Combinatorial Delivery of Dual and Triple TLR Agonists via Polymeric Pathogen-like Particles Synergistically Enhances Innate and Adaptive Immune Responses

Ranjna Madan Lala, Emory University
Pallab Pradhan, Georgia Institute of Technology
Krishnendu Roy, Emory University

Journal Title: Scientific Reports
Volume: Volume 7, Number 1
Publisher: Nature Publishing Group: Open Access Journals - Option C | 2017-05-31, Pages 2530-2530
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1038/s41598-017-02804-y
Permanent URL: https://pid.emory.edu/ark:/25593/s37t1

Final published version: http://dx.doi.org/10.1038/s41598-017-02804-y

Copyright information:
© The Author(s) 2017
This is an Open Access work distributed under the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/).

Accessed January 10, 2021 11:31 PM EST
Combinatorial Delivery of Dual and Triple TLR Agonists via Polymeric Pathogen-like Particles Synergistically Enhances Innate and Adaptive Immune Responses

Ranjna Madan-Lala, Pallab Pradhan & Krishnendu Roy

Despite decades of research very few vaccine-adjuvants have received FDA approval. Two fundamental challenges plague clinical translation of vaccine-adjuvants: reducing acute toxicities that result from systemic diffusion of many soluble adjuvants, and delivering multiple adjuvants at the same time to mimic the synergistic immune-stimulation of pathogens, while being safe. In order to address these barriers, we evaluated combinations of four clinically relevant immune-agonists, specifically Toll-like receptor (TLR) ligands, using biodegradable, polymer microparticles. We tested them alone and in combinations of 2 or 3, for a total of 10 unique conditions. We evaluated primary bone-marrow-derived Dendritic Cell phenotypes and functionality, and identified several synergistic combinations. We picked a dual and a triple adjuvant combination, TLR4/TLR9 and TLR4/TLR7/TLR9, for further evaluation and found that both combinations promoted antigen cross-presentation in vitro. Studies in mice using the model antigen Ovalbumin, showed that both combinations enhanced lymph node germinal center and T follicular helper cell responses. The triple adjuvant combination showed increased antigen-specific antibody titer with an overall balanced Th1/Th2 response, while the dual combination promoted Th1-polarized IgG responses. Our results show how polymeric particulate-carriers can be adopted to safely deliver combinatorial adjuvants and selectively synergize specific types of immune responses for vaccine applications.

Vaccine development remains largely an empirical process. Currently there are very few adjuvants licensed for use with human vaccines, and these are not able to induce the diverse immune responses required to protect against chronic and emerging diseases1. It is critical to develop new adjuvants that are safe, and provide varied immunomodulatory characteristics to enable fine-tuning of disease-specific immune responses. For example, there has been considerable effort towards developing vaccines that induce cellular immunity, which is needed to protect against intracellular pathogens2–4. In order to develop customized multicomponent vaccines with specific immune-function, rational design of adjuvants and adjuvant-delivery strategies is critical5.

Adjuvants operate by engaging innate immune cells, such as dendritic cells (DCs), and shape the subsequent adaptive immunity6–8. Pathogen associated molecular patterns (PAMPs) and danger signals are potent adjuvants; they activate DCs by stimulating cell-surface or intracellular pathogen recognition receptors (PRRs), causing enhanced levels of surface costimulatory markers, e.g. CD86 and CD40, and key secretory cytokines, such as IL12 and IL10, that play a central role in shaping the ensuing adaptive immune responses9, 10. Differentially activated DCs help polarize the T helper (Th) cell response, for example to Th1 or Th2, which further guide the nature of cell-mediated or humoral immune responses9, 11. Toll like receptors (TLRs) are one the most characterized members of the PRR family12, 13. There are ten (human) or twelve (mouse) different TLRs, which are expressed on the cell surface or intracellular compartments of a number of immune cells, including DCs12, 14–16. Lipopeptides and peptidoglycans (TLR1, −2, and −6), lipopolysaccharides (TLR4), flagellin (TLR5), dsRNA (TLR3), ssRNA
Specific combinations of PLPs act synergistically to modulate DC activation. In the following studies comparison with soluble adjuvants, either alone or in combination, were not made. As discussed in the Introduction section, soluble combinations have little clinical or in vivo relevance due to acute systemic toxicities.
of many soluble adjuvants (e.g. CpG) that prevent clinical use. Furthermore, soluble combinations optimized in vitro are also not relevant for in vivo applications due to differential retention kinetics at the site of injection. Therefore, combination adjuvants only in the context of PLPs are compared in this study.

To evaluate the effects of different PLP combinations on DC activation, we first defined sub-saturating dose for each PLP, in order to avoid saturating the individual TLR signaling pathways with high single adjuvant doses, which might prevent detection of any potential combinatorial effects of these adjuvants. We studied dose response for each adjuvant by stimulating BMDCs with increasing doses of soluble or PLP formulations of Pam3CSK4 (P), MPLA (M), R837 (R), and CpG (C). Supernatants isolated at 24 h post stimulation were assayed for IL12p70 by ELISA (Fig. 1). Based on these findings, we selected sub-saturating PLP doses (100 ng mL\(^{-1}\), 10 ng mL\(^{-1}\), 1 μg mL\(^{-1}\), and 100 ng mL\(^{-1}\) for P, M, R and C respectively) for subsequent experiments.

