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Immune Response Modulation of Conjugated Agonists with Changing Linker Length

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Abstract

We report immune response modulation with linked Toll-like receptor (TLR) agonists. Conjugating two agonists of synergistic TLRs induce an increase in immune activity compared to equal molarity of soluble agonists. Additionally, varying the distance between the agonists by changing the linker length alters the level of macrophage NF- κ B activity as well as primary bone marrow derived dendritic cell IL-6 production. This modulation is effected by the size of the agonists and the pairing of the stimulated TLRs. The sensitivity of linker-length-dependent immune activity of conjugated agonists provides the potential for developing application specific therapeutics.

Graphical abstract

Current research in immunotherapies focuses on the function of the innate and adaptive immune system to combat diverse diseases such as malaria, cancer, HIV, and Q-fever.¹ Pattern recognition receptors such as Toll-like receptors (TLRs) of innate immune cells recognize a host of chemical species.² Thereafter, the receptors induce a signaling cascade initiating the production of cytokines and costimulatory molecules in innate immune cells,

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Supporting Information

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Author Contributions

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Notes

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ultimately activating the adaptive immune system.³ Activating multiple TLRs on innate immune cells can lead to synergies that direct the adaptive immune response.⁴ Controlling these synergies to enhance and direct immune signals is an ongoing challenge. In addition to using soluble agonists,^{5–14} nanoparticle encapsulation¹⁵ and formation of TLR agonist homodimers¹⁶ all increase immune activity. Recently, we found that linking combination of synergistic TLR agonists also increases immune activity.¹⁷

Here, we show that altering the distance between agonists can modulate the synergy between two covalently conjugated agonists. We explore this principle for agonists of different molecular weights (500–9000 Da) and different TLRs (TLR2, -4, and -9). The guidelines governing these linkages change for each agonist pair. We report that the activity of conjugated agonists depends on a combination of the synergistic TLR pair, the linker length, and the agonist size, with size and linker length being most pronounced (Figure 1a). As a result, for certain TLR pairs and cell types, one can increase agonist activity and potency by controlling the average distance between two conjugated agonists. These data show that a portion of the inflammatory response can be modulated using chemical synthesis of multi-agonist systems to control cytokine levels. This information may be useful as one of the first steps toward developing immunotherapeutics with application-specific, customized immune responses, as well as informing the design of future polymeric activators of the innate immune system for delivery purposes.

Previously, we showed the increased agonist activity of the heterodimeric cross-linked agonist system where lipoteichoic acid (LTA, average MW = 6000 Da, a TLR2/6 agonist)¹⁸ and CpG_1826 (MW = 7112.7 Da, a TLR 9 agonist)¹⁹ were conjugated to form a heterodimer of two immune agonists (LTA_PEG₆_CpG). To understand the importance of average agonist distance in linked systems, we synthesized a series of heterodimers of covalently conjugated LTA and CPG_1826 with each agonist separated by a fixed distance *via* a heterotelechelic polyethylene glycol (PEG) linker. The synergistic agonist complexes were synthesized using a PEG linker bearing a terminal *N*-hydroxysuccinimide (NHS) ester and maleimide groups (Figure 1b). To form the conjugates, the free amines of the side chains of LTA²⁰ from *Bacillus subtilis* were reacted with the NHS terminus of the PEG linkers in PBS (pH 7.5) for 12 h. 3'-thiol, 5'-fluorescein phosphoramidite (FAM) modified CpG_1826 was then conjugated to the maleimide terminus of the LTA_PEG_{*n*} (*n* = 6, 12, or 24) compounds in PBS at RT for 12 h (Figure 1c). The resulting LTA_PEG_{*n*}_CpG heterodimer was purified *via* gel electrophoresis and quantified by the absorbance of the FAM tag of the CpG at 495 nm. The average intermolecular distance varied by changing the length of the PEG linker: PEG₆ (31.7 Å), PEG₁₂ (53.3 Å), and PEG₂₄ (95.2 Å).

