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A novel synthetic adjuvant enhances dendritic cell function

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Summary

The lipid core peptide (LCP) is a novel, synthetic, self-adjuvanted vaccine delivery system that neatly incorporates the adjuvant, carrier and antigenic peptides of a vaccine into a single molecular entity. This system has been previously shown to efficiently deliver vaccines and induce immunity. Because adjuvants target sentinels of the immune response, such as dendritic cells (DCs), that are widely distributed throughout the body to initiate specific immune responses, we investigated the effects of the adjuvant on DCs. Here we show that LCP targets vaccines to DCs and induces their activation.

Keywords: adjuvant; dendritic cells; LCP system; Toll-like receptor 2; vaccine delivery

Introduction

The lipid core peptide (LCP) is a synthetic adjuvant system that efficiently delivers vaccines. It neatly incorporates an adjuvant, a carrier and peptides from the vaccine into a single molecular unit. The adjuvant portion consists of a lipid section which mimics the PamCys system, and contains three fatty-acyl chains (Fig. 1a). This system was previously synthesized to carry antigenic peptide sequences from *Chlamydia* and group A streptococcus (GAS), and was found to induce high antibody titres in experimental animals.[1–4]

An effective adjuvant should target the accompanying vaccine towards dendritic cells (DCs), to improve antigen presentation and activation of immune responses. DCs are leucocytes that excel in the uptake, processing and presentation of antigen to T cells. Appropriately activated DCs interact with $CD4^+$ T lymphocytes through cell surface receptors such as major histocompatibility complex class II (MHCII), CD80 and/or CD86 and secrete cytokines such as interleukin (IL)-12 that induce activation of T cells.

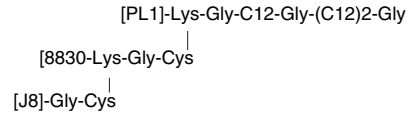

Figure 1. A schematic representation of the synthetic lipid core peptide construct.

In mice, DCs are characterized by the expression of CD11c and can be divided into broad populations based on the expression of CD8α and CD4, accordingly termed $CD8\alpha^+$, $CD4^+$ and the double-negative (DN) DC ($CD8\alpha^-$ $CD4^-$).[5,6] In humans, CD4 and CD8 do not differentiate DC subpopulations. DCs also express pattern recognition molecules known as the Toll-like receptors (TLRs) which provide activation signals to DCs. The TLR family consists of 11 members (TLR1–11) that recognize different varieties of pathogen-associated molecular patterns,[7] generally leading to the maturation and migration of DCs to the lymph nodes to initiate an immune response. While mRNA for TLR2 and TLR4 has been found in all murine DC subsets,[8] TLR9 protein expression and function have been shown in these cells.[9,10]

TLR2 is unique in that it recognizes a rather broad range of lipid ligands derived from different types of microbes (e.g. bacteria). Among these, TLR2 binds to lipoproteins/

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lipoteichoic acids derived from both natural (various pathogens) and synthetic sources, and also recognizes atypical types of lipopolysaccharide (LPS).[11] TLR2 heterodimerizes with either TLR1 or TLR6 for the recognition tri- or di-acylated lipids, respectively,[12–19] but this is not a strict requirement as synthetic lipopeptides such as macrophage-activating lipopeptide (MALP2)-SK_4, Pam_2C-SK_4 and Pam_3C-SK_4 can signal in the absence of both receptors,[20,21] leading to the notion that TLR2 may form functional homodimers for signalling.

In the present study, given that all adaptive immune responses are initiated by DCs, we investigated the effect of LCP on DC phenotype and function *in vivo* and whether the effects could be mediated by TLR2 signalling.

Materials and methods

Lipid core peptide

The LCP construct was synthesized as previously described,[1] dissolved in sterile, endotoxin-free phosphate-buffered saline (PBS) and stored in aliquots at −80° until required.