To examine the effects of simultaneous stimulation of TLR2, −4, −7 or −9 by using dual or triple combinations of the respective PLPs on DC activation, we exposed BMDCs to media alone or individual PLPs (P, M, R

| Formulation         | Size (μm) | Zeta Potential (mV) | Loading Method | Loading Efficiency |
|---------------------|-----------|---------------------|----------------|-------------------|
| PLGA-Pam3CSK4       | 1.5 ± 0.14| −7.96 ± 0.68        | Encapsulation  | 6.4 μg/mg (69.9 ± 0.9%) |
| PLGA-MPLA           | 1.69 ± 0.29| −7.59 ± 0.15        | Encapsulation  | 12.5 μg/mg (100%)  |
| PLGA-R837           | 1.79 ± 0.29| −6.82 ± 0.13        | Encapsulation  | 7.7 μg/mg (50.5 ± 1.3%) |
| PLGA-PEI-CpG        | 1.72 ± 0.37 (before PEI modification) | −20.56 ± 4.62 | Surface Loading  | 10.4 μg/mg (86.6 ± 12%) |
| PLGA-PEI-Ova        | 1.72 ± 0.37 (before PEI modification) | 18.43 ± 2.09  | Surface Loading  | 25.8 μg/mg (51.7 ± 3.0%) |

Table 1. Size, Zeta potential, and representative encapsulation/loading efficiency of PLGA-microparticles (PLP). The data shown are average of at least 2 (Pam3CSK4), 3 (Ovalbumin) or 5 (MPLA, R837, and CpG) batches.
or C) or their double (P+M; P+R; P+G; M+R; M+C; R+C) or triple (P+M+R; P+M+G; P+R+C; M+R+C) combinations. Cell-free supernatants were collected at 24 h post exposure, and assayed for IL12p70 by ELISA (Fig. 2a). As shown in Fig. 2a, P+M, M+R, M+C, P+M+C, and M+R+C PLP combinations elicited significantly higher IL12p70 as compared to the individual PLPs, and the IL12p70 synergy ratio (calculated as described in methods) for these combinations was 2.1, 2.9, 2.4, 1.2 and 1.2 respectively. We also measured IL10 in the culture supernatants by ELISA. As shown in Fig. 2b, PLP combinations P+R, P+M+R, P+M+C and P+R+C induced significantly higher IL10 than the individual adjuvants, and the IL10 synergy ratios for these combinations was 1.5, 1.9, 2.1 and 1.4 respectively. IL10 synergy ratio for combinations that synergistically enhanced IL12p70, i.e. P+M, M+R, M+C, P+M+C, and M+R+C, was 1.6, 1.7, 2.8, 2.1 and 1.2 respectively. We decided to select two combinations for further studies into their potential to promote specific Th polarization phenotypes. Thus, we defined the ratio of IL12p70 to IL10 elicited by these combinations, which is a predictor of Th1/Th2 polarizing responses (Fig. 2c). As seen in Fig. 2c, M+C combination had the highest IL12p70 to IL10 ratio (0.48). Of the triple combinations, M+R+C showed the highest IL12p70 to IL10 ratio (0.19), and we used this combination for further in depth investigation.

Further, we also analyzed these BMDCs for their surface activation markers, CD40 and CD86 by flow cytometry. Median fluorescence intensity (MFI) and percentage of co-stimulatory markers CD40 and CD86 on CD11c+ gated cells were measured (Fig. 3a and b). All PLP combinations elicited statistically significantly difference (p < 0.05) in MFI and percentage of CD40 and CD86 as compared to their individual components, except P+M and P+M+C, which did not induce significantly different CD86 MFI as compared to M or C individually; and P+R for which percentage of CD86 cells was not significantly different than P alone.

**M+C and M+R+C combinations enhance antigen cross presentation in vitro.** Robust antigen presentation by APCs is critical for generation of adaptive immune response. The results reported above indicate
that PLP combinations of M+C and M+R+C synergistically induce IL12p40, and modulate the Th1/Th2 shaping cytokine levels (Fig. 2). To examine the ability of these adjuvant combinations on priming and polarization of T cells, we co-cultured BMDCs stimulated with these PLPs with TCR-Tg CD4 T isolated from OT-II mice for 72 h. Cell free culture supernatants were assayed for IFN-γ and IL4. Surprisingly, we did not observe significant Th1 or Th2 bias since DCs exposed to M+C or M+R+C combinations did not specifically promote higher IFN-γ or IL4 production by T cells or higher T cell proliferation as compared to single PLPs (Supplementary Fig. S1).