To determine how linking these agonists changed the response of innate immune cells, we tested the linked agonists on a reporter macrophage cell line (RAW 264.7) to show the change in NF- κ B activity. The LTA_PEG_{*n*}_CpG agonists (50 nM) showed not only higher NF- κ B activity compared to equimolar soluble agonists (50 nM of each agonist) but also increasing linker length increased NF- κ B activity (Figure 1d). In addition, we tested cytokine (IL-6, IL-12, and CD86 (see SI)) production and cell surface marker upregulation of primary bone-marrow derived dendritic cells (BMDCs) induced by the heterodimers. When stimulating BMDCs with LTA_PEG_{*n*}_CpG heterodimer, the linked agonists showed a

higher immune response, as measured by IL-6, compared to the activation induced by equimolar doses of individual agonists or combined soluble agonists (Figure 1e). However, when comparing the different lengths of the PEG linkers in BMDCs, we observed no linker-length-dependent increase in IL-6 levels. The weakest IL-6 production was from PEG₁₂, but there was no significant difference between PEG₆ and PEG₂₄. While there was a clear correlation between linker length and NF- κ B activity for reporter macrophages, the lack of a linker length effect for primary cells showed the heterodimers can have different activities depending on the characteristics of the agonists. Therefore, we sought to determine if other agonists of different size or TLR pairing showed an increase in linked agonist activity but a lack of linker-length-dependent activity.

To determine if the synergy we observed for the LTA_PEG₆-CpG system was specific to the synergistic TLR agonist pair or depended on the size of the agonists, we synthesized a TLR2/6 and TLR9 pair that varied only by the size of the TLR2/6 agonist. To vary agonist size, we chose Pam₂CSK₄C (PAM, 1373 Da)²¹ and covalently coupled it to CpG₁₈₂₆ (Figure 2a). The Pam_PEG_n-CpG compounds were synthesized with a 3'-amino-terminated CpG₁₈₂₆ (5' fluorescein phosphoramidite) conjugated to the NHS ester of the PEG linker. The synthetic diacylated lipopeptide, PAM, was modified with the addition of a terminal cysteine for conjugation to the terminal maleimide of the CpG_PEG_n complex. The conjugated heterodimer was purified by gel electrophoresis.

Unlike the LTA_PEG_n-CpG system, linked Pam₂CSK₄C and CpG₁₈₂₆ (Pam_PEG_n-CpG, 50 nM) agonists did not induce higher NF- κ B activity than the equimolar soluble agonists in the RAW 264.7 cells (Figure 2b). However, there was a linker-length-dependent activity where PEG₁₂ decreased the level of activity while PEG₆ was the most comparable to the soluble agonists. In primary cells, the Pam_PEG_n-CpG did not show higher IL-6 production or a linker-length-dependent increase. However, a similar trend in drop of activation with PEG₁₂ was observed, congruent with the trends in NF- κ B activity. Therefore, the linker-length-dependent cell stimulation may change NF- κ B activity, but this change in transcription factor activity does not translate directly into increased transcription and production of cytokines as measured by IL-6 and IL-12 levels (Figure 2c, SI).

After comparing both linker length and agonist size with the TLR2/6_TLR9 linked agonists system, we set out to test agonist pairing as a potential influence on linked TLR synergies. We chose TLR4 and TLR9 both to consolidate our synthetic route and because TLR4 is a membrane bound receptor like TLR2.^{22–24} While membrane bound, TLR4 is active at both the cell surface and in endosomes but stimulated by a small molecule agonist (447 Da ; Figure 3a). The TLR4_PEG_n-TLR9 construct was synthesized in an analogous manner to the TLR2_PEG_n-TLR9 molecules. The TLR4 agonist, pyrimidoindole (Ind), was modified from previously reported SAR studies²⁵ to bear a free amine for conjugation to the heterodimer. The amine handle was conjugated to the NHS ester of the PEG linkers, and the resulting Ind_PEG_n maleimide was reacted with 3'-thiol CpG₁₈₂₆. The resulting heterodimer conjugates, Ind_PEG_n-CpG, were quantified by the absorbance of the FAM-labeled CpG₁₈₂₆. Through the new pairs, we sought to compare the effect of agonist spacing in relation to TLR pair (TLR4+TLR9 vs TLR2/6+TLR9) and the effect of a small

molecule (MW < 500 Da) compared to larger molecules (MW > 1300) such as Pam₂CSK₄C or LTA.

