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Authors
Albin, Tyler J
Tom, Janine K
Manna, Saikat
et al.

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Linked Toll-Like Receptor Triagonists Stimulate Distinct, Combination-Dependent Innate Immune Responses

Tyler J. Albin,[†,⊥] Janine K. Tom,[†,⊥] Saikat Manna,[†,‡] Adrienne P. Gilkes,[§,∥] Samuel A. Stetkevich,[†] Benjamin B. Katz,[†] Medalyn Supnet,[§,∥] Jiin Felgner,[§,∥] Aarti Jain,[§,∥] Rie Nakajima,[§,∥] Algis Jasinskas,[§,∥] Albert Zlotnik,[§,∥] Eric Pearlman,[§,∥] D. Huw Davies,[§,∥] Phillip L. Felgner,[§,∥] Amanda M. Burkhardt,*[,§,∥] and Aaron P. Esser-Kahn*[,†,‡]

[†]Department of Chemistry, University of California, Irvine, California 92617, United States

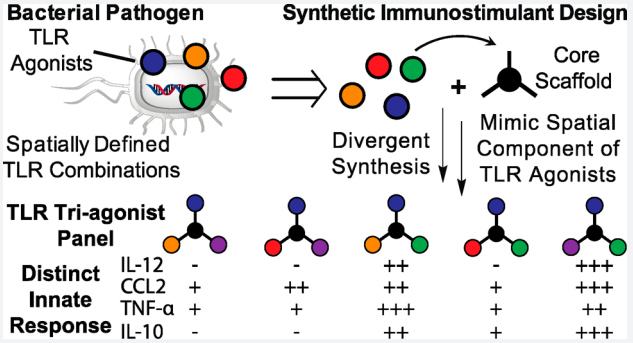
[‡]Pritzker School of Molecular Engineering, University of Chicago, Chicago, Illinois 60637, United States

[§]Vaccine Research and Development Center, Department of Physiology & Biophysics, University of California, Irvine, California 92617, United States

[∥]School of Medicine, Institute for Immunology, University of California, Irvine, California 92617, United States

Supporting Information

ABSTRACT: Traditional vaccination strategies have failed to generate effective vaccines for many infections like tuberculosis and HIV. New approaches are needed for each type of disease. The protective immunity and distinct responses of many successful vaccines come from activating multiple Toll-like receptors (TLRs). Vaccines with multiple TLRs as adjuvants have proven effective in preclinical studies, but current research has not explored two important elements. First, few multi-TLR systems explore spatial organization—a critical feature of whole-cell vaccines. Second, no multi-TLR systems to date provide systematic analysis of the combinatorial space of three TLR agonists. Here, we present the first examination of the combinatorial space of several spatially defined triple-TLR adjuvants, by synthesizing a series of five triple-TLR agonists and testing their innate activity both *in vitro* and *in vivo*. The combinations were evaluated by measuring activation of immune stimulatory genes (Nf-κB, ISGs), cytokine profiles (IL12-p70, TNF-α, IL-6, IL-10, CCL2, IFN-α, IFN-β, IFN-γ), and *in vivo* cytokine serum levels (IL-6, TNF-α, IL12-p40, IFN-α, IFN-β). We demonstrate that linking TLR agonists substantially alters the resulting immune response compared to their unlinked counterparts and that each combination results in a distinct immune response, particularly between linked combinations. We show that combinations containing a TLR9 agonist produce more Th1 biasing immune response profiles, and that the effect is amplified upon conjugation. However, combinations containing TLR2/6 agonist are skewed toward T_H2 biasing profiles despite the presence of TLR9. These results demonstrate the profound effects that conjugation and combinatorial administration of TLR agonists can have on immune responses, a critical element of vaccine development.



INTRODUCTION

Although vaccines are one of the greatest achievements of modern medicine, there are still many diseases that lack vaccines. Many exciting candidate antigens exist for target diseases, but they lack effective adjuvants that provide appropriate responses. Toll-like receptor (TLR) agonists are utilized as adjuvants, which are added to vaccine antigens to control an immune response.[1,2] To date, TLR-agonist-based adjuvants have largely been administered as a single agonist eliciting a single type of response. However, combinations of TLR agonists are both synergistic and inhibitory—altering cellular and antibody responses—yielding customized responses suited to specific pathogens. Combinations of TLRs are, in many ways, the original vaccination approach, as the natural adjuvanticity of whole-cell vaccines relies on TLR combinations (Figure 1A). A prime example is the successful whole-cell yellow fever virus vaccine,[3,4] which uses multiple TLR agonists to generate protective immunity.[5,6] While specific TLR combinations have been validated in current vaccines, few studies have explored the combinatorial space of linked TLR agonists.

The few examples of multi-TLR agonist systems in polymer particles and whole-cell vaccines show great promise, but most studies are done in solution and only with two agonists.[5,7−11] Recently, we showed that linked multi-TLR agonists alter immune responses. In our work, covalently linked agonists exhibit more potent activation compared to a solution of

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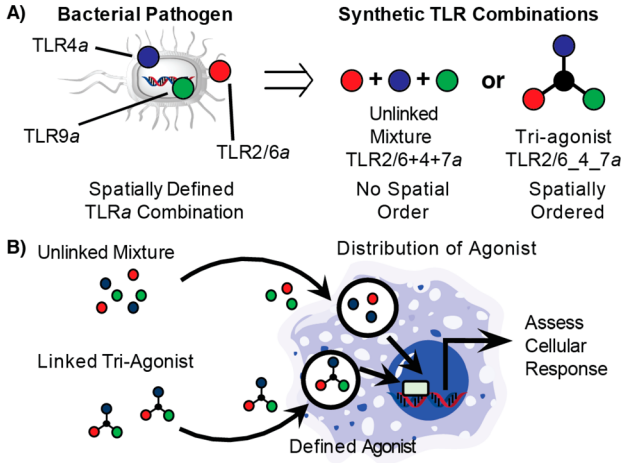

Figure 1. (A) TLR agonists are naturally present in pathogens. To recapitulate TLR activation by a pathogen, they can be added as a mixture or conjugated to mimic the spatial confinement in pathogens. (B) Conjugating the agonists ensures that all of the agonists are taken up by immune cells into the same endosome, leading to differing immune responses compared to the analogous unlinked combination.

unlinked agonists, and a TLR triagonist increased antibody titers and expanded antibody breadth.[12−14] Beyond our work, others have showed the following: (1) Spatial organization of TLR agonists with antigens enhances the immune response.[15−18] (2) The signaling architecture of TLRs is multimeric.[19] (3) Particles with multiple TLR agonists improve immune responses in vaccine applications.[20−22] (4) Single molecules which activate multiple TLRs provide a unique response (Figure 1B).[23] Despite these promising results, to date, no study has created a systematic comparison of even a small set of multi-TLR combinations *in vivo*.