Next, we tested the ability of these BMDCs for their capacity for antigen cross presentation by co-culturing DCs stimulated with PLPs in the presence of increasing soluble antigen (Ovalbumin) doses, with TCR-Tg CD8 T isolated from OT-I mice for 72 h. While we did not see significant effects of the combinatorial PLPs on CD8 T cell proliferation as measured by CFSE dilution assay (Supplementary Fig. S2), DCs matured with M+R+C PLP combinations were more efficient at antigen cross presentation amongst all groups tested at all antigen doses, M+C combination promoted better antigen cross presentation than M and C at specific antigen concentrations (Fig. 4).

**Vaccinations with M+C and M+R+C PLPs promote enhanced humoral response.** We next tested how M+C and M+R+C PLP combinations affect antigen specific adaptive immune responses in vivo. C57BL/6 J mice were immunized with Ovalbumin-PLP with or without the PLPs M, R, C, M+C or M+R+C at day 0, weeks 2 and 4. Blood sera were collected at week 5 post 1st vaccination and assayed for IgG1 and IgG2c by Ova specific ELISA, as described in methods. As shown, M+R+C PLP combination increased the production of IgG1 and IgG2c antibodies, while mice vaccinated with M+C combination had high levels of IgG2c, but not IgG1 (Fig. 5a), as compared to the respective individual PLPs, suggesting a Th1 polarized response. Next, we investigated the effects of combinatorial adjuvants on germinal center (GC) formation in the lymph node (site of activated B cell proliferation, somatic hypermutation and immunoglobulin class switching) and T follicular helper cell (Tfh) response, which provide crucial help for humoral response. Flow cytometry analyses of cells from draining lymph nodes harvested at day 35 post immunization revealed that both PLP-combination vaccinated mice had significantly higher B220+ GL7+ activated germinal center B cells (Fig. 5b). We also saw an enhanced Th response in the lymph nodes as measured by an increase in CXCR5+Bcl6+ cells (gated on CD3+CD4+) in draining lymph nodes (Fig. 5c), suggesting that the enhanced antibody levels elicited by the combinatorial adjuvants may be due to higher germinal center and Thf response.

**Discussion**
Emerging evidence demonstrates the ability of specific combinations of adjuvants to synergistically enhance vaccine induced immune response. However, most research in this field has focused on using soluble adjuvants, which have several disadvantages; for example, their potential to diffuse systemically can result in serious side effects. Furthermore, differences in retention kinetics of small molecules with different hydrophilicity and hydrophobicity severely restrict the adjuvant combinations that can be used effectively with predicted outcomes. Biomaterial carrier based delivery of adjuvants has proven to be an effective strategy for diminishing their toxic effects and improving the overall efficacy. In this study, we carried out an extensive comparison of PLGA microcarrier based dual and triple combinations of TLR2, −4, −7 and −9 agonists for their potential to enhance specific immune responses in vitro and in vivo. Given the well established and previously described limitations of...
immune responses, while IL10 boosts Th2 oriented immune responses, and dampens the Th1 response. Thus, Th1/Th2 balance determines the overall nature of the adaptive immune response. IL12p70 promotes Th1 biased and humoral immune responses.

− agonists for TLR2, 3, and 9 greatly improves HIV envelope peptide vaccine response in mice, underscoring than two TLRs has great potential in enhancing selective immune responses. A study has shown that combining TLR agonist combinations on modulating the immune response; however, simultaneous triggering of more than two TLRs has great potential in enhancing selective immune responses. A study has shown that combining more than two TLR agonists to synergistically boost the immune response. Hence, finding adjuvants that improve antigen cross presentation is really significant, especially for developing vaccines needed for multi-adjuvant delivery, we deliberately decided not to include any soluble agonists in our study, and focused on comparing the effects of various PLP combinations. We exposed DCs to individual, dual or triple PLP combinations, and tested how they modulate DC responses and the subsequent adaptive immunity. Our findings reveal new insights into the effects of specific combinations of four highly clinically relevant TLR adjuvants on immune response, and highlight the potential of two specific PLP combinations, M+C and M+R+C, in inducing enhanced antigen cross presentation and humoral immune responses.

IL12p70 and IL10 are key inflammatory cytokines that are critical in polarizing Th response to Th1 or Th2; and Th1/Th2 balance determines the overall nature of the adaptive immune response. IL12p70 promotes Th1 biased immune responses, while IL10 boosts Th2 oriented immune responses, and dampens the Th1 response. Thus, we decided to primarily focus on assaying IL12p70 and IL10 secretion by DCs, and evaluated the quantitative and qualitative differences in induction of these cytokines by various PLP combinations. Our results showed that PLP combinations of P+M, M+R, M+C, P+M+C, and M+R+C led to synergistic enhancement IL12p70 secretion over their individual components. P+R, P+M+R, P+M+C and P+R+C significantly and synergistically increased IL10 induction. We found that M+C combination significantly altered the IL12p70/IL10 ratio, as well as synergistically enhanced the IL12p70 levels. Most of the research on combinatorial TLR adjuvants has focused on using dual combinations. For example, combination of MPLA and CpG was shown to induce significantly higher T cell responses against a Leishmania vaccine candidate as compared to single adjuvants. Another study has shown that polymeric particles carrying ligands for TLR4 and TLR7 synergistically enhance antigen specific production as compared to either single ligand carrying particles. Very little is known about the effects of triple TLR agonist combinations on modulating the immune response; however, simultaneous triggering of more than two TLRs has great potential in enhancing selective immune responses. A study has shown that combining agonists for TLR2, −3, and −9 greatly improves HIV envelope peptide vaccine response in mice, underscoring the importance of combining more than two TLR agonists to synergistically boost the immune response. Hence, in addition to a dual combination, we also studied a triple combination to ascertain its potential in modulating the immune responses. Amongst the triple combinations, M+R+C significantly altered the IL12p70/IL10 ratio, in addition to synergistically enhancing the IL12p70 secretion. Thus, we chose two combinations, i.e. M+C and M+R+C, for further detailed studies on their effects on adaptive immune responses.

