Immunology

DOI: 10.1002/ange.201306551

Covalently Coupled Immunostimulant Heterodimers**

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Abstract: *We report increased stimulation of dendritic cells via heterodimers of immunostimulants formed at a discrete molecular distance. Many vaccines present spatially organized agonists to immune cell receptors. These receptors cluster suggesting that signaling is increased by spatial organization and receptor proximity, but this has not been directly tested for multiple, unique receptors. In this study we probe the spatial aspect of immune cell activation using heterodimers of two covalently attached immunostimulants.*

Herein we present the increased immunostimulatory effects of a polymer-linked heterodimer composed of dendritic-cell agonists (Figure 1). These agonists, termed pathogen-associated molecular patterns, are molecular signals used by the immune system to distinguish self from non-self.[1] The use of multiple agonists is emerging as the key aspect in robust activation of the immune system in applications ranging from vaccine formulation[2] to cancer immunotherapy.[3] For example, in many attenuated whole-virus vaccines, agonist "synergies" are used to generate durable immune responses and lifelong immunity.[4] These agonist combinations activate a range of receptors found throughout dendritic cells (DCs).[5]

The toll-like receptor (TLR) family is the most highly studied DC receptor subset.[6] The molecular basis for the activation of TLRs relies on the formation of higher-order complexes.[7] Inside a DC, the trans-membrane domains of these receptor complexes induce oligomerization of the signaling protein, myeloid differentiation primary response gene 88 (MYD88).[8] These MYD88 oligomers assemble with additional signaling proteins to activate an immune response.[9] We hypothesized that the spatial confinement in heterodimers of receptor agonists would increase activation. This could potentially occur through the confined association of multiple receptors thereby promoting MYD88 oligomerization and immune-cell activation.

Activation of any one receptor on a DC leads to stimulation. Simultaneous activation of multiple TLRs results in increased stimulatory effects broadly called synergies that can direct the polarization of the immune response.[10] Stimulation of DCs by varied agonist combinations directs amplification of the immune response, whereby a specific immunotherapy (e.g. vaccine, cancer) requires control over different combinations of pathways.[11] Several approaches, including the use of virus, nanoparticle, and dendrimer motifs, have combined multiple agonist signals.[12] Additionally, in other immune-cell systems, multivalent ligands enhance activation.[13] Increased synergistic activity appears to be correlated with proximity or multivalency, but this observation has never been directly tested until this study. Our operating hypothesis is that multivalent molecules might stimulate higher-order TLR structures. In this study, we found that the coupling of TLR agonists results in increased stimulation, but we cannot yet conclude that this behavior is due exclusively to TLR ordering.

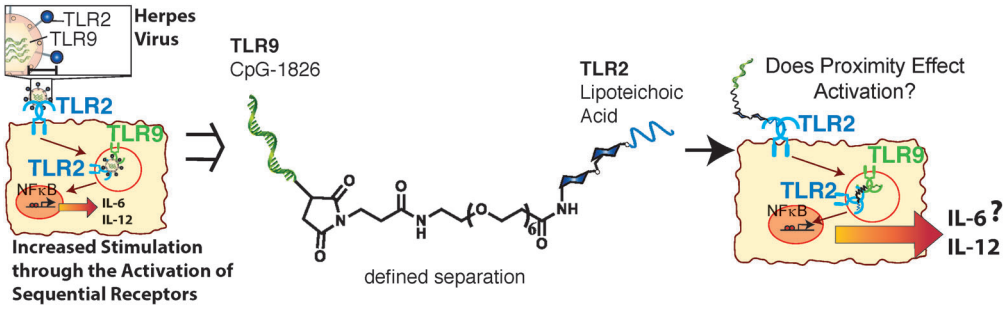

Figure 1. In native pathogens, such as the herpes virus, an inter-agonist spacing exists between different agonists. In this study, we probed the immunostimulatory effects of two different agonists, LTA and CpG, conjugated at a discrete molecular distance.

Well-defined and short inter-agonist spacings of imidazoquinoline homodimers results in modulation of immunostimulation through TLRs 7 and 8.[14] Similar to approaches used to investigate other biological heterodimers,[15] we sought to explore the effect of inter-agonist proximity on the activation of multiple TLRs with different agonist combinations. We used an α,ω-heterotelechelic poly(ethylene glycol) (PEG) linker to synthesize agonist heterodimers consisting of immunostimulants (known to have a mild synergy) from two different signaling pathways. These agonists activate corresponding TLRs in two different cellular locations, on the cell surface and in endosomal compartments.

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[**] We thank the Cahalan, Tsai, and Guan laboratories for reagents and instrument use. J.K.T acknowledges the NSF (DGE-0808392) for a fellowship. We acknowledge UC-Irvine for startup funds and an NIH New Innovator Award (DP2 AI112194).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201306551.