Here, we demonstrate the synthesis of five distinct TLR triagonist combinations and compare their immunological response for both spatially organized and unorganized systems. The combinations were evaluated by measuring activation of immune stimulatory genes, cytokine profiles from stimulated primary cells, and serum cytokine levels and weight loss after model vaccinations *in vivo*. The linked TLR triagonists elicit distinct innate immune responses compared to their unlinked counterparts.

RESULTS

Panel of Five TLR Triagonists Synthesized via a Set of Bioconjugation Reactions. To explore the potential of unique TLR combinations, we needed to synthesize a series of TLR triagonists. This represents a major synthetic challenge as agonists encompass a diverse range of molecular structures, including lipopolysaccharides, peptidoglycans, flagellin protein, single-stranded DNA, and RNA.[24] To undertake this task, we designed a novel, divergent set of bioconjugation reactions. Each agonist was functionalized with an amine, carboxylic acid, thiol, or azide and linked to a triazine core functionalized with an amine, an alkyne, and either a carboxylic acid or maleimide (Figure 2B). We previously reported a TLR4_7_9*a* triagonist synthesis using this core.[14] In this study, we expanded the design of the triazine core scaffold to access multiple TLR agonist combinations by interchanging TLR agonists functionalized with the appropriate chemical handle—creating a panel of linked TLR agonists. TLR triagonists were synthesized containing different combinations of three of the following TLR agonists (TLR*a*): TLR1/2*a* (Pam_3CSK_4), TLR2/6*a* (Pam_2CSK_4), TLR4*a* (pyrimido-indole derivative),[25] TLR7*a* (imidazoquinoline derivative),[26,27] and TLR9*a* (ODN 1826 CpG DNA containing a phosphorothioate backbone) (Figure 2A). These agonists were chosen for their synthetic accessibility and synergistic effects. The pyrimido-indole TLR4*a* is less potent than the conventional MPLA agonists for TLR4 but is more well-suited for conjugation.[25] Similarly, the TLR7*a* imidazoquinoline was selected for its free amino moiety for conjugation, although its activity compared to the conventional R848 molecule is similar.[28] The five combinations synthesized were TLR1/2_4_7*a*, TLR2/6_4_7*a*, TLR1/2_4_9*a*, TLR2/6_4_9*a*, and TLR4_7_9*a* (Figure S1). In our

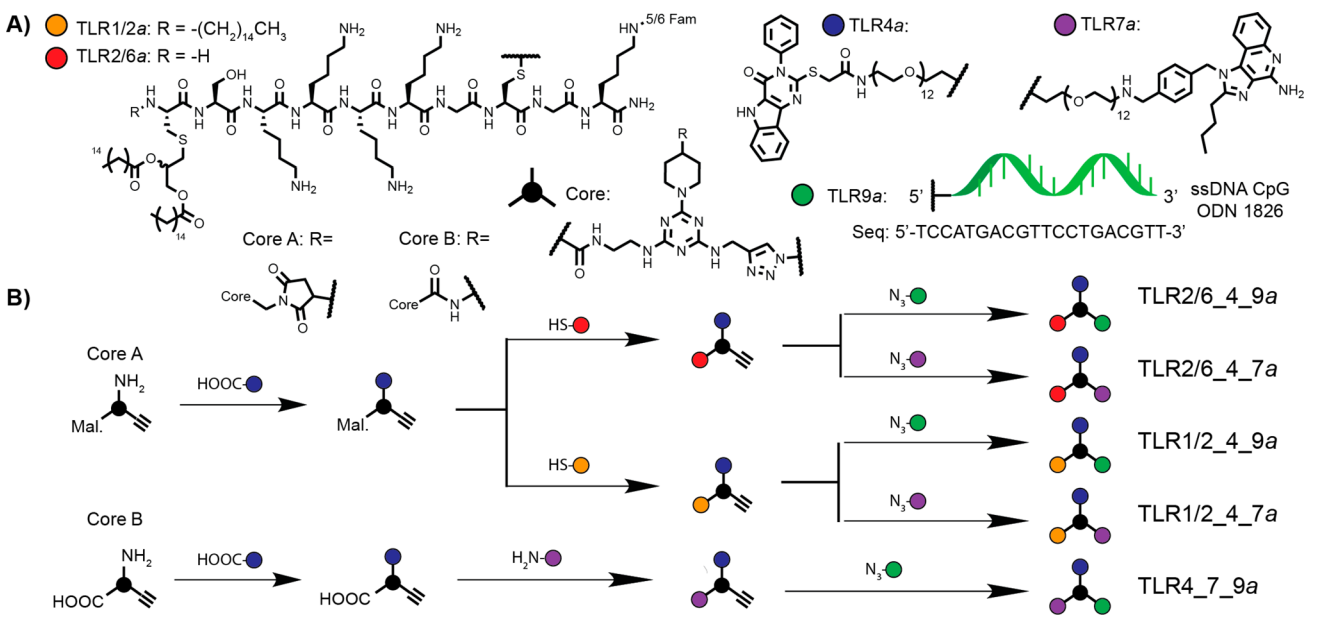

Figure 2. (A) Chemical structures of individual TLR agonists with covalent linkage sites shown. (B) General synthetic strategy to create five distinct TLR triagonists. Mal. = Maleimide.