Surprisingly, despite a significantly altered BMDC cytokine response as compared to the respective individual adjuvants, M+C and M+R+C PLP combinations did not exhibit any notable difference in promoting CD4 T cell antigen presentation potential by DCs. When we tested the ability of BMDCs for their capacity for antigen cross presentation by co-culturing DCs stimulated with these adjuvants in the presence of different antigen (Ova) doses, with TCR-Tg CD8 T isolated from OT-I mice, we found that both M+C and M+R+C PLP combinations showed enhanced IFNγ secretion, suggesting that these PLP combinations improve cross presentation of soluble antigens. Presentation of exogenous antigens in APCs is mainly carried out via class II MHC pathway, and their class I MHC presentation is very inefficient, resulting in overall weak priming of CD8 T cells. Hence, finding adjuvants that improve antigen cross presentation is really significant, especially for developing vaccines needed to elicit robust cellular immune responses.

Using an Ova immunization model, we showed that both M+C and M+R+C combinations induced potent germinal center and Th response in the draining lymph nodes, as compared to the respective individual PLPs. In
In summary, this study provides significant insights into an array of specific immune responses that can be elicited by using defined adjuvant combinations, and expands our current knowledge on the effects of clinically relevant adjuvants. Our results highlight the capacity of MPLA/CpG and MPLA/R837/CpG adjuvant combinations for enhanced antigen cross presentation and humoral immune responses, and underscore the potential of

terms of antibody response, while M + R + C showed an overall increase in antigen specific IgG1 and IgG2c titers, suggesting a balanced Th1/Th2 response, M + C PLPs promoted enhanced IgG2c but not IgG1, indicating that this combination promotes Th1 biased response. Surprisingly, we did not observe significant antigen specific CD4 or CD8 T cell responses in this model (data not shown), which could be a limitation of the antigen dose and the immunization schedule tested for this study, and future studies will be directed with different antigen doses and immunization schedules to test the impact of these adjuvant combinations on T cell responses.

Figure 5. MC and MRC combinations promote robust humoral responses. Mice were immunized s.c. with PBS, PLP-Ova (O), with or without PLP-M, R, C, M+C, or M+R+C. At day 35, draining lymph nodes and blood sera were analyzed. (a) Ova specific IgG1 and IgG2c response in sera is depicted. Graphs and a representative figure from each group showing frequencies of germinal center (b) and Th1 cells (c) in draining lymph nodes are shown. Data are represented as mean ± SEM of at least 5 replicates. Statistical analysis was performed by 1 way Anova followed by Tukey's multiple comparison tests. (b and c): The following symbols indicate significant differences (p < 0.05) with different groups: *(All); *(UT); *(Blank); *(M); *(R); *(C); *(MC); *(MRC).
using PLP based delivery systems to synergistically augment specific immune responses. Overall, these findings can be leveraged for devising specific strategies for vaccine design and immunotherapy applications.