Mice

Female C57BL/6 mice were purchased from the Central Animal Breeding House (Pinjarrah Hills, Qld, Australia) or from the Herston Medical Centre (Herston, Qld, Australia). Female OT-II mice were kindly provided by Dr Adriana Baz of the Queensland Institute of Medical Research (QIMR, Herston, Qld, Australia).

Immunizations and tissue collection

In these studies, we compared the effects of LCP alone and in conjunction with an immunogenic protein ovalbumin (OVA) on DC phenotype and function. Mice were injected intraperitoneally (i.p.) either with 50 μg of LCP or 50 μg of LCP + 50 μg of OVA (Sigma-Aldrich, St Louis, MO) or with PBS alone. Blood was obtained via tail-snipping after 4 and 7 days, and the serum was collected and stored at −20° until it was used. Spleens were harvested at different time-points post-injection as shown in Fig. 2 or after 7 days for functional assays.

Isolation of splenic DCs and T cells

Spleens were digested as previously described[22,23] and DCs were isolated using anti-CD11c magnetic antibody cell sorting (MACS) beads according to the manufacturer's instructions (Miltenyi Biotech, Bergish Gladbach, Germany). The isolated DCs were always labelled with anti-CD11c-phycoerythrin (PE) for fluorescence-activated cell sorter (FACS) analysis to confirm purity. The T cells were isolated using MACS anti-Thy-1 beads (Miltenyi Biotech). The labelled cell preparations were passed through two columns to give 95–98% purity. Viability was assessed by trypan blue or labelling with 7-actinomycin D.

Flow cytometry of DCs

To determine whether $CD11c^+$ DCs or DC subpopulations were affected by exposure to LCP, their maturation was assessed by their expression of MHCII, CD80 or CD86 following exposure to LCP *in vivo* compared with DCs from naïve mice. DCs were purified as described above and then labelled for flow cytometry.[23] To minimize non-specific labelling, DCs were always pre-incubated with purified rat immunoglobulin in 5% bovine serum albumin (BSA)/PBS for 20 min prior to cell labelling and monoclonal antibodies used were directly conjugated with fluorescein (FITC), PE or allophycocyanin (APC). DCs were routinely analysed for MHCII (M5/115·15·2), CD80 (16-10A1), CD86 (GL1), CD11c (HL3), CD4 (GK1·5) and CD8 (H35-17·2) expression using reagents purchased from Pharmingen (Franklin Lakes, NJ). Samples were analysed on FACS Calibur™ (BD Biosciences, Franklin Lakes, NJ), gating on viable cells, using CELLQUEST™ software (version 3·3; BD Biosciences) or WINMDI (version 3·1; freeware written by Joe Trotter). Approximately 10^4 cells from each sample were analysed for accurate measurement.

DC-allogeneic T-cell mixed lymphocyte cultures

Purified, naïve OT-II $CD4^+$ T cells were labelled with 5 μg of carboxyfluorescein succinimidyl ester (CFSE) as per the manufacturer's instructions (CellTrace™ CFSE cell proliferation kit from Molecular Probes, Carlsbad, CA), and dispensed into round-bottomed 96-well plates at a concentration of $2{\cdot}5 \times 10^5$ cells/well. Purified splenic DCs were added in titrating numbers, in triplicate, to these T cells, isolated from OTII mice. DCs were added to the wells at cell densities ranging from 5×10^3 to $2{\cdot}5 \times 10^4$ cells/well. The cultures were supplemented with 50 μg/ml OVA and incubated at 37° for 4 days before FACS analysis.

Serum cytokine measurements

Cytokines were assessed in the sera of immunized mice. The levels of cytokines in the serum were measured using the Becton Dickenson Inflammation Bead Array (Franklin Lakes, NJ) according to the manufacturer's instructions.