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design, we incorporated PEG linkers to improve triagonist solubility and reduce potential steric effects between the agonist and TLR during binding. Each triagonist was purified by either HPLC or gel extraction and confirmed by MALDI-TOF or ESI-MS. More detailed information on the synthesis of the triagonists can be found in the Materials and Methods section and the Supporting Information.

The aggregation state of TLR agonists can influence their immunostimulatory activity. For example, CpG ODN (TLR9*a*) sequence and aggregation has been shown to influence activity.[29] In addition, Pam_3CSK_4 (TLR1/2*a*) and Pam_2CSK_4 (TLR2/6*a*) have been shown to form self-assembled nanostructures.[30] TLR7 and TLR8 agonist oligomerization has also been shown to play a role in modulating activation.[31] Finally, TLR agonist aggregation has also been shown to affect activity when paired with antigens in conjugate vaccines.[32,33] Thus, we characterized the individual, linked, and unlinked TLR agonist combination's particle size following dilution in PBS by dynamic light scattering (DLS) to gain insight into their particulate state. The small molecule TLR4*a* and TLR7*a* did not show any clear signs of aggregation by DLS (Figure S2). TLR1/2*a* and TLR9*a* appeared to show some signs of aggregation, although it did not appear to be consistent, potentially indicating unstable or heterogeneous associations. TLR2/6*a* showed a consistent particle size that was approximately 100 nm in size. All of the synthesized TLR triagonists showed some degree of aggregation by DLS intensity; however, only the non-TLR9*a* containing combinations, TLR1/2_4_7*a* and TLR2/6_4_7*a*, showed the major species being aggregated material by number (Figure S3). We speculate that this may be due to their hydrophobic character, whereas when TLR9*a* is included, the hydrophilic, single-stranded DNA limits aggregation of the triagonist. This is notable when comparing the aggregated states of TLR2/6*a* and TLR2/6_4_7*a* to TLR2/6_4_9*a*. The unlinked TLR9*a* containing combinations all showed more aggregated character relative to the linked compounds, resembling that of the most aggregation prone agonist in the formulation (Figure S4). For example, TLR2/6+4+9*a* aggregation resembled that of TLR2/6*a* rather than TLR2/6_4_9*a*.

Evaluation of TLR Activation of NF-κB by TLR Triagonists. Upon synthesis of these five distinct TLR triagonists, we investigated their ability to elicit immune responses. One element of synergistic activity from TLR combinations is the activation of transcription factor activity. We initially examined stimulation of the transcription factor nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). NF-κB responses are a broad measure of TLR agonist adjuvanticity which is correlated, but not directly related, to overall adjuvanticity.[34] NF-κB activity was compared in RAW-Blue NF-κB macrophage reporter cells stimulated either with the synthesized TLR triagonists or with analogous unlinked combinations. At the highest concentration tested (100 nM), TLR2/6_4_7*a*, TLR1/2_4_9*a*, and TLR2/6_4_9*a* stimulated the highest NF-κB activity of the five, linked triagonists and were not statistically significant from one another ($p > 0.05$, Figure 3A). TLR4_7_9*a* showed an intermediate level of NF-κB activity, and TLR1/2_4_7*a* stimulated the least NF-κB activity. The trend in NF-κB activity generally followed the order of potency of individual agonists alone, where TLR2/6*a* was the most potent, followed by TLR1/2*a* and TLR9*a* (Figure S5). With the exception of TLR1/2_4_7*a*, all of the linked compounds stimulated similar levels of NF-κB activity compared to their unlinked counterparts.

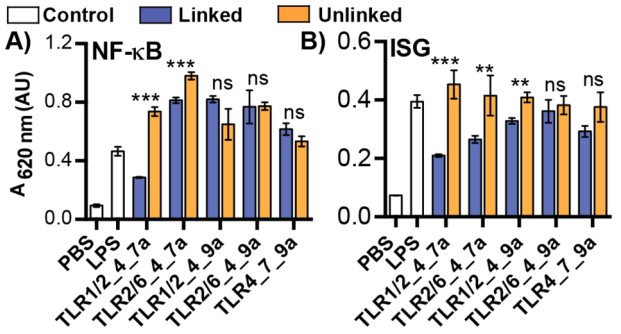

Figure 3. Innate immune activation as measured by (A) NF-κB and (B) ISG activity in RAW-Blue 264.7 macrophage cell assay treated with TLR triagonists or a 1:1:1 mixture of the analogous unlinked agonists. Cells were incubated with each compound (100 nM) for 18 h at 37 °C. Supernatant was removed and incubated with QUANTI-Blue for 1 h (NF-κB) or 4 h (ISG), and the absorbance at 620 nm was measured. Error bars represent SD. Samples were run in triplicate, where ** $p < 0.01$; *** $p < 0.001$; ns, not significant as determined by a two-tailed student *t* test. All noted statistical analyses represent the asterisked compound compared to the analogous unlinked compounds. See the SI for a full data set. AU = absorbance units.

After these initial responses, we became concerned that conjugating individual TLR agonists to a triazine scaffold could potentially affect their biological activity by disrupting binding to the receptor. Thus, we compared the activity of the triazine core conjugated to single agonists with that of the individual agonist without any linkers or core. All TLR agonists, except TLR9*a* (which was unaffected), had decreased activity upon conjugation to the triazine core as follows: TLR1/2*a* (2-fold decrease), TLR2/6*a* (20-fold decrease), TLR4*a* (10-fold decrease), and TLR7*a* (50-fold decrease, Figure S5). One *might* assume that this loss in activity would lead to drastically lower activity of the triagonists compared to the additive effect of mixing the unlinked agonists, which were not attached to the core. However, apart from TLR1/2_4_7*a*, the compounds stimulated similar activity to the unlinked mixture. Because all of the individual agonists lost activity when linked to the core, we hypothesized that the activity is partially restored due to amplified synergistic effects from conjugation compared to the mixed, unlinked agonists.