Methods

Preparation and characterization of PLGA microparticle formulations. PLPs used in this study were prepared with PLGA using a water-oil-water double emulsion, solvent evaporation method as reported earlier. MPLA (detoxified lipid A, Avanti Lipids) was dissolved in chloroform at 5 mg mL⁻¹, TLR7 ligand R837 (Invivogen) was dissolved at 10 mg mL⁻¹ in DMSO with heating; Pam3CSK4 (Invivogen) was dissolved in water at 6.6 mg mL⁻¹. For encapsulating these agonists, 200 µg of PLGA (RG502H, Sigma Aldrich, MO) was dissolved in 7 ml dichloromethane (DCM) (Sigma Aldrich, MO). 250 µl MPLA followed by 150 µl water; 250 µl R837 mixed with 150 ul water; 300 µl Pam3CSK4 or 300 µl water was added to PLGA dissolved in DCM, and homogenized at a speed of 10,000 rpm for 2 min. The emulsion was washed with 50 ml of 1% PVA (Sigma Aldrich, MO) and homogenized again for 2 min at 10,000 rpm and stirred for 3.5–4 h for complete evaporation of DCM. The PLPs were collected by centrifugation and washed 3 times with deionized water, followed by lyophilization, and stored at −20°C until further use. As described previously, R837 encapsulation was estimated by measuring absorbance at 325 nm using a standard curve of R837 in DMSO. Based on hydrophobicity of the molecule and previous publications, MPLA encapsulation was taken to be 100%. Pam3CSK4 encapsulation was estimated by Fluorodehyde OPAs (Thermofisher Scientific) assay using a Pam3CSK4 standard curve, as per the manufacturer’s protocol. Surface functionalization of the synthesized PLGA microparticles was done by covalent conjugation of branch PEI (MW 70,000, Polyscience, PA) with the acid group on the surface of PLGA microparticles using EDC/NHS chemistry as also reported earlier by our group. CpG ODN 1826 (Invivogen, San Diego, CA) or Ovalbumin (EndoGrade Ovalbumin BioVedor, NC) were surface loaded on PLGA-PEI particles by electrostatic interaction. Briefly, PLGA-PEI particles at 5 mg mL⁻¹ (for CpG) or 5 mg (0.5 mL⁻¹) (Ova) and CpG ODN at 60 µg mL⁻¹ or Ovalbumin at 250 µg (4.5 mL⁻¹) were resuspended in PBS in two separate DNase RNase free tubes. The PLGA-PEI solution was added drop-wise to the CpG or Ova solution while vortexing and the mixture was incubated on an end to over shaker overnight at 4°C. The particles were collected by centrifugation and the supernatant was analyzed for CpG at 260 nm wavelength using a 3 Ta micro-volume plate in a BioTek reader (BioTek, VT) and Ova by micro bca assay (Thermo Scientific, IL). The amount of nucleic acid or protein present in the supernatant was subtracted from the initially added amount to quantify the total amount of surface loaded nucleic acid onto PLGA-PEI microparticles. Particles were characterized for their size and zeta potential using a Zetasizer Nano ZS instrument (Malvern, MA).

Mice. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology and all methods were performed in accordance with the relevant guidelines and regulations. Mice were housed under pathogen-free conditions in the physiological research laboratory (PRL) at the Institute of Bioscience Bioengineering (IBB), Georgia Institute of Technology, and provided with sterile water and food ad libitum. C57BL/6 J, OTI Tg mice (specific for Ova 257–264) were purchased from the Jackson Laboratory.

BMDCs stimulation and activation assays. Bone marrow cells from C57BL/6 J mice were grown in RPMI 1640 medium (Lonza, Walkersville, MD) with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM glutamine, β-ME, 10 mM HEPES, 1 mM sodium pyruvate, 1X nonessential amino acids, and 20 mg mL⁻¹ murine recombinant GM-CSF (Peprotech, Rocky Hill, NJ) for generating BMDCs. Incubations were carried out at 37°C with 5% CO2. Fresh medium with GM-CSF was added on days 2, 4 and 6, and cells were used on day 7. 3 × 10⁵ cells per well in 96 well polypropylene plates were stimulated with adjuvants at various concentrations as mentioned in results. Cell free supernatants were assayed for IL12p70 and IL10 by ELISA using kits from Ebiosciences as per the manufacturer’s instructions. To estimate synergy in cytokine induction, synergy ratio was calculated = Amount of cytokine induced by the micro-PLP combination/Sum of total cytokine amounts induced by the individual micro-PLPs of that combination. Staining for cell-surface markers (CD11c, CD40 and CD86) was done by resuspending cells in 200 μl FACS buffer (PBS with 2% FBS) with anti-mouse CD16/CD32 Fc block at 4°C for 10 min, followed by incubation at 4°C for 30 min with murine anti-CD11c APC, anti-CD40 PE, anti-CD86 FITC antibodies (Ebiosciences). Cells were washed with FACS buffer, stained with 7-AAD viability stain (BD Biosciences) and data were acquired using an BD Accuri or BD LSRII (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, San Carlos, CA).

Antigen presentation assays. CD8 T cells were purified from single-cell suspensions of spleen from OTI Tg mice using the CD8 T cell isolation kit and AutoMACS column as per the manufacturer’s instructions (Miltenyi Biotec). 3 × 10⁵ BMDCs per well in 96 well polypropylene plates were incubated with 10 µg mL⁻¹, 100 µg mL⁻¹ or 1 mg mL⁻¹ soluble Ovalbumin in the presence or absence of various adjuvants at concentrations specified in results for 24 h. DCs were washed with PBS and co-cultivated with Ag specific CD8 T cells at a ratio of 1:4 for 72 h. Supernatants collected from these cells were analyzed for IFNγ and IL4 by ELISA using ready-set-go kits (Ebioscience) according to the manufacturer’s instructions.