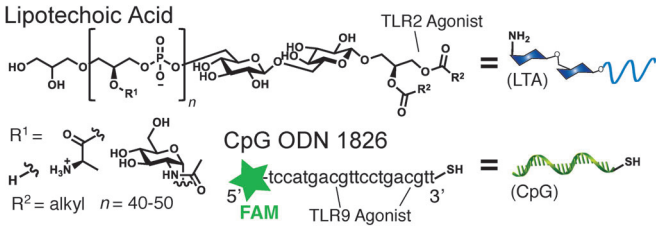

Figure 2. Lipoteichoic acid (LTA) is composed of alanine, GlcNAc, and hydroxy moieties linked along a phosphate backbone that is terminated with alkyl chains of varied structures. The CpG DNA, CpG ODN 1826, contained both a fluorescent FAM tag and a thiol group.

We covalently conjugated lipoteichoic acid to a DNA fragment (Figure 2) by using an α,ω-heterotelechelic PEG-based linker. The DNA is the endosomal TLR9 agonist, CpG ODN 1826 (CpG),[16] modified to contain a thiol and 6-fluorescein phosphoramidite (FAM) tag at the 3′ and 5′ ends, respectively. CpG was attached to the cell-surface TLR2/6 agonist, lipoteichoic acid (LTA), a class of glycosylated lipopolypeptide that contains a variable phosphate-linked polymeric backbone.[17]

The two agonists were conjugated using a heterotelechelic PEG-based linker (Figure 3) bearing *N*-hydroxysuccinimide (NHS) and maleimide end groups. LTA was first coupled to the PEG linker before conjugation of the maleimide moiety to the thiol on CpG. Strict control of the solution pH near 7.4 was required during all stages of the bioconjugation process as LTA is susceptible to hydrolysis under acidic or basic conditions. LTA from *Bacillus subtilis* was conjugated to the PEG linker through coupling of the alanine side chain in LTA to the NHS ester end-group of the PEG linker (see the Supporting Information for the experimental procedure). The resulting conjugate was characterized by NMR spectroscopy (see Figure S2 in the Supporting Information), UV/Vis spectroscopy, SDS-PAGE, and fast protein liquid chromatography (FPLC; see Figure S3). The LTA_PEG conjugate was quantified on the basis of the absorption maximum at 256 nm for use in the next step.

We further elaborated the LTA_PEG conjugate to synthesize the LTA_CpG heterodimer by treatment with CpG. The LTA_CpG heterodimer was purified by FPLC (Superdex G75, Dulbecco's phosphate-buffered saline, 0.2 mL min^{-1}) and characterized by UV/Vis spectroscopy (see Figure S4), SDS-PAGE (see Figure S5), and dynamic light scattering (DLS; see Figure S6). Stable particles were not observed by DLS. However, the LTA_CpG heterodimer was found to agglomerate over time; this is similar to LTA alone. The conjugate was quantified as the corresponding CpG by mass according to the FAM absorbance at 495 nm.

The LTA_CpG heterodimer was tested with two different cell lines, murine macrophage RAW-Blue cells and murine bone-marrow-derived dendritic cells (BMDCs). RAW-Blue is a reporter cell line for the activation of NFκB, a transcription factor and general measure of immune-cell stimulation. RAW-Blue cells secrete embryonic alkaline phosphatase (SEAP) upon activation of the NFκB pathway, thus enabling quantification of cell stimulation. RAW-Blue cells were stimulated with LTA, CpG, an unconjugated mixture of LTA and CpG, or the LTA_CpG heterodimer (see Figure S7). For comparison, lipopolysaccharide (LPS) was also tested. Concentrations were varied from 10 to 100 ng mL^{-1} with respect to CpG in the case of the LTA_CpG heterodimer and agonist mixture. The LTA_CpG heterodimer activated the RAW-Blue cell line to a greater extent than an unconjugated mixture of CpG and LTA. For concentrations greater than 25 ng mL^{-1}, the LTA_CpG heterodimer exhibited increased stimulation relative to LPS, one of the most potent known immunostimulants (Figure 4a).