In Ind_PEG_n_CpG systems, the linked agonists showed higher NF- κ B activity in the reporter RAW-Blue cell line than stimulation by equimolar soluble agonists at 100 nM (Figure 3b). [The molarity used is different from LTA_PEG_n_CpG and Pam_PEG_n_CpG due to the weaker potency of the pyrimidoindole agonist.] There was also a distinct linker-length-dependent increase in activity for the two systems. In the Ind_PEG_n_CpG system, NF- κ B activity was highest when the agonists were separated by PEG₁₂. This suggests there is an optimal spatial distance required for conjugating multiple TLR agonists as the NF- κ B activity of LTA_PEG_n_CpG and Pam_PEG_n_CpG reached a maximum at different linker lengths. In primary BMDCs, Ind_PEG_n_CPG heterodimers showed higher activity than the indole and CpG₁₈₂₆ agonists combined as soluble agonists. Further, clear linker-length-dependence was observed in contrast with the previously described TLR2_TLR9 heterodimers. PEG₁₂ had higher levels of IL-6 than those observed for PEG₆ and PEG₂₄ (Figure 3c). PEG₆ had the second most potent activity followed by PEG₂₄ with the lowest activity of the three linked agonists.

To better understand the distinct differences in the synergistic activation between multiple linked TLR agonist pairs, we sought to understand the mechanism through which these different activations were proceeding. TLR 2 knockout (KO), TLR 4 KO, and TLR 9 KO cells were used to study the effect of not only covalently conjugating the agonists but also the linker length dependence on immune cell activity to determine how steric constraints of different size molecules balanced with activation of unique receptor pairs. The BMDCs of the KO mice were incubated overnight with the heterodimers at the same concentrations as the wildtype BMDCs, and the IL-6 levels were measured *via* flow cytometry and intracellular cytokine staining. [As the changes we were expecting might be subtle, in each case, we included a corresponding dose of CpG1826 as a guide for differences from experimental data in base-level cytokine production. The dosage of CpG varied between LTA and PAM2 (50 nM) and pyrimido-indole (100 nM).]

For TLR9 KO BMDCs, both the LTA_PEG_n_CPG and PAM_PEG_n_CPG showed reduced cytokine production for all compounds (Figure 4). For LTA_PEG_n_CpG, there was a distinct decrease in CpG₁₈₂₆-dependent cytokine production with increasing linker length (Figure 4a, d). While CPG₁₈₂₆ had high levels of activation in TLR2 knockout BMDCs, the linked systems had lower activation, and within the heterodimers, the shortest linker length—smallest heterodimer—showed the highest activity. The linker-length-dependent decrease in activity can be due to the steric bulk of the agonist dimer. Compared to CpG₁₈₂₆ alone, the additional LTA_PEG_n conjugation on CpG₁₈₂₆ may be hindering CpG₁₈₂₆ activation of TLR9. As such, the larger linker length, PEG₂₄, provided the most additional steric bulk to CpG₁₈₂₆, resulting in lowest immune cell activation. In this case, the increased size of the linker hindered activation of the receptors pointing to a biochemical rationale (i.e., synergistic pathways) for increased activity seen previously from longer linker lengths.

The same trend was observed with Pam_PEG_n_CpG (Figure 4b, e). Interestingly, Pam_PEG_n_CpG had higher IL-6 production compared to LTA_PEG_n_CpG in TLR 2 KO

BMDCs (Figure 4b). This result may be explained by the smaller size of Pam (1.3 kDa) compared to LTA (average 6 kDa). The smaller size of PAM₂CSK₄C reduces the steric hindrance in Pam-PEG_n and is therefore less disruptive to CpG_1826 induced TLR9 activation—leading to higher IL-6 production. Thus, synergistic activation of TLRs depends on the size of the agonist, and the increased immune activity of heterodimers depends on the synergistic activity of both TLRs.

Agonist size dependent activation for Ind-PEG_n-CpG had closer agreement with the activation of RAW-Blue 264.7 macrophages and activation of wild type BMDCs (Figure 3). For both the TLR 4 KO and TLR9 KO cells, PEG₁₂ showed the highest activity. Likely, because the pyrimido-indole is a small molecule, the additional size added to CpG_1826 is less than that of adding PAM₂CSK₄C or LTA. Therefore, the activity of the CpG is not affected as drastically as was observed for Pam-PEG_n-CpG and LTA-PEG_n-CpG and is similar to the wild type BMDCs (Figure 4c, f).