IL-12 secretion

DCs were isolated as described above from the different treated groups and cultured on Multiscreen-HA sterile

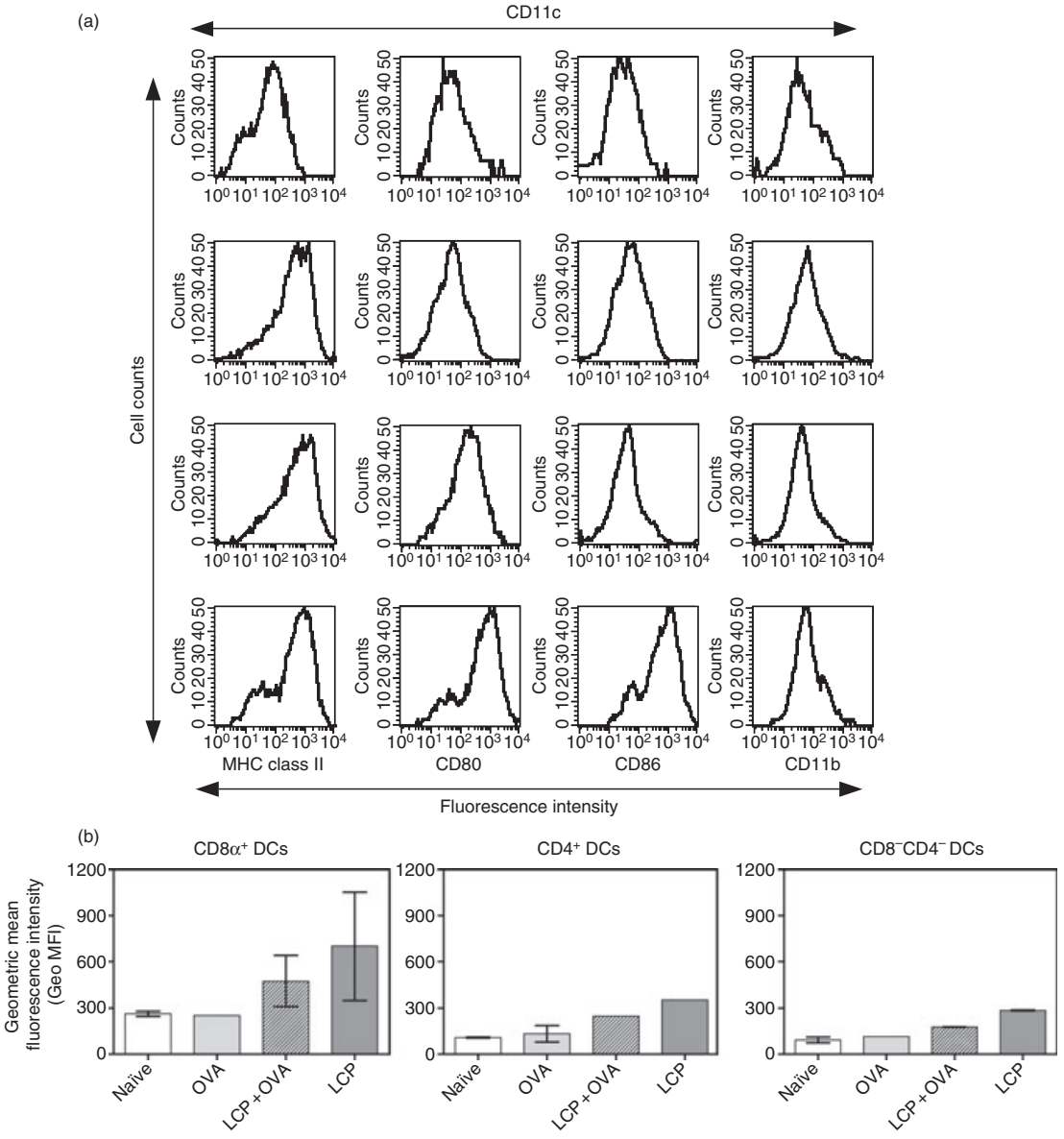

Figure 2. Flow-cytometry profiles of murine splenic dendritic cells (DCs) from lipid core peptide (LCP)-treated mice. (a) Multiple cohorts of mice were administered with either the synthetic compound LCP or phosphate-buffered saline (PBS) and CD11c$^+$ DC isolated after 3, 7 and 11 days. DCs were labelled for major histocompatibility complex (MHC) class II, CD80, CD86 and CD11b expression and analysed by flow cytometry. (b) Multiple cohorts of mice were treated with LCP alone, LCP with ovalbumin (OVA) or OVA alone and DC isolated from naïve or treated mice after 7 days. The DCs were labelled for expression of MHC class II and DC subpopulation markers, CD8 and CD4, for flow cytometry analysis. The error bars shown are the standard error of the mean (SEM) and the data shown are an example of one from two experiments with three or four mice in each cohort.