Mouse Immunization and tissue harvests. 6–8 week old C57BL/6 J mice were immunized with various PLP formulations (10 µg Ova, 37 µg MPLA, 50 µg R837 or 20 µg CpG ODN per injection respectively), subcutaneously (inguinal) in 200 μl volume three times, at 2 weeks interval. PBS or antigen alone injected mice were used as negative controls. Blood serum, spleen and lymph nodes were harvested at 5 weeks post 1st immunization. Serum antibody ELISAs were carried out as described elsewhere with some modifications: 96 well Nunc maxisorp plates were coated with 100 ng Ova (100 µl)⁻¹ in carbonate buffer at 4°C overnight. Plates were washed with washing buffer (PBS + 0.05% Tween20), and blocked with 4% BSA in PBS + 0.05% Tween20 for
1 h at 37 °C. Blocking buffer was removed and blood sera dilution 10 fold serially (100x, 1000x, 10000x, 100000x) in PBS + 0.05% Tween20 were added to the plates, and incubated at 37 °C for 2 h. Plates were washed 5 times with washing buffer and incubated with 5000 fold dilution of IgG1-HRP (Southern Biotech, AL) or IgG2c-HRP (Southern Biotech, AL) at 37 °C for 1 h. Plates were washed and developed using TMB (tetramethylbenzidine) substrate (Southern Biotech, AL) and were stopped using 2 N sulfuric acid. Absorbance was read at 450 nm using a BioTekplate reader. Anti-mouse antibodies for Flow cytometry: GL7 FITC (Biolegend), B220 PerCP-Cy5.5 (biotecnique), CD3 PE/Cy7 (Biolegend), CD4 APC (biotecnique), Bc6 PE (Biolegend) (intracellular staining), CXC5 PE/Dazzle (Biolegend) were used in this study. For surface staining single cell suspensions from draining lymph nodes were washed with FACS buffer and stained for 30 min at 4 °C, followed by washing with FACS buffer. For intracellular staining, established protocol from BD Biosciences was used. Briefly, surface stained cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and stained for intracellular antibodies in 1x BD perm/wash solution at 4 °C for 30 min. Cells were washed with perm wash followed by FACS buffer. Stained cell samples were fixed with BD Cytofix (BD Biosciences) and acquired on a LSR-II cytometer (BD Biosciences). FACS data were analyzed on Flow JO software.

Statistics. 1 way Anova followed by Tukey’s multiple comparison tests were performed. The following symbols indicate significant differences: * (p < 0.05), ** (p < 0.01), *** (p < 0.001). Where indicated **** (p < 0.0001).

Data Availability. All relevant data supporting the results of this study are included in the current article and supplementary information. Raw data and additional supporting data can be made available to the authors upon reasonable request.