The magnitude and polarization of the increased immune response was further examined in BMDCs to better understand the effect of the LTA_CpG heterodimer on the stimulation of primary antigen-presenting cells. The LTA_CpG heterodimer provided greater up-regulation of cell-surface markers and release of cytokines associated with amplification of the immune response. By antibody staining, we monitored changes in cell-surface proteins on cells containing the BMDC phenotype CD11c+. T-cell adhesion (CD 40, 80, and 86) and antigen-presentation (MHC-II) proteins were measured by flow cytometry. These proteins, always present on BMDCs, are up-regulated upon stimulation. Exposure to the LTA_CpG heterodimer increased the expression of each protein;

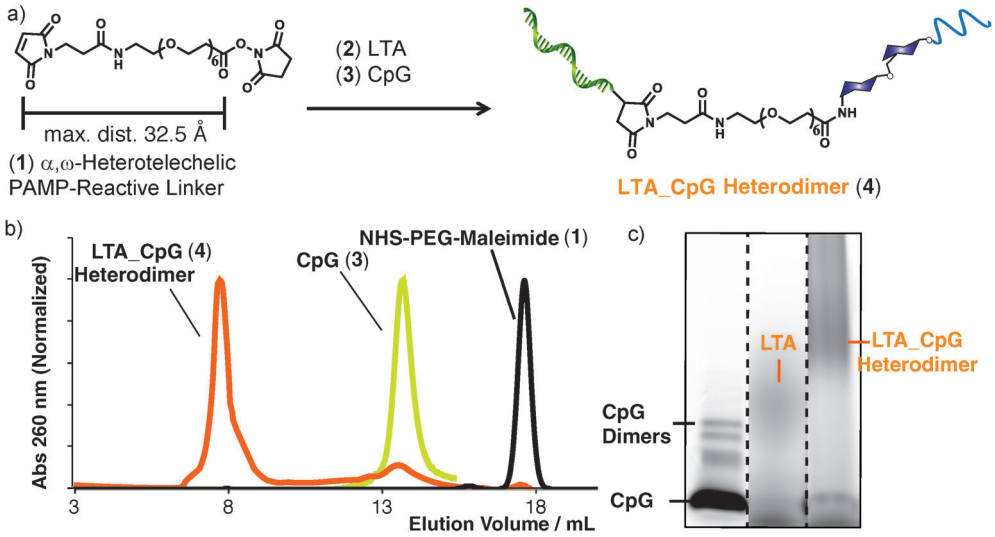

Figure 3. a) An α,ω-heterotelechelic PEG-based linker was conjugated first to a free amine on LTA and then to a thiol on CpG. b) The resulting crude product was purified by fast protein liquid chromatography. Characterization of the purified conjugate included c) SDS-PAGE (lane 1: CpG, lane 2: LTA labeled with fluorescein isothiocyanate, lane 3: purified LTA_CpG heterodimer).

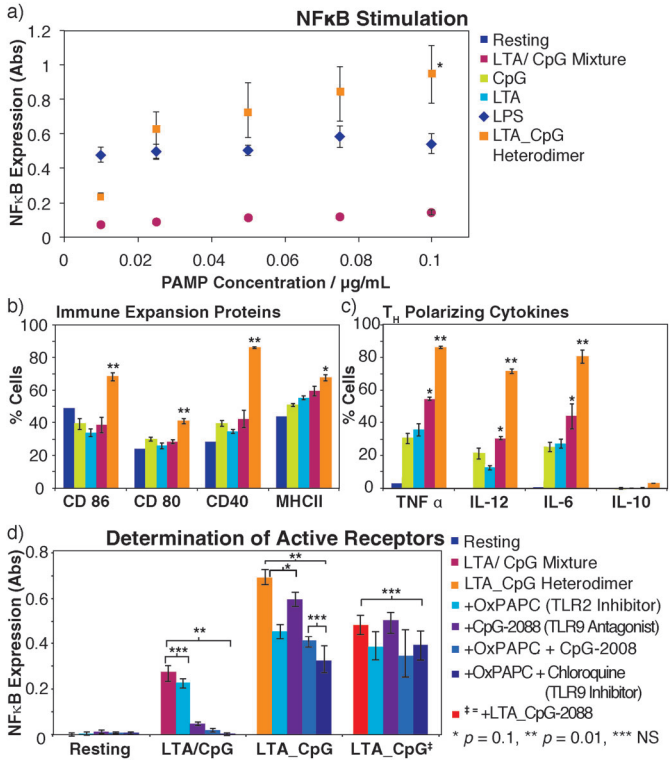

Figure 4. a) RAW-Blue cells were stimulated by the addition of an unconjugated mixture of CpG/LTA, LPS, or the LTA_CpG heterodimer. Activation was quantified by using the Quanti-Blue assay of NFκB (*$p < 0.005$ for the heterodimer relative to LPS and *$p < 0.001$ for the heterodimer relative to the LTA/CpG mixture). b) Bone-marrow-derived dendritic cells (BMDCs) were incubated with the same agonist combinations at 100 ng mL^{-1}. Antibody staining and flow cytometry were used to determine changes in cell-surface proteins involved with T-cell adhesion and antigen presentation as well as the expression of c) polarizing cytokines. d) The effects of antagonists and inhibitors of TLR2 and TLR9 were also examined in RAW-Blue cells.

this increase was most evident with CD 40, in which case surface expression was increased by over 40% for the LTA_CpG heterodimer relative to the unconjugated mixture (Figure 4b). The stimulation profile we observed is indicative of an increase in antigen cross-presentation and T-cell expansion based on increases in T-cell adhesion proteins and MHC-II. We therefore expect that the LTA_CpG heterodimers will perform as superior immunostimulants relative to either agonist alone or an unconjugated mixture.