plates (Millipore, Billercia, MA) previously coated with 10 μg/ml anti-mouse IL-12 (p70) antibody (Pharmingen) overnight and washed, and unbound sites were blocked with 5% fetal calf serum (FCS). 2×10^5 cells were cultured in medium alone or with either 1·0 μM phosphorothioate modified CpG oligonucleotide 1668 (5′-TCC ATG ACG TTC CTG ATG CT-3′)[24] or 10 μg/ml of lipopolysaccharide (LPS) (0111:B4, *Escherichia coli*).[24] After overnight culture at 37°, plates were washed with 0·05% Tween-PBS and incubated with 0·5 μg/ml biotin-anti-IL-12 (Pharmingen) followed by alkaline phosphatase (AKP)-labelled-streptavidin (Pharmingen). Nitroblue tetrazolium dye (NBT)/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (BCIP) tablets (Sigma-Aldrich) were used to visualize spots.

TLR cell line culture

The stably transfected TLR2-293HEK cell line was maintained in Dulbecco's modified Eagle's minimal essential

medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% FCS (Cambrex, East Rutherford, NJ) and 500 μg/mL G418 (Invitrogen, Carlsbad, CA). The 293HEK cell line was maintained under the same conditions without the G418. All cells were kept in an incubator at 37° with 5% CO_2.

Plasmids, transfections, luciferase assays and protein assays

Cells were plated in OptiMEM-I (Gibco) in six-well plates at 1×10^6 cells/well and transfected with 1 μg/well pNKkB-Luc. The transfection medium consisted of lipofectamine (DOTAP-chloride: DOPE) (Sigma-Aldrich) made up in a ratio of 1 : 1 in sterile, endotoxin-free water. Transfected cells were pooled, re-seeded in 24-well plates, stimulated at 44 hr post-transfection and harvested at 48 hr. The lysates were assayed for luciferase activity using the luciferase assay kit (Promega, Madison, WI), read on a luminometer (Turner Designs, Sunnyvale, CA) and assayed for protein content using the Bio-Rad Bradford assay (Bio-Rad Laboratories, Hercules, CA). The luciferase activity of every sample was normalized to protein content. The bar chart shows the ratio of the reading for each stimulated sample to the reading for theTLR2+ cell lines cultured in medium only (un-stimulated).

Statistical analyses

Statistical analyses were conducted using Student's paired, two-tailed *t*-test, and results were significant at $P = 0{\cdot}0001$. Bar charts shown are mean ± standard error of the mean (SEM). *P*-values were calculated using Student's unpaired *t*-test, with a two-sided tail, based on pooled data from replicate experiments.

Results

Treatment with LCP induces DC maturation

To determine whether the synthetic compound LCP was recognized by DCs *in vivo*, cohorts of mice were administered either LCP or PBS by i.p. injection. Splenic DCs were isolated 3, 7 or 11 days post-injection, and the maturation assessed based on MHCII expression. These studies found that the expression of MHCII increased by day 3 and started to decline after 11 days, while CD80 was up-regulated from day 7 onwards and expression of CD86 was not consistently induced (Fig. 2a). The expression on the intergrin CD11b was not affected.

To determine whether a particular DC subpopulation was affected, cohorts of mice were administered LCP alone, LCP and OVA (to represent a vaccine), OVA alone, or PBS, all given intravenously to target the spleen. After 7 days, when maturation had peaked (Fig. 2a), DCs were isolated from the spleens and labelled for expression of MHCII, CD4 and CD8, to identify activation of DC subpopulations. The expression of MHCII was consistently increased on all DC subpopulations but most notably increased on $CD8^+$ DCs following LCP treatment with or without OVA compared with DCs from naïve or OVA-treated mice (Fig. 2b).