References
1. Mbow, M. L., De Gregorio, E., Valiante, N. M. & Rappuoli, R. New adjuvants for human vaccines. Curr. Opin. Immunol. 22, 411–416, doi:10.1016/j.coi.2010.04.004 (2010).
2. Appay, V., Douek, D. C. & Price, D. A. CD8 T cell efficacy in vaccination and disease. Nat. Med. 14, 623–628, doi:10.1038/nm.1777 (2008).
3. Epstein, J. E. et al. Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity. Science 334, 475–480, doi:10.1126/science.1112514 (2011).
4. Seder, R. A. & Hill, A. V. Vaccines against intracellular infections requiring cellular immunity. Nature 406, 793–798, doi:10.1038/35021239 (2000).
5. Rueckert, C. & Guzman, C. A. Vaccines: from empirical development to rational design. PLoS Pathog. 8, e1003001, doi:10.1371/journal.ppat.1003001 (2012).
6. Banchereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. Nature 392, 245–252, doi:10.1038/35032888 (1998).
7. Steinman, R. M. & Hemmi, H. Dendritic cells: translating innate to adaptive immunity. Immunol. Rev. 40, 642–656, doi:10.1111/j.1600-065X.2014.01416.x (2014).
8. Pulendran, B. Modulating vaccine responses with dendritic cells and Toll-like receptors. Immunol. Rev. 199, 227–290, doi:10.1111/j.1600-0590.2004.00414.x (2004).
9. Kumar, H., Kawai, T. & Akira, S. Pathogen recognition by the innate immune system. Int. Rev. Immunol. 30, 16–34, doi:10.3109/083018510.12059976 (2011).
10. Moser, M. & Murphy, K. M. Dendritic cell regulation of TH1-TH2 development. Nat. Immunol. 1, 199–205, doi:10.1038/79734 (2000).
11. Kawai, T. & Akira, S. Pathogen recognition with Toll-like receptors. Curr. Opin. Immunol. 17, 338–344, doi:10.1016/j.coi.2005.02.007 (2005).
12. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11, 373–384, doi:10.1038/ni.1863 (2010).
13. Takeda, K., Kaisho, T. & Akira, S. Toll-like receptors. Annual review of immunology 21, 355–376, doi:10.1146/annurev.immunol.21.120601.141126 (2003).
14. Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. Cell 124, 783–801, doi:10.1016/j.cell.2006.02.015 (2006).
15. Kawasaki, T. & Kawai, T. Toll-like receptor signaling pathways. Front. Immunol. 5, 461, doi:10.3389/fimmu.2014.00461 (2014).
16. Hayashi, F. et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410, 1099–1103, doi:10.1038/35074106 (2001).
17. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J. & Gusovsky, F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J. Biol. Chem. 274, 10689–10692, doi:10.1074/jbc.M104.01689 (1999).
18. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. Recognition of double-stranded RNA and activation of NF-kappab by Toll-like receptor 3. Nature 413, 732–738, doi:10.1038/35099560 (2001).
19. Heil, F. et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526–1529, doi:10.1126/science.1093620 (2004).
20. Hemmi, H. et al. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740–745, doi:10.1038/35047123 (2000).
21. Duthie, M. S., Windish, H. P., Fox, C. B. & Reed, S. G. Use of defined TLR ligands as adjuvants within human vaccines. Immunol. Rev. 239, 178–196, doi:10.1111/j.1600-065X.2010.00978.x (2011).
22. Schwarz, K. et al. Role of Toll-like receptors in costimulating cytotoxic T cell responses. Eur. J. Immunol. 33, 1465–1470, doi:10.1002/eji.2002323919 (2003).
23. Mitchell, D. et al. Dual stimulation of MyD88-dependent Toll-like receptors induces synergistically enhanced production of inflammatory cytokines in murine bone marrow-derived dendritic cells. J. Infect. Dis. 202, 318–329, doi:10.1086/563499 (2010).
24. Tousi, D. N. & Massari, P. Immune Adjuvant Efect of Molecularly-defined Toll-Like Receptor Ligands. Vaccines (Basel) 2, 323–353, doi:10.3390/vaccines20200323 (2014).
25. Caproni, E. et al. MF59 and Pam3CSK4 boost adaptive responses to influenza subunit vaccine through an IFN type I-independent mechanism of action. J. Immunol. 188, 3088–3098, doi:10.4049/jimmunol.188126 (2012).
26. Casella, C. R. & Mitchell, T. C. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. Cellular and molecular life sciences: CMLS 65, 3231–3240, doi:10.1007/s00018-008-8228-6 (2008).
27. Baldridge, J. R. et al. Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. Expert Opin. Biol. Ther. 4, 1129–1138, doi:10.1517/14712598.4.7.1129 (2004).
63. Pai Kasturi, S.
66. Storni, T., Kundig, T. M., Senti, G. & Johansen, P. Immunity in response to particulate antigen-delivery systems.
64. Pradhan, P.
58. Moon, J. J.
56. Lynn, G. M.
59. Danhier, F.
55. Kasturi, S. P.
48. Makela, S. M., Strengell, M., Pietila, T. E., Osterlund, P. & Julkunen, I. Multiple signaling pathways contribute to synergistic TLR
46. Bagchi, A.
41. Klinman, D. M. Immunotherapeutic uses of CpG oligodeoxynucleotides.
38. Demento, S. L.
35. Adams, S.
33. Vasilakos, J. P. & Tomai, M. A. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants.
31. Hemmi, H.
26. Huang, J. J. et al. Adjuvant-carrying synthetic vaccine particles augment the immune response to encapsulated antigen and exhibit strong local immune activation without inducing systemic cytokine release. Vaccine 32, 2882–2895, doi:10.1016/j.vaccine.2014.02.027 (2014).
29. Mata-Haro, V. et al. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 316, 1628–1632, doi:10.1126/science.1138963 (2007).
28. Moon, J. J., Han, S., Hamada, S. et al. Enhancing humoral responses to a malaria antigen with nanoparticle vaccines that expand Th1 cells and promote germinal center induction. Proc. Natl. Acad. Sci. USA 109, 1080–1085, doi:10.1073/pnas.1112648109 (2012).
27. Demento, S. L. et al. Safety and immunogenicity of an AS01-adjuvanted varicella-zoster virus subunit candidate vaccine against herpes zoster in adults >=50 years of age. J. Infect. Dis. 208, 1953–1961, doi:10.1093/infdis/jit365 (2013).
26. Huang, J. J. et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur. J. Immunol. 34, 251–262, doi:10.1002/eji.200324032 (2004).
25. Ilinsky, P. O. et al. Adjuvant-carrying synthetic vaccine particles augment the immune response to encapsulated antigen and exhibit strong local immune activation without inducing systemic cytokine release. Vaccine 32, 2882–2895, doi:10.1016/j.vaccine.2014.02.027 (2014).