We report examples of linked TLR agonists and the change in immune cell activation with changing linker length. Through the TLR KO BMDC IL-6 presentation, we found the importance of activation of both TLRs in increasing activity through conjugated agonists. The conjugated TLR agonists increased the NF- κ B activity. Many heterodimer pairs have an optimal spatial distance between the agonists for effective activation of some innate immune cells. LTA-PEG_n-CpG showed the greatest activity with the largest distance, while PAM-PEG_n-CpG showed good activity at PEG₆ and PEG₂₄ but a decrease in activity with PEG₁₂. Ind-PEG_n-CPG heterodimers had the highest cytokine presentation at PEG₁₂.

Our operating hypothesis of the discrepancies in agonist spacing can be attributed to the size of the paired agonists. As seen in KO BMDC activity, with smaller agonists, the distance between agonists can be shorter and still induce robust immune signaling. In contrast, larger agonists created steric bulk that interfered with single agonist dependent activity. Therefore, depending on the linker length and spatial presentation of two agonists, the activity of the synergistic agonists can be limited by access to the receptor. Additionally, the change in synergistic pairing can add to the changes of the effect of conjugated agonists. Though TLR synergy has been shown for multiple TLR pairs, the effects of synergy are not universal as the changes are subtle, depending on the combination of stimulated TLRs. Therefore, the change in response from one TLR pair to another can be attributed to the mechanism of the TLR synergy involved.

In this study, we present evidence that spatial distance between agonists can alter and control innate immune cell activity, but the control depends on TLR agonists, their distance, and steric sizes. Contrasting that the spatial organization increased activity, we found that spatially constrained TLR agonists limited their ability to activate individual TLRs in knockout studies. As different agonist pairs require different spatial presentations of agonists, we plan to further explore the direct mechanism of linked agonists and the TLR synergy mechanism as well as the effect of conjugated agonists *in vivo* in future work. As an increasing number of spatially constrained multiagonists are developed as therapeutic agents and tools for adjuvanting activity *in vivo*, it is important to consider the spatial elements which can allow customized immune responses. Changing the spatial constraint can both

enhance and mitigate the synergistic effects of linked TLR agonists, potentially lowering the effective dose or adjusting the downstream activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- (1) Katsikis P, Schoenberger S, Pulendran B. Probing the 'labyrinth' linking the innate and adaptive immune systems. *Nat Immunol.* 2007; 8:899–901. [PubMed: 17712338] (2) Offersen R, Nissen S, Rasmussen T, Ostergaard L, Denton P, Sogaard O, Tolstrup M. A novel Toll-like receptor 9 agonist, MGN1703, enhances HIV-1 transcription and NK cell-mediated inhibition of HIV-1 infected autologous CD4+ T cells. *J Virol.* 2016; 90:4441–4453. [PubMed: 26889036] (3) Honstetter A, Ghigo E, Moynault A, Capo C, Toman R, Akira S, Takeuchi O, Lepidi H, Raoult D, Mege J. Lipopolysaccharide from *Coxiella burnetii* is involved in bacterial phagocytosis, filamentous actin reorganization, and inflammatory responses through Toll-like receptor 4. *J Immunol.* 2004; 172:3695–703. [PubMed: 15004173]
- Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol.* 2001; 2:675–680. [PubMed: 11477402]
- Mancini RJ, Stutts L, Ryu KA, Tom JK, Esser-Kahn AP. Directing the immune system with chemical compounds. *ACS Chem Biol.* 2014; 9:1075–1085. [PubMed: 24690004]
- Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc Natl Acad Sci U S A.* 2000; 97:13766–13771. [PubMed: 11095740]
- Querec T, Bennouna S, Alkan S, Laouar Y, Gordon K, Flavell R, Akira S, Ahmed R, Pulendran B. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med.* 2006; 203:413–424. [PubMed: 16461338]
- Duggan JM, You D, Cleaver JO, Larson DT, Garza RJ, Guzman Pruneda FA, Tuvim MJ, Zhang J, Dickey BF, Evans SE. Synergistic Interactions of TLR2/6 and TLR9 induce a high level of resistance to lung infection in mice. *J Immunol.* 2011; 186:5916–5926. [PubMed: 21482737]
- Theiner G, Rössner S, Dalpke A, Bode K, Berger T, Gessner A, Lutz MB. TLR9 cooperates with TLR4 to increase IL-12 release by murine dendritic cells. *Mol Immunol.* 2008; 45:244–252. [PubMed: 17599410]
- Moody MA, Santra S, Vandergrift NA, Sutherland LL, Gurley TC, Drinker MS, Allen AA, Xia S-M, Meyerhoff RR, Parks R, Lloyd KE, Easterhoff D, Alam SM, Liao H-X, Ward BM, Ferrari G, Montefiori DC, Tomaras GD, Seder RA, Letvin NL, Haynes BF. TLR-7/8 and 9 agonists cooperate to enhance HIV-1 envelope antibody responses in rhesus macaques. *J Virol.* 2014; 88:3329–3339. [PubMed: 24390332]
- Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defense. *Nat Rev Immunol.* 2007; 7:179–190. [PubMed: 17318230]