DC function is up-regulated following treatment with LCP

To determine whether the increased maturation of $CD11c^+$ DCs induced by LCP translated into an improved capacity to induce proliferation of T cells, $CD11c^+$ DCs were isolated from cohorts of untreated mice or mice administered LCP alone, LCP and OVA, or OVA alone and co-cultured with OTII T cells labelled with CFSE (Fig. 3a). After 4 days, the cultures were labelled to identify CD4 T cells and the proliferation was measured by the level of CSFE expression (Fig. 3a). As the CSFE label is lost as the cells divide, T cells that had proliferated had

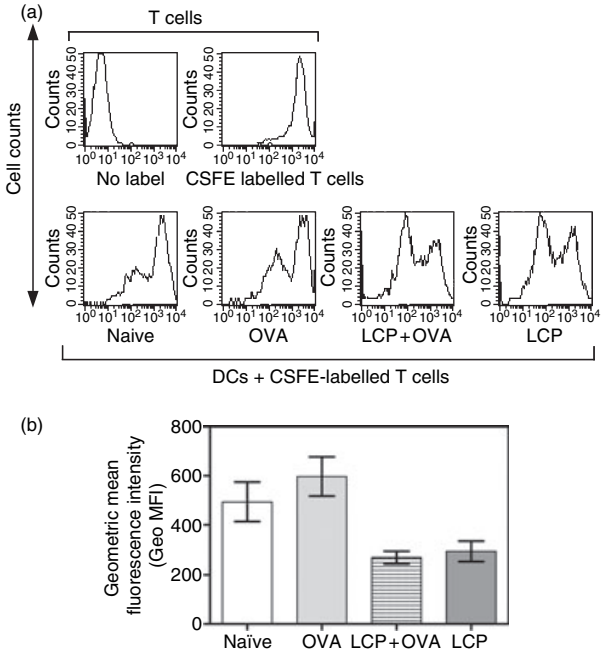

Figure 3. Function of murine splenic dendritic cells (DCs) from lipid core peptide (LCP)-treated mice. Splenic DCs were isolated from mice 7 days after the administration of ovalbumin (OVA), LCP + OVA, LCP alone or phosphate-buffered saline (PBS), and co-cultured for 4 days with unlabelled or carboxyfluorescein succinimidyl ester (CFSE) labelled, naïve $CD4^+$ T cells. (a) Examples of flow cytometry profiles showing the proliferation frequency of the CFSE-labelled T cells. (b) Bar chart showing the proliferation frequency of the CFSE-labelled T cells from cohorts of mice, in replicate experiments. The error bars shown are the standard error of the mean (SEM) and the data shown are an example of one from two experiments with three or four mice in each cohort.

a lower level of CSFE expression. The flow cytometry profiles show that T cells cultured with DCs from LCP-treated mice, with and without OVA, compared with T cells cultured with DCs from mice treated with OVA only or naïve mice, had lower CSFE levels, indicating improved proliferation (Fig. 3a). Analysis of the data from multiple mice in repeated experiments showed that DCs from mice given LCP and OVA were significantly better at inducing proliferation of antigen-specific T cells compared with DCs from naïve mice ($P < 0{\cdot}0382$) or mice treated with OVA alone ($P < 0{\cdot}0079$) (Fig. 3b). Moreover, treatment of mice with LCP alone also improved DC-mediated proliferation of T cells compared with DCs from mice treated with OVA alone ($P < 0{\cdot}0155$).

