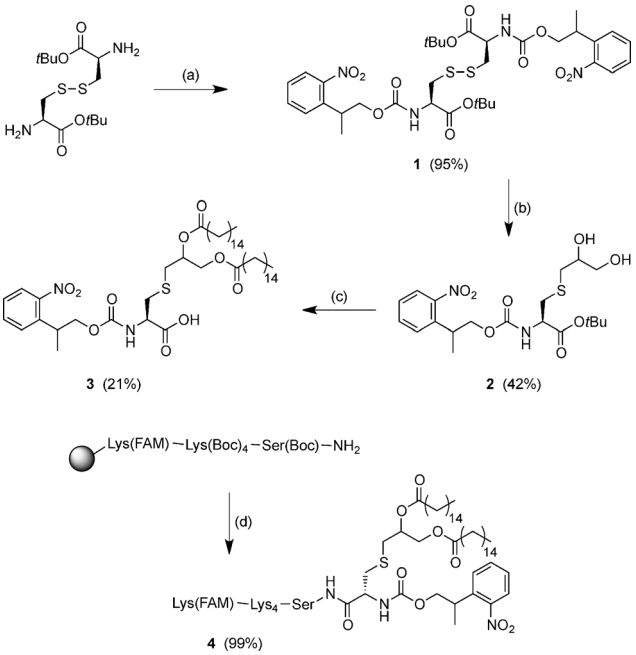


Figure 1. Design of the TRIGIR probe and light-mediated activation of tagged cells. A) The TRIGIR probe can associate with TLR2 without subsequent TLR2/6 signal transduction. Upon irradiation, the photolabile protecting group is released, thus activating the TLR2/6 complex and resulting in inflammatory signaling. B) Crystal structure of TLR2/6 bound to Pam_2CSK_4. Interactions of the N-terminus of the peptide with L318′ on TLR6 and intercalation of palmityl chains into the TLR2 binding pocket are highlighted. The probe associates to TLR2 through interactions with the palmityl chains, and the photolabile 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 with 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.



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 equiv), TEA, DMF, 80°C, 15 min; c) 1. Palmityl chloride, TEA, DCM, 0°C, 12 h; 2. TFA, TIPS, DCM, RT, 3 h; d) 1. (**3**, 3 equiv) DIC, DMAP, RT, 24 h; 2. TFA, DCM, TIPS, RT, 6 h. DIC = 1,3-diisopropylcarbodiimide, DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, DTT = 1,4-dithiothreitol, TEA = triethylamine, TIPS = triisopropylsilane.

activation.[24] This hypothesis was supported by the TLR2/6 crystal structure, which implies that hydrogen bonding of the *N*-terminus of the peptide with L318′ is the strongest interaction of Pam_2CSK_4 with TLR6.[25] Second, intercalation of the palmitic acid chains of Pam_2CSK_4 with TLR2 suggested that our probe could bind TLR2 without activating the TLR2/6 complex, resulting in a tagged population of photosensitized cells (Figure 1 B). Third, the four lysine residues of Pam_2CSK_4 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 1 C).

To develop our TRIGIR probe, we synthesized a photocaged dipalmitylated cysteine in 8% yield from cystine di-*tert*-butyl ester over five steps (Scheme 1). The synthesis began with the addition of 2-(2-nitrophenyl)propyl chloroformate (NPPOC-Cl) to di-*tert*-butyl cystine to generate **1**. This reaction was followed by reduction of the disulfide and addition of an excess (10 equiv) of 1-bromo-2,3-propanediol to afford **2** in 42% yield (two steps).

Subsequent palmitylation and removal of the *tert*-butyl protecting group provided the terminal amino acid NPPOC-di-palmityl-cysteine **3** in 21% yield over two steps. Wang resin preloaded with *N*-ε-FAM-lysine was used to build the serine-tetra-lysine (SK_4) sequence before capping with **3** over 24 h. Global deprotection and resin cleavage provided the fluorescent, photocaged sequence NPPOC-C(Pam)$_2$SK$_4$K(FAM)-OH (**4**) with the *N*-terminal nitrogen, which is critical for TLR6 activity, blocked by the photolabile protecting group (see the Supporting Information for complete synthetic details). Exposure to long-wave UVA light (Chromato-Vue TL-33, 1.8 A, 115 V, 60 Hz, 365 nm, 10 min) resulted in more than 75% conversion to the parent Pam_2CSK_4 immunostimulant, both in solution and complete cell media formulations ($t_{1/2} = 5.1$ min). Photolysis was subsequently 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 that express p65-dsRed fusion protein[12] were used to observe light-mediated stimulation by confocal microscopy. Lastly, the relationship between the origin of stimulation and the resulting inflammatory response was studied in murine 3T3 fibroblasts and primary BMDCs.

Light-dependent inflammation (as measured by the inflammatory transcription factor NF-κB) was first quantified upon direct addition of the TRIGIR probe to cell culture media and was comparable to cells treated with equimolar amounts of the parent Pam_2CSK_4 immunostimulant. Prior to irradiation, cells remained in the resting state across TRIGIR probe concentrations varying by at least two orders of magnitude (typically 0.1–100 nM). Upon irradiation (365 nm,

10 min), both RAW-Blue and HEK hTLR2$^+$ cell lines exhibited increases in NF-κB (Figure S8 and S9). Similar results were obtained for the direct treatment of BMDCs, and light-mediated nuclear translocation of NF-κB was observed in p65-dsRed fibroblasts (Figure S10). For every cell type that was tested, the TRIGIR probe demonstrated light-mediated stimulation with only basal levels of stimulation for cells kept in the dark (Figure 1 D).