To determine the polarization of the increased stimulation, we measured the expression of polarizing cytokines. Five cytokines were screened, including TNF α, ILs 6, 10, and 12, (Figure 4c), and interferon-γ (see Figure S8). CpG, LTA, and the mixture of CpG and LTA all induced production of cytokines associated with a T_H1-type response (TNF α, IL-6, IL-12). The LTA_CpG heterodimer induced over a 30% increase in the production of each of these cytokines within this same T_H1 profile and also induced the production of IL-10 at low (3% of cells expressed IL-10) but significant levels ($p < 0.001$, for the heterodimer as compared to the resting state).

Mechanistic studies were performed with TLR2 and TLR9 antagonists (OxPAPC, TLR 2 and ODN2088, TLR 9), an endosomal-protease inhibitor to block TLR 9 (chloroquine), and LTA conjugated to ODN2088 (Figure 4d). First, we used OxPAPC (Invivogen, CA) to competitively inhibit the TLR2 pathway. The resulting decrease in stimulation confirmed that CpG_LTA acts partially through TLR2. The addition of either ODN2088 or chloroquine further decreased stimulation, thus confirming that the activity was dependent on both TLR2 and TLR9. Stimulation also decreased upon the addition of ODN2088 or chloroquine alone, thus showing that activation was partially dependent on the TLR9 pathway. A combination of ODN2088 and chloroquine produced an additive effect in decreasing stimulation (see the Supporting Information). Cumulatively, these results indicate that stimulation by LTA_CpG proceeds through traditional TLR2/6 and TLR9 pathways. To test whether the increased activity of LTA_CpG was due to activation of both TLRs, LTA was conjugated to the antagonist CpG sequence ODN2088. This sequence competitively binds TLR9, thereby inhibiting activity while retaining TLR9 binding. Stimulation with the antagonist construct was less than that observed with the LTA_CpG heterodimer but greater than that observed with a mixture of the two PAMPs. Taken together, these results indicate that although the ability of the LTA_CpG heterodimer to access the TLR2/6 and TLR9 pathways is partially responsible for the synergies, there may be a second mechanism at work, as inhibitor and antagonist controls did not completely return baseline activity.

Our current operating hypothesis is that a molecular-level synergy between TLR2 and TLR9 is enhanced by tethering the TLR2/6 agonist LTA to the TLR9 agonist CpG. This hypothesis is supported by synergies found in the herpes virus, which successively activates TLR2 and then TLR9, and by the synergistically decreased activity when inhibitors are added.[18] We are not yet sure of the mechanism of this synergy. One possibility is that the LTA_CpG heterodimer creates an avidity effect for each TLR, thereby promoting formation of the myddosome and increasing stimulation. Although we have shown that the LTA_CpG heterodimer is sensitive to both TLR2 and TLR9 antagonists, we have not fully characterized the mechanism of increased stimulation. Therefore, this rationalization remains only one possible explanation. We also observed the RAW-Blue cell line stimulated with CpG or the LTA_CpG heterodimer by confocal microscopy. Cell binding and entry differed in length and location for CpG vs. the LTA_CpG heterodimer. CpG localized more rapidly, and LTA_CpG showed a greater, but more diffuse presence (see Figure S9). This evidence in conjunction with the antagonist/inhibitor assay provides support for the presence of a molecular-level synergy.

In summary, we synthesized a covalently coupled LTA_CpG heterodimer and observed increases in immune-cell stimulation with the LTA_CpG heterodimer relative to a mixture of LTA and CpG. This increased stimulation was conserved across two different cell types and among T-cell adhesion proteins, as well as polarizing cytokines. Our working hypothesis is that spatial patterning of agonists is

enhancing the formation of higher-order, myddosome (MYD88 oligomer) structures, but an as yet unknown effect that operates outside of TLRs cannot be ruled out. Increases in cell adhesion proteins and antigen-presentation proteins were observed for the LTA_CpG heterodimer relative to a mixture of the two agonists. The corresponding cytokine profile is also larger in magnitude for the LTA_CpG heterodimer and is indicative of a T_H1 response. These results indicate that controlling the spatial presentation of agonists to dendritic-cell receptors alters the stimulation of dendritic cells. We plan to further probe this amplification in T-cell expansion assays. In further experiments, we will attempt to confirm a molecular-level effect by exploring different linker lengths and other combinations of agonists.

Received: July 26, 2013
Revised: October 16, 2013
Published online: November 20, 2013

Keywords: bioconjugation · dendritic cells · heterotelechelic polymers · immunology · vaccines

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