Cytokine production by DCs is up-regulated by LCP

To determine whether the increased maturation of $CD11c^+$ DCs induced by LCP translated into an improved capacity to secrete IL-12p70, an important cytokine for initiating immune responses, $CD11c^+$ DCs were isolated from cohorts of untreated mice or mice administered LCP alone, LCP and OVA, or OVA alone. The DCs were cultured with either LPS or CpG or left unstimulated for the assessment of IL-12 secretion in an enzyme-linked immunosorbent spot-forming cell assay (ELISPOT) (Fig. 4). A significantly higher number of LCP-experienced DCs secreted IL-12 when stimulated with CpG ($P < 0{\cdot}0003$) or LPS ($P < 0{\cdot}015$) compared with DCs from naïve mice. Sera from mice given LCP with and without OVA were tested on days 4 and 7 post-injection for cytokines using the CBA mouse inflammation kit (BD Biosciences) but cytokine levels in the serum were not increased consistently (data not shown).

The LCP construct is able to signal via TLR2

To determine whether the LCP construct could signal DCs via TLR2, an *in vitro* system was used. The HEK293 cell lines, either wild type or stably expressing human TLR2, were transiently transfected with an NFKKB-Luc plasmid. The TLR2-transfected cells were treated in culture with 2, 10 and 50 μM LCP construct and harvested, and the levels of luciferase activity were determined. Cultures of TLR2-transfected cells without LCP or given a positive control agonist, or the untransfected cells were used as controls. The untransfected cells and transfected cells in media were minimally stimulated compared with the positive control (Fig. 5). The TLR2-transfected cell line cultured with LCP showed an increase of approximately 60% relative to the positive control level. There was no significant difference among results for the three concentrations of LCP.

Discussion

The LCP system has been shown to induce high antibody titres in previous studies involving group A streptococcus.[1] This led us to elucidate the mechanisms through which LCP enhances immunity. DCs are positioned at the interface of the innate and adaptive immunities and thus play a pivotal role in vaccine efficiency. Our *in vivo* data show that the LCP has an effect on the entire population

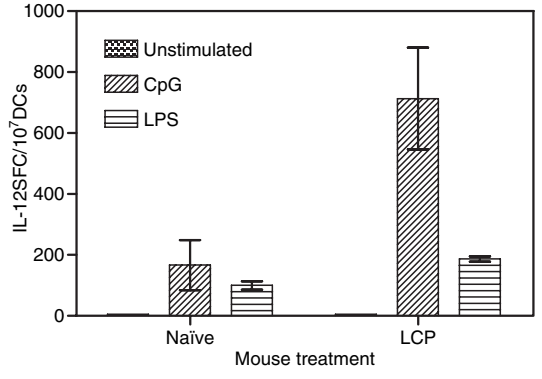

Figure 4. Interleukin (IL)-12 secretion by murine splenic dendritic cells (DCs) from lipid core peptide (LCP)-treated mice. Splenic DCs were isolated from mice 7 days after the administration of ovalbumin (OVA), LCP + OVA, LCP alone or phosphate-buffered saline (PBS), and were re-stimulated *in vitro* with either LPS or CpG. The numbers of DCs secreting IL-12 were then assessed by enzyme-linked immunosorbent spot-forming cell assay (ELISPOT). The error bars shown are the standard error of the mean (SEM) and the data shown are an example of one from two experiments with three or four mice in each cohort.

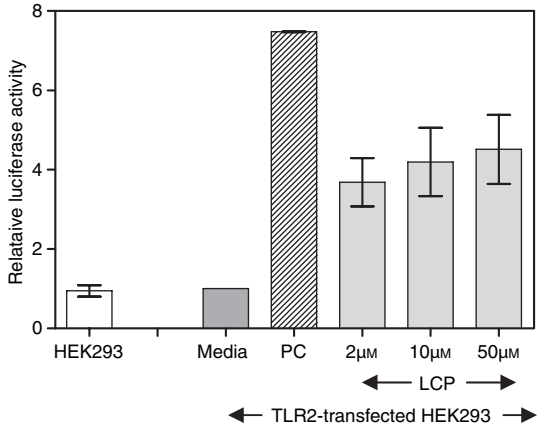

Figure 5. The lipid core peptide (LCP) construct is able to stimulate Toll-like receptor 2 (TLR2) activity. Cells lines expressing TLR2 were transfected with a plasmid carrying the luciferase gene under control of the nuclear factor (NF)-κB promoter, or mock transfected and then treated with the LCP construct or with a positive control (PC), Pam2Cys. Luceferase activity was then measured to assess TLR2 signalling PC, positive control.