To further assess the contributions of specific TLR agonists when linked to the core, we evaluated the deconstructed components of each triagonist combination (Figures S6 and S7). Overall, we found that the activity of each triagonist typically followed that of the most potent individual TLR agonist in the combination. For example, TLR2/6_4_7*a* demonstrated high NF-κB activity similar to that of the potent individual TLR2/6*a*. However, linkage-dependent, synergistic effects were observed. In one case, TLR2/6_7*a* had 41% higher activity, despite the negative effects of being linked, as compared to the unlinked combination of TLR2/6*a* and TLR7*a* (Figures S6 and S7B). In other cases, inhibitory effects were observed. For example, the unlinked TLR1/2+4+7*a* and linked TLR1/2_4_7*a* both resulted in lower NF-κB activity than the predicted additive activity of the three individual agonists (Figures S6 and S7A). For further details on the NF-κB stimulatory activity of the triagonist components, please refer to the Supporting Information. We also validated that none of the compounds caused a decrease in cell viability

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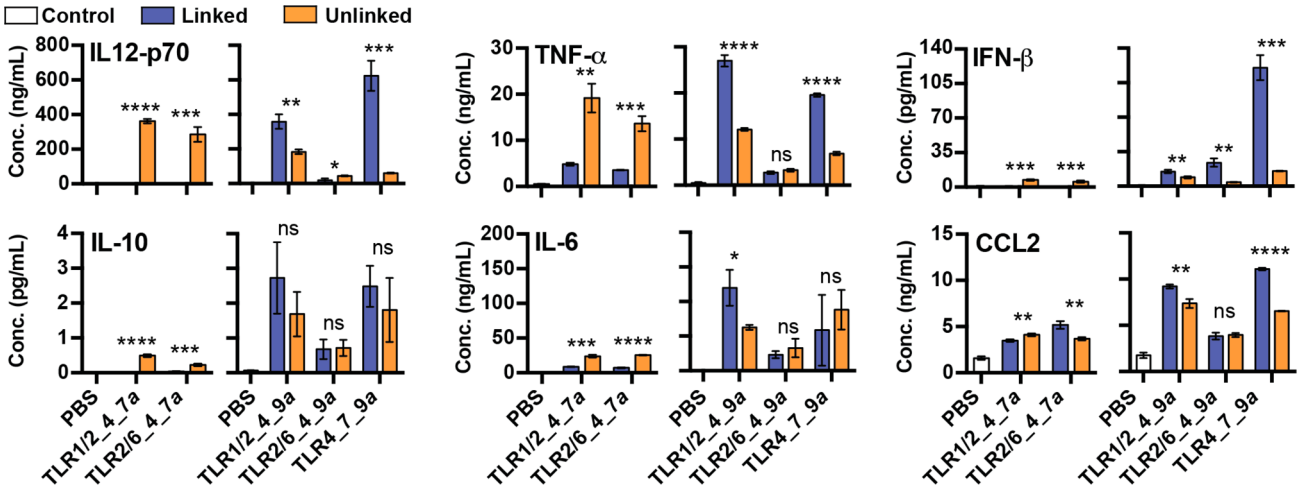

Figure 4. *In vitro* cytokine expression from BMDCs as measured by CBA (TNF-α, IL-10, and CCL2) and ELISA (IL-12p70, IL-6, and IFN-β). Cells were incubated with each TLR triagonist (100 nM) or a 1:1:1 (molar ratio) mixture of the analogous unlinked TLR agonists (100 nM each) for 6 h at 37 °C. Error bars represent standard deviation of the mean. Samples were run in triplicate, where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, not significant. Statistical analysis is between the linked vs unlinked agonists, performed by the two-tailed Student *t* test. TLR1/2_4_7*a* and TLR2/6_4_7*a* were tested separately from TLR1/2_4_9*a*, TLR2/6_4_9*a*, and TLR4_7_9*a* due to assay size constraints. No measurable levels of IFN-γ or IFN-α were detected.

compared to PBS (Figure S8). In addition, we assessed compound stability *in vitro* following incubation in fetal bovine serum (FBS). We found that in general compounds retained most of their NF-κB activity following 24 h of FBS incubation, where TLR1/2_4_7*a* had the lowest drop in activity, 77% of the un-FBS treated triagonist activity (Figure S9). From this we concluded that conjugation changes the activity of NF-κB partially between a balance of decreasing potency and increasing activation.

Evaluation of TLR Activation of ISGs by TLR Triagonists. Beyond activating NF-κB and subsequent inflammatory cytokines, the activation of interferons can be critical for a successful adjuvant, particularly in stimulating the differentiation of antigen specific $CD4^+$ and $CD8^+$ T cells.[35] We investigated interferon stimulatory genes (ISGs) activity following TLR triagonist activation using an ISG-Blue RAW macrophage reporter cell line.

Generally, the unlinked triagonist combinations stimulated higher ISG activity, measured by the absorbance at 620 nm (absorbance units, AU), as compared to the linked triagonists (Figure 3B). The triagonists lacking TLR9*a*, TLR1/2_4_7*a* (0.21 AU) and TLR2/6_4_7*a* (0.27 AU), induced 50% less ISG stimulatory activity compared with their unlinked counterparts, TLR1/2+4+7*a* (0.46 AU) and TLR2/6+4+7*a* (0.42 AU). The triagonists containing TLR9*a* (TLR1/2_4_9*a* (0.33 AU), TLR2/6_4_9*a* (0.37 AU), and TLR4_7_9*a* (0.30 AU)) stimulated similar levels of ISG activity compared to unlinked counterparts. Thus, ISG activity was enhanced with the addition of TLR9*a* in the linked compounds but did not change when unlinked.

TLR Triagonists Elicit Distinct Cytokine Profiles *in Vitro*. Although the triagonists induced NF-κB and ISG activity, the largest difference we observed was the triagonist's ability to elicit distinct cytokine profiles, consistent with our initial study of triagonist activity.[14] Synergistic or inhibited secretion of specific cytokines is another element of stimulation from multi-TLR systems. These distinct cytokine profiles can potentially define the type of immune response generated.[36] Thus, we analyzed the cytokine profiles elicited by TLR triagonist stimulation of murine bone-marrow-derived dendritic cells (BMDCs) *in vitro* (Figure 4). BMDCs were stimulated with linked or unlinked TLR agonists for 6 h. The supernatant was analyzed by cytometric bead array (CBA) for IL-12p70, TNF-α, and IFN-γ (T_H1 promoting) and IL-6, IL-10, and CCL2 (T_H2 promoting) cytokine and chemokine concentrations, or by ELISA if the detected cytokines were near the limit of CBA detection (IL-6, IL-12p70, IFN-β, IFN-α) (Figure 4).