10. Warger T, Osterloh P, Rechtsteiner G, Fassbender M, Heib V, Schmid B, Schmitt E, Schild H, Radsak MP. Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo. *Blood*. 2006; 108:544–550. [PubMed: 16537810]
11. Goff PH, Hayashi T, Martínez-Gil L, Corr M, Crain B, Yao S, Cottam HB, Chan M, Ramos I, Eggink D, Heshmati M, Krammer F, Messer K, Pu M, Fernandez-Sesma A, Palese P, Carson DA. Synthetic Toll-Like receptor 4 (TLR4) and TLR7 ligands as influenza virus vaccine adjuvants induce rapid, sustained, and broadly protective responses. *J Virol*. 2015; 89:3221–3235. [PubMed: 25568203]
12. Fox CB, Sivananthan SJ, Duthie MS, Vergara J, Guderian JA, Moon E, Coblenz D, Reed SG, Carter D. A nanoliposome delivery system to synergistically trigger TLR4 and TLR7. *J Nanobiotechnol*. 2014; 12:17.
13. Bergmann-Leitner ES, Leitner WW. Adjuvants in the Driver's Seat: How Magnitude, Type, Fine Specificity and Longevity of Immune Responses Are Driven by Distinct Classes of Immune Potentiators. *Vaccines*. 2014; 2:252–296. [PubMed: 26344620]
14. Siefert AL, Caplan MJ, Fahmy TM. Artificial bacterial biomimetic nanoparticles synergize pathogen-associated molecular patterns for vaccine efficacy. *Biomaterials*. 2016; 97:85–96. [PubMed: 27162077]
15. Kasturi SP, Skountzou I, Albrecht RA, Koutsonanos D, Hua T, Nakaya HI, Ravindran R, Stewart S, Alam M, Kwissa M, Villinger F, Murthy N, Steel J, Jacob J, Hogan RJ, García-Sastre A, Compans R, Pulendran B. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature*. 2011; 470:543–547. [PubMed: 21350488]
16. Shukla NM, Mutz CA, Malladi SS, Warshakoon HJ, Balakrishna R, David SA. Toll-Like Receptor (TLR)-7 and -8 modulatory activities of dimeric imidazoquinolines. *J Med Chem*. 2012; 55:1106–1116. [PubMed: 22239408]
17. Mancini RJ, Tom JK, Esser-Kahn AP. Covalently coupled immunostimulant heterodimers. *Angew Chem Int Ed*. 2014; 53:189–192.
18. Ginsburg I. Role of lipoteichoic acid in infection and inflammation. *Lancet Infect Dis*. 2002; 2:171–179. [PubMed: 11944187]
19. Klinman DM, Currie D, Gursel I, Verthelyi D. Use of CpG oligodeoxynucleotides as immune adjuvants. *Immunol Rev*. 2004; 199:201–216. [PubMed: 15233736]
20. Fischer W, Mannsfeld T, Hagen G. On the basic structure of poly (glycerophosphate) lipoteichoic acids. *Biochem Cell Biol*. 1990; 68:33–43. [PubMed: 2350496]
21. (1) Spohn R, Buwitt-Beckmann U, Brock R, Jung G, Ulmer AJ, Wiesmüller K-H. Synthetic lipopeptide adjuvants and Toll-like receptor 2–structure-activity relationships. *Vaccine*. 2004; 22:2494–2499. [PubMed: 15193414] (2) Buwitt-Beckmann U, Heine H, Wiesmüller K-H, Jung G, Brock R, Ulmer AJ. Lipopeptide structure determines TLR2 dependent cell activation level. *FEBS J*. 2005; 272:6354–6364. [PubMed: 16336272]
22. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol*. 2004; 5:987–995. [PubMed: 15454922]
23. Kagan JC. Signaling organelles of the innate immune system. *Cell*. 2012; 151:1168–1178. [PubMed: 23217704]
24. Maisonneuve C, Bertholet S, Philpott DJ, De Gregorio E. Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants. *Proc Natl Acad Sci U S A*. 2014; 111:12294–12299. [PubMed: 25136133]
25. Chan M, Hayashi T, Mathewson RD, Nour A, Hayashi Y, Yao S, Tawatao RI, Crain B, Tsigelny IF, Kouznetsova VL, Messer K, Pu M, Corr M, Carson DA, Cottam HB. Identification of substituted pyrimido[5,4-b]indoles as selective Toll-Like receptor 4 ligands. *J Med Chem*. 2013; 56:4206–4223. [PubMed: 23656327]