of CD11c$^+$ DCs, but in particular on CD8α^+ DCs, with maturation peaking at 7 days post-injection. Maturation was evidenced by an increase in both MHCII and CD80 expression in comparison to naïve and day 3 DCs, while CD86 was not consistently induced at any time. By day 11, expression of MHCII and CD80 began to decline. The length of time taken for the up-regulation of CD80 is in agreement with previous studies[25] and also indicates a bias towards a T helper type 1 (Th1) immune response. CD8α^+ DCs are known for their ability to drive Th1 responses, and the strong expression of TLR2, taken together with the up-regulation of CD80, indicates that the LCP system should drive a Th1-weighted immune response. CD80 and CD86 contribute equally to the activation of T cells, and can even substitute for each other.[25]

The DCs obtained from *in vivo* treated mice were able to drive the proliferation of antigen-specific CD4$^+$ T cells. The LCP treatment was also able to drive a greater percentage of CD4$^+$ T cells into later generations, particularly generations 6, 8 and 10. A strong CD4$^+$ T-cell response would provide robust help for antibody responses,[26] perhaps leading to the generation of long-term memory. Moreover, IL-12 is a key cytokine in driving the proliferation of naïve CD4$^+$ T cells[27] and so we measured the production of this cytokine. When DCs were isolated from the spleens of naïve or LCP-treated mice they did not secrete detectable IL-12. These DCs were therefore re-stimulated *in vitro*, with either CpG or LPS, to measure the 'potential' of these DCs to secrete IL-12. DCs that had experienced LCP *in vivo* produced higher levels of IL-12 compared with their naïve counterparts, indicating that the adjuvant improved the capacity of DCs to secrete IL-12. Moreover, a greater number of DCs from LCP-treated mice exposed to CpG in culture secreted IL-12 compared with those stimulated with LPS. This disparity in IL-12 production between DCs stimulated with CpG and LPS may be attributed to the fact that CpG targets TLR9, which is expressed on all murine DC subsets, while LPS targets TLR4, which is only expressed on myloid DCs.[9,10]

Our study also found that DCs from mice given LCP alone (without OVA) were able to take up OVA from the cultures for presentation to OTII T cells as efficiently as the DCs from mice given OVA with the LCP. This suggests that, even though LCP had induced DC maturation *in vivo*, these mature DCs were able to take up OVA from the cultures, possibly using DEC-205[28,29] for presentation to CD4$^+$ T cells. Moreover, plasmacytoid DCs develop fully in the bone marrow and then enter the bloodstream and thus are also able to present antigen when mature.[30,31] This observation indicates that, while LCP induces DC maturation, the presentation of other antigens is still possible following immunization.

Finally, we have also determined that the LCP is capable of signalling through human TLR2 in an *in vitro* cell line-based assay. Treatments starting at 2 μM were sufficient to induce a response, while responses increased slightly with increasing concentrations of LCP. This finding is supported by a previous study that found that another synthetic lipid-based adjuvant signalled a DC cell line via TLR2.[32] In several other studies, bone marrow-derived DCs (BMDCs) stimulated by Pam3Cys were able to secrete IL-12, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, IL-6 or IL-10 and promote Th1, Th2 or T-regulatory responses.[33–36] The TLR2 ligand OpriI stimulates DCs to secrete IL-12 and TNF-α and promote a Th1 environment.[37] However, the effects of synthetic lipid-based adjuvants have not been studied on *in vivo* splenic DCs. Here we show that LCP induces maturation of splenic DCs, where all three subsets of conventional DCs (CD8α, CD4 and DN) strongly express TLR2,[8,38] and that LCP can signal via TLR2.

In conclusion, we have determined that LCP has a moderate and significant adjuvant activity capable of inducing the maturation of murine DCs and improving their function, potentially by signalling via TLR2.

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Disclosures

All the authors have no potential conflicts of interest.

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