In order to generate photosensitized cells, we used TRIGIR to tag each cell type individually (see the Supporting Information for the complete TRIGIR procedure). The TRIGIR probe tagged cells in a dose-dependent manner over 1–3 h with strong preference for TLR2$^+$ (> 95 % tagging) compared to TLR2$^-$ (< 10 % tagging) cells (Figures S11 and S12 in the Supporting Information). Next, tagged cells were washed. Removal of unbound TRIGIR probe was confirmed by irradiating the wash buffer before addition to RAW-Blue cells (Figure S13). To test activation, tagged cells were irradiated to effect transduction of TLR2/6, as measured by light-mediated production of tumor necrosis factor (TNF; BMDCs) or NF-κB transcription (HEK hTLR2$^+$, RAW-Blue, p65-dsRed fibroblasts; Figures 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 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 (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 2 A). In inflammation, it is particularly challenging to precisely 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 E_2.[32]

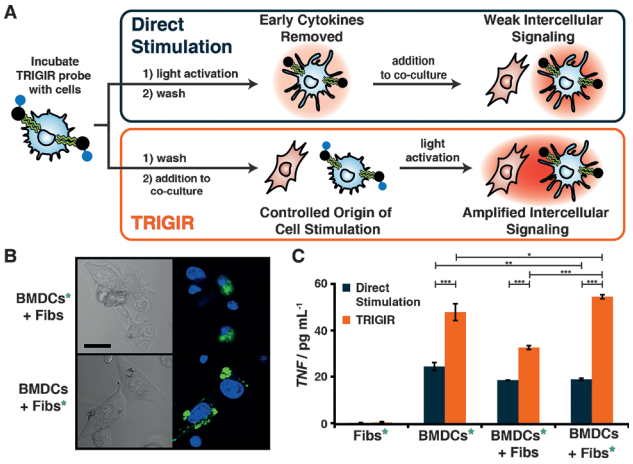


Figure 2. The TRIGIR technique compared to direct stimulation provides a method to study *trans* activation as initiated by tagged cells. A) TRIGIR preserves early signaling that is removed in experiments involving direct stimulation followed by washing. B) Bright-field (left) and fluorescent (right, blue DAPI and green TRIGIR probe) images for mixtures of BMDCs and fibroblasts (Fibs). * denotes cells tagged with the TRIGIR probe. Scale bar: 20 μm. 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: standard deviations for experiments repeated six times each (*$p < 0.025$, **$p < 0.01$, ***$p < 0.001$).

We measured TNF obtained from mixed populations of BMDCs and fibroblasts relative to isolated populations, while using TRIGIR to vary the location of TLR signal transduction, thereby varying the origin of inflammation (Figure 2 B). This approach allowed quantification of TNF production arising from 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 Pam_2CSK_4 or irradiated TRIGIR probe), washed, and then added to co-culture. Bulk inflammation in co-culture was quantified as serum TNF after 20 h for both TRIGIR and direct stimulation cultures.

The TRIGIR technique produced marked differences depending on the initiating cell type; indicative of the different role each cell type plays in an inflammatory response. These differences were not as pronounced or not discernible using the standard direct-stimulation technique. In direct-stimulation experiments, all cell combinations involving BMDCs produced TNF, with stimulated BMDCs alone producing the most (21 pg mL^{-1}). Slightly lower levels of TNF were observed for direct stimulation of fibroblasts with unstimulated BMDCs or direct stimulation of BMDCs with unstimulated fibroblasts (Figure 2 C).

Conversely, TRIGIR co-cultures displayed significantly higher TNF levels with clear differences among experiments. This demonstrates the enhanced signal obtained when cells are stimulated post-washing. TRIGIR BMDCs alone pro-

duced TNF in appreciable amounts (48 pg mL^{-1}), and addition of untagged fibroblasts to TRIGIR BMDCs resulted in attenuated TNF (30 pg mL^{-1}). Fibroblasts do not secrete TNF, however, TRIGIR fibroblasts mixed with untagged BMDCs produced the most TNF (56 pg mL^{-1}). This result indicates that TNF production is sensitive to the origin of inflammation; BMDCs are more sensitive to *trans* activation from TRIGIR fibroblasts than to direct transduction of TLR2/6 on BMDCs. While these results do not conflict with the current 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 is a valuable 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. The TRIGIR method can be used to direct the inflammation initiated from BMDCs and fibroblasts. By tagging isolated populations of each cell type and subjecting them to TRIGIR in co-culture, we determined that the combined proinflammatory immune response generated from fibroblasts and BMDCs is amplified by transduction of TLR2/6 on fibroblasts to a greater extent than the direct transduction of TLR2/6 on BMDCs for cell populations subjected to TRIGIR. We expect that this class of photosensitizing immunostimulants and the TRIGIR technique will provide a valuable method to understand the spatial, temporal, and intercellular contributions to inflammation.

Keywords: immunoassays · inflammation · lipids · peptidomimetics · photochemistry

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