24. Ilono, J. O. et al. Phase I trial of subcutaneously administered recombinant human interleukin-2 in patients with metastatic melanoma. Cancer 95, 127–134, doi:10.1002/cncr.10631 (2002).
23. Portielje, J. E. et al. Phase I study of subcutaneously administered recombinant human interleukin 12 in patients with advanced renal cell cancer. Clin. Cancer Res. 5, 3983–3989 (1999).
22. Engel, A. L., Holt, G. E. & Lu, H. The pharmacokinetics of Toll-like receptor agonists and the impact on the immune system. Expert Rev. Clin. Pharmacol. 5, 275–289, doi:10.1586/epc.11.5 (2011).
21. Carson, W. E. et al. Coadministration of interleukin-18 and interleukin-12 induces a fatal inflammatory response in mice: critical role of natural killer cell interferon-gamma production and STAT-mediated signal transduction. Blood 96, 1465–1473 (2000).
20. Kasturi, S. P. & Tomai, M. A. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. Expert Rev. Vaccines 10, 499–511, doi:10.1586/erv.10.174 (2011).
19. Kasturi, S. P. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol. 4, 249–258, doi:10.1038/nri1329 (2004).
18. Volpiner, J. et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur. J. Immunol. 34, 251–262, doi:10.1002/eji.200324032 (2004).
17. Makela, S. M., Strengell, M., Pietila, T. E., Ostendorf, P. & Julkunen, I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. J. Leukoc. Biol. 85, 664–672, doi:10.1189/jl.2008.0503 (2009).
16. Hanson, M. C. et al. Nanoparticle STING agonists are potent lymph node-targeted vaccine adjuvants. J. Clin. Invest. 125, 2532–2546, doi:10.1172/JCI77995 (2015).
15. Kwong, B., Liu, H. & Irvine, D. J. Induction of potent anti-tumor responses while eliminating systemic side effects via liposome-anchored combinatorial immunotherapy. Biomaterials 32, 5134–5147, doi:10.1016/j.biomaterials.2011.03.067 (2011).
14. Lynn, G. M. et al. In vivo characterization of the physicochemical properties of polymer-linked TLR agonists that enhance vaccine immunogenicity. Nat. Biotechnol. 33, 1201–1210, doi:10.1038/nbt.3371 (2015).
13. Iliopoulos, I. et al. Characterization of the physicochemical properties of polymer-linked TLR agonists that enhance vaccine immunogenicity. Nat. Biotechnol. 33, 1201–1210, doi:10.1038/nbt.3371 (2015).
12. Portielje, J. E. et al. Phase I study of subcutaneously administered recombinant human interleukin 12 in patients with advanced renal cell cancer. Clin. Cancer Res. 5, 3983–3989 (1999).
11. Eton, O. et al. Phase I trial of subcutaneously administered recombinant human interleukin-2 in patients with metastatic melanoma. Cancer 95, 127–134, doi:10.1002/cncr.10631 (2002).
10. Portielje, J. E. et al. Phase I study of subcutaneously administered recombinant human interleukin 12 in patients with advanced renal cell cancer. Clin. Cancer Res. 5, 3983–3989 (1999).
9. Enged, A. L., Holt, G. E. & Lu, H. The pharmacokinetics of Toll-like receptor agonists and the impact on the immune system. Expert Rev. Clin. Pharmacol. 4, 275–289, doi:10.1586/ecp.11.5 (2011).
8. Carson, W. E. et al. Coadministration of interleukin-18 and interleukin-12 induces a fatal inflammatory response in mice: critical role of natural killer cell interferon-gamma production and STAT-mediated signal transduction. Blood 96, 1465–1473 (2000).
7. Kasturi, S. P. & Tomai, M. A. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. Expert Rev. Vaccines 10, 499–511, doi:10.1586/erv.10.174 (2011).
6. Kasturi, S. P. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol. 4, 249–258, doi:10.1038/nri1329 (2004).
5. Volpiner, J. et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur. J. Immunol. 34, 251–262, doi:10.1002/eji.200324032 (2004).
4. Makela, S. M., Strengell, M., Pietila, T. E., Ostendorf, P. & Julkunen, I. Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells. J. Leukoc. Biol. 85, 664–672, doi:10.1189/jl.2008.0503 (2009).
3. Hanson, M. C. et al. Nanoparticle STING agonists are potent lymph node-targeted vaccine adjuvants. J. Clin. Invest. 125, 2532–2546, doi:10.1172/JCI77995 (2015).
2. Kwong, B., Liu, H. & Irvine, D. J. Induction of potent anti-tumor responses while eliminating systemic side effects via liposome-anchored combinatorial immunotherapy. Biomaterials 32, 5134–5147, doi:10.1016/j.biomaterials.2011.03.067 (2011).
1. Lynn, G. M. et al. In vivo characterization of the physicochemical properties of polymer-linked TLR agonists that enhance vaccine immunogenicity. Nat. Biotechnol. 33, 1201–1210, doi:10.1038/nbt.3371 (2015).
67. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3, 133–146, doi:10.1038/nri1001 (2003).
68. Zhu, Q. *et al.* Using 3 TLR ligands as a combination adjuvant induces qualitative changes in T cell responses needed for antiviral protection in mice. *J. Clin. Invest.* 120, 607–616, doi:10.1172/JCI39293 (2010).
69. Maldonado Galdeano, C. *et al.* Impact of a probiotic fermented milk in the gut ecosystem and in the systemic immunity using a non-severe protein-energy-malnutrition model in mice. *BMC Gastroenterol.* 11, 64, doi:10.1186/1471-230X-11-64 (2011).

**Acknowledgements**

This work was partially supported by the National Institutes of Health (NIH) through Grant U01AI124270 (PI: Roy), The Georgia Tech Foundation and the Georgia Research Alliance through an Immuno-Engineering seed grant, and through funding from the Carol Ann and David D. Flanagan Professorship as well as the Robert A. Milton Chair to K.R.

**Author Contributions**

R.M.L. designed and performed experiments, collected and analyzed all data, and wrote the manuscript. P.P. helped in designing experiments as well as in performing and collecting data. K.R. helped design experiments, supervised overall work and helped write the manuscript. All authors reviewed and approved the final version of the manuscript.

**Additional Information**

**Supplementary information** accompanies this paper at doi:10.1038/s41598-017-02804-y

**Competing Interests:** The authors declare that they have no competing interests.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017