TLR1/2_4_9*a* and TLR4_7_9*a* elicited high levels of IL-12p70 (TLR1/2_4_9*a*, 358 pg/mL; TLR4_7_9*a*, 624 pg/mL) and TNF-α (TLR1/2_4_9*a*, 27.0 ng/mL; TLR4_7_9*a*, 19.6 ng/mL), which is consistent with the reported role for TLR9*a* in selectively inducing T_H1 cells.[37] For these two compounds, the linked agonists stimulated significantly higher levels of IL-12p70 (TLR1/2_4_9*a*, 95% increase; TLR4_7_9*a*, 900% increase) and TNF-α (TLR1/2_4_9*a*, 123% increase; TLR4_7_9*a*, 184% increase) as compared to the analogous unlinked mixtures. In addition, the linked TLR4_7_9*a* produced relatively high levels of IFN-β (120 pg/mL) compared to the unlinked mixture (15 pg/mL). In contrast, compounds containing TLR2/6*a*, including TLR2/6_4_7*a* and TLR2/6_4_9*a*, stimulated relatively more IL-10, IL-6, and CCL2. Thus, TLR2/6*a* shifted the cytokine profile toward a T_H2 type response, overcoming the T_H1 biasing effect of TLR9*a*, as observed when comparing TLR1/2_4_9*a* (IL12-p70: 358 pg/mL) and TLR2/6_4_9*a* (IL12-p70: 18 pg/mL). The cells stimulated by TLR2/6_4_7*a* also displayed a more T_H2 biasing cytokine profile. TLR1/2_4_7*a* produced relatively low levels of all cytokines compared to the other triagonist combinations and the unlinked agonists—suggesting that this linked combination suppresses agonist activity. While we observed changes in cytokine expression for both the linked and unlinked combinations, we did not see statistically significant, reproducible changes in cell surface activation markers between linked and unlinked combinations, although they were higher than PBS alone (data not shown). From these results, we conclude that both the trimeric combination and its spatial ordering can drastically alter the activity of stimulated

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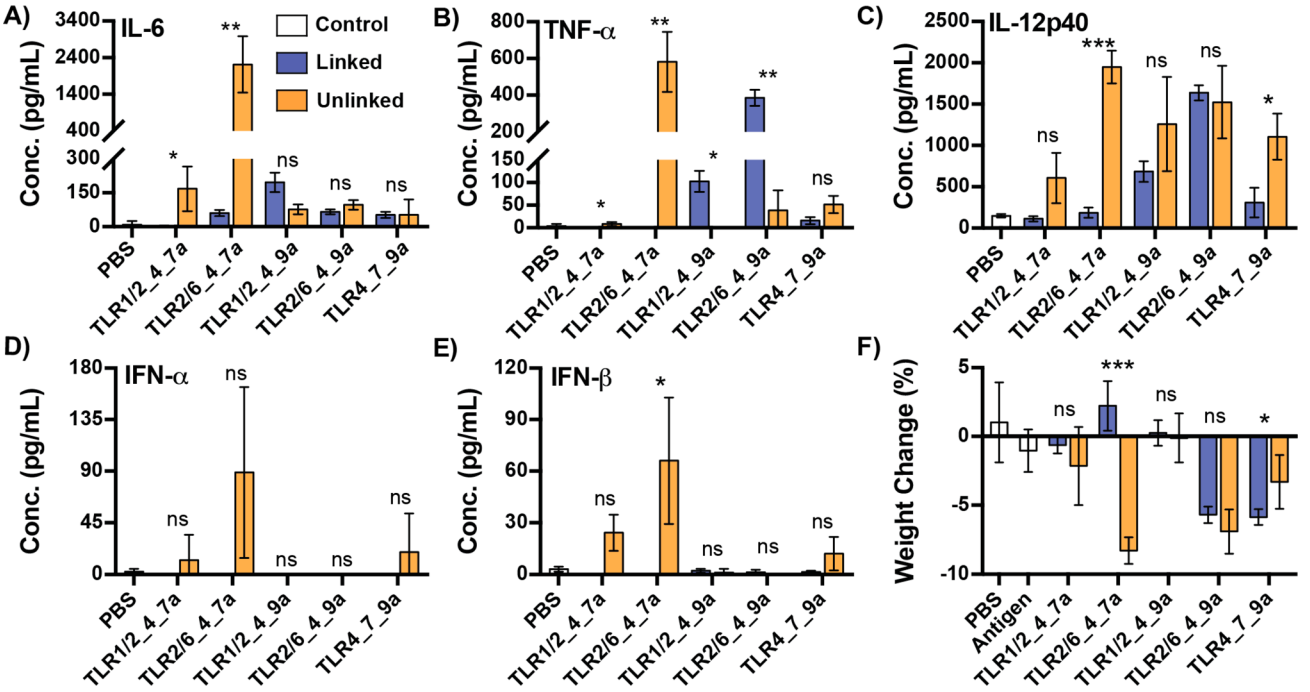

Figure 5. (A) *In vivo* IL-6, (B) TNF-α, (C) IL-12p40, (D) IFN-α, and (E) IFN-β serum levels in C57BL/6 mice 3 h (IL-6 and IL-12p40) and 1 h (TNF-α, IFN-α, and IFN-β) postinjection as measured by ELISA. Mice were injected via IM with TLR triagonists (1 nmol) or a 1:1:1 molar ratio mixture of the analogous unlinked TLR agonists (1 nmol each). (F) Percent weight change 24 h post-triagonist-injection mixed with 0.5 nmol CBU_1910 antigen. Samples were run in triplicate for ELISA experiments, and in $n = 8$ for the percent weight experiment (except PBS and TLR2/6_4_7*a* where $n = 5$), where * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant. Statistical analysis is between the linked versus unlinked agonist for the indicated compound, performed using a two-tailed Student *t* test.

cells with the greatest increases/decreases being changes of over 900%. These results point to the significant opportunities provided by these compounds to elicit unique responses in cell culture and potentially *in vivo*.