Figure 1.

(a) Agonist characteristics affecting immune cell activity when conjugated. (b) Synthesis of agonist conjugation with PEG linker. (c) Molecular structure and average mass of lipoteichoic acid (LTA) and representation of TLR2/TLR9 agonist heterodimer LTA_PEG_n-CpG. Stimulation of 50 nM of LTA, CpG_1826, equimolar LTA and CpG_1826, and LTA_PEG_n-CpG measured by (d) RAW-Blue activation *via* NF-κB stimulation after 24 h incubation at 37 °C. (e) BMDC intracellular cytokine production when incubated with agonists for 8 h at 37 °C. Cytokine expression measured *via* flow cytometry. Each result is from three independent experiments. **p* > 0.05, ***p* < 0.05.

Figure 2.

(a) Molecular structure and mass of Pam₂CSK₄C (PAM) and representation of TLR2/TLR9 agonist heterodimer Pam-PEG_n-CpG. Stimulation of 50 nM of Pam₂CSK₄, CpG_1826, equimolar Pam₂CSK₄ and CpG_1826, and Pam-PEG_n-CpG measured by (b) RAW-Blue activation *via* NF- κ B stimulation after 24 h incubation at 37 °C of (c) BMDC intracellular cytokine production when incubated with agonists for 8 h at 37 °C. Cytokine expression measured *via* flow cytometry. Each result is from three independent experiments. * $p > 0.05$, ** $p < 0.05$.

Figure 3.

(a) Molecular structure and mass of pyrimido-indole (Ind) and representation of TLR4/TLR9 agonist heterodimer Ind_PEG_n_CpG. Stimulation of 100 nM of pyrimido-indole, CpG_1826, equimolar pyrimido-indole and CpG_1826, and Ind_PEG_n_CpG measured by (b) RAW-Blue activation *via* NF- κ B stimulation after 24 h incubation at 37 °C of (c) BMDC intracellular cytokine production when incubated with agonists for 8 h at 37 °C. Cytokine expression measured *via* flow cytometry. Each result is from three independent experiments. * $p > 0.05$, ** $p < 0.05$.

Figure 4.

TLR knockout BMDC IL-6 expression measured for each heterodimer. (a, d) 50 nM of LTA, CpG_1826, equimolar LTA and CpG_1826, and LTA_PEG_nCpG. (b, e) 50 nM of Pam₂CSK₄, CpG_1826, equimolar Pam₂CSK₄ and CpG_1826, and Pam_PEG_nCpG. (c, f) 100 nM of pyrimido-indole, CpG_1826, equimolar pyrimido-indole and CpG_1826, and Ind_PEG_nCpG. Each result is from three independent experiments. * $p > 0.05$, ** $p < 0.05$.