To further analyze the origin of characteristic cytokine profiles generated by the different triagonists, we compared how individual TLR agonists contributed to the response (Figures S10−S16). We determined that the cytokine profile of a TLR agonist could be dramatically altered by being linked with another agonist. For example, TLR2/6*a* linked to TLR9*a* (TLR2/6_9*a*) resulted in a reduction of IL-12p70 (100% decrease), TNF-α (81% decrease), CCL2 (58% decrease), IL-6 (44% decrease), and IL-10 (60% decrease) levels compared to TLR9*a* alone. This result shows that the cytokine profile observed for TLR2/6_4_9*a* more closely resembled that of TLR2/6*a* than TLR9*a* (Figures S14 and S16D). In another case, conjugation of TLR1/2*a* with TLR9*a* (TLR1/2_9*a*) induced a similar effect as TLR2/6*a* conjugation. However, linking TLR4*a*, a very weak agonist on its own, to the triagonist TLR1/2_4_9*a* resulted in a restored TLR9*a*-like cytokine profile, unlike TLR2/6_4_9*a* (Figure S16C,D). For a more in-depth description of the individual contributions of TLR agonists please refer to the Supporting Information. In summary, chemical attachment and linking of these agonists have strong abilities to alter agonist activity.

***In Vivo* Serum Cytokine Profiles and Weight Loss.** After finding that spatial arrangement alters *in vitro* cytokine activity, we sought to determine how this changed *in vivo* activity. We measured systemic cytokines (IL-6, TNF-α, IL12-p40, IFN-α, IFN-β) levels in the blood of C57BL/6 mice via ELISA (Figure 5A−E). We also monitored the mouse weight shortly after IM injection of the compounds (Figure 5F). Injection of individual TLR agonists often results in high levels of systemic cytokines, which can have adverse health effects as indicated by weight loss in the animals.[38] We hypothesized that the linked triagonists might cause less systemic cytokine production due to their distinct immune responses as well as the changes in biophysical properties.

The differences observed in systemic cytokines varied between linked and unlinked compounds and between different combinations (Figure 5). We observed that TNF-α, IFN- α, and IFN-β expression reached its maximum 1 h postinjection, while IL-6 and IL-12p40 expression continued to increase at 3 h postinjection, consistent with previous reports of systemic cytokine detection following TLR stimulation.[39] We observed that many compounds elicited modest concentrations of serum cytokines (Figure 5A−E). Only unlinked TLR2/6+4+7*a* produced high levels of IL-6 (2215 pg/mL), IFN-α (90 pg/mL), and IFN-β (66 pg/mL) while both unlinked TLR2/6+4+7*a* and linked TLR2/6_4_9*a* resulted in high TNF-α serum levels (582 and 385 pg/mL, respectively). Several combinations stimulated high levels of IL-12p40 (Figure 5C). Generally, the unlinked compounds stimulated similar levels of IL-12p40, where unlinked TLR2/6_4_7*a* stimulated the highest (1949 pg/mL). Of the linked combinations, only the TLR9*a* containing combinations showed appreciable levels of IL12p40. Interestingly, TLR2/6_4_9*a* stimulated the highest amounts of IL12-p40 (1637 pg/mL) of the linked combinations, the opposite trend observed *in vitro* for the expression of the functional IL12-p70.

Where we saw the biggest correlation was in weight loss. TLR2/6+4+7*a* resulted in the most dramatic weight loss (8.3% loss) in mice 24 h postinjection (Figure 5F) correlating well with its large increase in serum cytokines. Therefore, the

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Table 1. Summary of Results Comparing TLR Triagonist Combinations, Linked (L) or Unlinked (U)

Measurement		TLR1/2_4_7*a*		TLR2/6_4_7*a*		TLR1/2_4_9*a*		TLR2/6_4_9*a*		TLR4_7_9*a*	
		L	U	L	U	L	U	L	U	L	U
In-Vitro Transcription Factors	NF-κB	+	++	+++	+++	+++	++	+++	+++	++	++
	ISGs	+	++	+	++	++	++	++	++	+	++
In-Vitro BMDC Cytokines	TNF-α	+	++	+	++	+++	++	+	+	++	+
	IL-12p70	-	++	-	++	++	+	-	-	+++	+
	IFN-β	-	-	-	-	+	-	+	-	++	+
	IL-10	-	+	-	-	+++	++	+	+	+++	++
	CCL2	+	+	++	+	++	++	+	+	+++	++
	IL-6	-	+	-	+	+++	++	+	+	++	++
In-Vivo Serum Cytokines	TNF-α	-	-	-	+++	++	-	+++	+	-	+
	IL-6	-	++	+	+++	++	+	+	+	+	+
	IL12-p40	-	++	-	+++	++	++	+++	+++	+	++
	IFN-α	-	-	-	+	-	-	-	-	-	-
	IFN-β	-	-	-	+	-	-	-	-	-	-
	Weight Loss	-	-	-	+++	-	-	++	++	++	+

***In-Vitro* Transcription Factors:** <2x increase: -, 2-4x increase: +, 4-8x increase: ++, >8x increase: +++
Fold over negative control (if statistically significant from control)
***In-Vitro* BMDC Cytokines:**
TNF-α: 2-10 ng/mL: +, 10-20 ng/mL: ++, >20 ng/mL: +++
IL-12p70: <50 pg/mL: -, 50-200 pg/mL: +, 200-400 pg/mL: ++, >400 pg/mL: +++
IFN-β: 10-25 ng/mL: +, >25 ng/mL: ++
IL-10: <0.3 ng/mL: -, 0.3-1 ng/mL: +, 1-2 ng/mL: ++, >2 ng/mL: +++
CCL2: <2.5 ng/mL: -, 2.5-5 ng/mL: +, 5-10 ng/mL: ++, <10 ng/mL: +++
IL-6: <20 ng/mL: -, 20-50 ng/mL: +, 50-100 ng/mL: ++, >100 ng/mL: +++
***In-Vivo* Serum Cytokines:**
TNF-α & IL-6:<25 pg/mL: -, 25-100 pg/mL: +, 100-300 pg/mL: ++, >300 pg/mL: +++
IL12-p70: <200 pg/mL: -, 200-500 pg/mL: +, 200-1500 pg/mL: ++, >1500 pg/mL: +++
IFN-α & IFN-β: <60 pg/mL: -, >300 pg/mL: +
Weight loss 24 h after injection: 0-2.5% loss: -, 2.5-5.0% loss: +, 5.0-7.5% loss: ++, >7.5% loss: +++

weight increase observed with TLR2/6_4_7*a* (2.2% increase) confirmed our hypothesis that agonist linkage can dampen systemic effects upon adjuvant administration and alter systemic responses. However, not all of the differences between compounds were as stark. For example, there was notable weight loss in mice following TLR2/6_4_9*a* or TLR4_7_9*a* injection, regardless of linkage, despite low levels of systemic cytokines for both. We included a more extensive description of *in vivo* systemic effects in the Supporting Information.

DISCUSSION

Developing novel vaccines that generate appropriate effector responses and protection against a pathogen remains a challenge. The process of generating a desired response is complicated by a lack of understanding between innate immune signaling and their long-term effect on protective adaptive responses. Innate immune system activation with individual TLR agonists has been studied. However, most pathogens and effective vaccines stimulate multiple TLRs, and the understanding of how these combinations of agonists affect the immune response is less clear. In addition, the TLR agonists in these natural systems are organized in a structural manner, which influences activity and necessitates consideration in vaccine formulation development.[40]

TLR Combinations. In this study, we present an initial study of combinations of three different TLR agonists and their influence on innate immune response. We found that the combination of TLR agonists in the linked triagonists drastically affected the immune response profile generated. Although most of the triagonists stimulated similar levels of NF-κB and ISG activity, the specific triagonist combinations stimulated distinct cytokine profiles, which can lead to discrete adaptive immune responses (Table 1).

Overall, the presence of TLR9*a* in a trimeric combination had the greatest effect on the resulting immune response. Two of the three TLR9*a* containing triagonists (TLR1/2_4_9*a* and TLR4_7_9*a*) showed a T_H1-biasing cytokine response, characterized by higher levels of IL-12p70, and TNF-*α*.[36] In addition, the linked TLR4_7_9*a* elicited relatively high levels of IFN-*β*. This is perhaps not surprising as TLR9*a* is reported to elicit a biased T_H1 cellular response compared to other TLR agonists in its use in clinical vaccines.[37] However, the presence of TLR2/6*a* diminished production of T_H1-inducing cytokines, even when TLR9*a* was present (TLR2/6_4_9*a*). This observation was consistent with previous reports that TLR2 stimulation exhibits inhibitory effects on IL-12 originating from CpG stimulation.[7] TLR1/2*a* also demonstrated this effect when conjugated with TLR9*a* (TLR1/2_9*a*). TLR1/2*a* is also known to display an inhibitory effect on T_H1-inducing cytokine secretion upon coadministration with CpG DNA.[7,41] However, further conjugation with TLR4*a* to afford the triagonist, TLR1/2_4_9*a*, restored cytokine profile activity similar to that of TLR9*a* alone. We speculate this could be a result of the relatively high potency of TLR2/6*a* compared to TLR1/2*a*, which further biases the immune response toward a T_H2 response (Figure 4). In addition, we investigated the aggregation state of the different triagonist combinations. The TLR9*a* containing combinations did not appear to form aggregates while the two non-TLR9*a* did. Although TLR1/2_4_9*a* and TLR4_7_9*a* showed dramatic differences in immune response type compared to the non-TLR9*a* containing combinations, it is unclear if the aggregation state of the combinations is correlated to the result as they both also showed dramatic differences compared to TLR2/6_4_9*a* despite sharing similar aggregation states.

This T_H1/T_H2 biasing property can be useful in designing adjuvants for vaccines. In further studies we have observed that adjuvanting a subunit vaccine with TLR2/6_4_7*a* generated a robust T_H2 antigen specific antibody response, while

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ACS Cent. Sci. 2019, 5, 1137−1145

TLR4_7_9*a* elicited the most potent antigen specific T_H1 T cell responses of all the triagonists, but low antibody titers (Gilkes et al. 2019, submitted). These results are consistent with the cytokine profiles observed in this study. Interestingly, when TLR1/2_4_9*a* is used as an adjuvant, a balanced T_H1/T_H2 response is observed. We hypothesize that, in this case, the TLR1/2*a* T_H2 biasing properties are sufficient to generate large antibody titers, while the TLR9*a* still influences generation of a T_H1 response.

Triagonists lacking TLR9*a* (TLR1/2_4_7*a* and TLR2/6_4_7*a*) generally elicited a T_H2 biased immune response, characterized by higher proportions of IL-6 and CCL2 compared to other cytokines (Figure 4). Following this result, these compounds generated larger proportions of IgG1 to IgG2c antibodies in a subunit vaccine (Gilkes et al. 2019, submitted). However, these triagonists were relatively weak at stimulating indicators of immune responses *in vitro* (Figures 3 and 4). The weaker potency of these compounds may be due to differences in biophysical properties upon linkage. Another possibility is that, when linked, this particular combination does not behave in a synergistic manner. For example, TLR1/2, TLR4, and TLR7 agonists all exhibit inhibitory effects on IL-6 expression when coadministered *in vitro*, which is supported by both the linked and unlinked TLR1/2_4_7*a* combination studied in this work (Figure 16A).[7]

These observations are particularly pertinent for vaccine development because they give insight into how linking particular TLR agonists in combination determines their immunological activity. In the case of TLR9*a* containing triagonists, the TLR9*a* guided the immune response, unless influenced by TLR2/6*a*, whereas TLR triagonists lacking TLR9*a* stimulated relatively fewer markers of immune activation.

In addition, the linked compounds varied greatly in their cytokine profiles, both *in vitro* and *in vivo*, from their unlinked counterparts, demonstrating that the linking of the agonists played a role in their activity (Table 1). Interestingly, there was less variation in the immune response profile between the unlinked agonists, regardless of TLR combination. This difference demonstrates that a wider range of innate immune responses are accessible by linking the agonists.

Linked vs Unlinked Triagonists. Under normal physiological conditions, TLR agonists interact with immune cells while structurally organized in the pathogen's cellular architecture.[8] Although stimulating cells with soluble mixtures of TLR agonists has been demonstrated, this approach lacks the spatial association of the agonists as they are displayed on the surface and within the pathogens. Administering TLR agonist combinations that are spatially confined to a particle has shown promise in vaccine applications.[20,21] To more accurately examine the role of spatial arrangement of TLR agonists, we linked combinations of three TLR agonists and evaluated the effects of linked versus unlinked TLR agonist combinations on immune system activation.

Covalently linking TLR agonists to the triagonist scaffold decreases the activity of an individual TLR agonist, most likely through steric interactions with the receptor. Most agonists displayed a 10- to 50-fold decrease in NF-κB activity, which should result in drastically lower activity of the linked combination. We found that although linking three TLR agonists in some cases resulted in slightly lower activity, generally linkage had little effect (within 20% of activity in more cases). We found this result surprising as single agonists linked to the core showed dramatic decreases in activity. Therefore, linking the combination of multiple TLR agonists compensated for the loss in activity of each individual agonist.

One of the major differences we noted regarding *in vivo* administration of linked triagonists was their effect on systemic cytokine levels in mice. We originally hypothesized that the larger molecular weight of the triagonists and change in biophysical properties compared to the individual agonists would result in higher local retention of the compound, and thus lower systemic cytokines. For example, Lynn et al. demonstrated that by linking TLR7*a* to a polymer, the compounds remain localized near the lymph nodes rather than in the bloodstream.[28] However, this hypothesis was not fully supported, as the linked combination resulted in higher serum cytokine levels, while in other instances those elicited by the unlinked agonists were higher. We now postulate that the level of systemic cytokines can vary between the specific combinations of triagonists. For example, all triagonists bearing TLR7*a* had higher systemic cytokines when unlinked than when linked (Table 1). TLR7*a* is known to quickly diffuse into the bloodstream, causing high levels of systemic cytokines.[28,38] By linking it to a larger molecule, its systemic diffusion may be hindered. However, the only case where linkage made a notable impact on mouse weight 24 h postinjection was for TLR2/6_4_7*a*. We speculate that the inflammatory properties of free TLR7*a* and TLR2/6*a* are synergistic in this case, but this activity is suppressed when the agonists are linked.

Linked TLR triagonists containing TLR9*a* generally showed an increase in immune stimulatory capabilities compared to unlinked combinations. The largest difference was observed with TLR1/2_4_9*a* and TLR2/6_4_9*a*, which had drastically higher T_H1-inducing cytokines compared to their unlinked counterparts (IL12p70: TLR1/2_4_9*a*, 95% increase; TLR4_7_9*a*, 900% increase). This result surprised us as many of the compounds did not show much difference in transcription factor activation. This result suggests that the increased cytokine levels may not stem from activating TLRs on the same cell (overall activity of NF-κB was similar) but perhaps helps activate distinct cell types via one of the attached agonists. The other two agonists might then alter the resulting cytokine profile generated by that cell type as we observed *in vitro*.

The unlinked mixtures of TLR agonists containing TLR9*a* showed a greater tendency toward aggregation than their analogous unlinked combinations. However, this distinct difference in particulate character did not appear to have a dramatic effect on activity, as the TLR2/6_4_9*a* combination was one of the most similar in activity between linked and unlinked combinations. In addition, the linked TLR2/6_4_7*a* and unlinked TLR2/6+4+7*a* showed similar aggregation profiles yet had drastically different *in vivo* cytokine and weight loss profiles. We speculate that although aggregation state of TLR agonists prior to administration has been shown to play an important role in some experiments, in this case, it did not correlate well with trends in activity. This suggests that the distinct immune response profiles we observed were influenced by factors other than aggregation.

As difficult and emerging diseases continue to threaten global health, there is a pressing need for new and effective vaccine strategies. In developing these vaccine strategies, eliciting a targeted immune response tailored for the pathogen of interest is of paramount importance. TLR agonists play a major role in this development as they can direct the type of

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host effector responses generated during vaccination. Thus, understanding the effects of TLR agonist combinations, both linked and unlinked, is vital to furthering vaccine technologies. We presented an initial set of results showing that spatial organization can controllably, if not predictably, shape immune responses toward desired outcomes. In further studies (Gilkes et al. 2019, submitted), we examine how these compounds can be used in a target subunit vaccine for Q-Fever and find that agonist conjugation helps improve immune responses in many cases, supporting this central idea. Methods to predictably tune immune responses are needed for reverse vaccinology approaches in the development of next-generation vaccines.[42] This work provides a roadmap to the design and immune response outcome upon chemical manipulation of TLR agonist combinations to expedite vaccine development.

MATERIALS AND METHODS

Complete details of reagents and methods for cell culture, chemical synthesis, cell assays, and *in vivo* experiments are provided in the SI, Materials and Methods section.

Safety Statement. No unexpected or unusually high safety hazards were encountered. Researchers should take precaution when handling immunostimulants to avoid potential adverse side effects.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00823.

Materials, methods, experimental data figures, synthesis, and characterization (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: burkhara@uci.edu.

*E-mail: aesserkahn@uchicago.edu.

ORCID

Saikat Manna: 0000-0002-0809-5695

Aaron P. Esser-Kahn: 0000-0003-1273-0951

Author Contributions

⊥T. J. Albin and J. K. Tom contributed equally to this work.

Notes

The authors declare no competing financial interest.

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