Incorporation of CpG into a Liposomal Vaccine Formulation Increases the Maturation of Antigen-Loaded Dendritic Cells and Monocytes To Improve Local and Systemic Immunity

Melanie R. Neeland, Martin J. Elhay, Jackie Nathanielsz, Els N. T. Meeusen and Michael J. de Veer

*J Immunol* 2014; 192:3666-3675; Prepublished online 19 March 2014;
doi: 10.4049/jimmunol.1303014
http://www.jimmunol.org/content/192/8/3666

Supplementary Material

http://www.jimmunol.org/content/suppl/2014/03/19/jimmunol.1303014.DCSupplemental

Why *The JI*?

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References

This article cites 54 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/192/8/3666.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Incorporation of CpG into a Liposomal Vaccine Formulation Increases the Maturation of Antigen-Loaded Dendritic Cells and Monocytes To Improve Local and Systemic Immunity

Melanie R. Neeland, Martin J. Elhay, Jackie Nathanielsz, Els N. T. Meeusen, and Michael J. de Veer

Liposomal vaccine formulations incorporating stimulants that target innate immune receptors have been shown to significantly increase vaccine immunity. Following vaccination, innate cell populations respond to immune stimuli, phagocytose and process Ag, and migrate from the injection site, via the afferent lymphatic vessels, into the local lymph node. In this study, we directly cannulate the ovine lymphatic vessels to detail the in vivo innate and adaptive immune responses occurring in the local draining lymphatic network following vaccination with a liposome-based delivery system incorporating CpG. We show that CpG induces the rapid recruitment of neutrophils, enhances dendritic cell–associated Ag transport, and influences the maturation of innate cells entering the afferent lymph. This translated into an extended period of lymph node shutdown, the induction of IFN-γ-positive T cells, and enhanced production of Ag-specific Abs. Taken together, the results of this study quantify the real-time in vivo kinetics of the immune response in a large animal model after vaccination of a dose comparable to that administered to humans. This study details enhancement of numerous immune mechanisms that provide an explanation for the immunogenic function of CpG when employed as an adjuvant within vaccines. The Journal of Immunology, 2014, 192: 3666–3675.

The online version of this article contains supplemental material.

*Biotechnology Research Laboratories, Department of Physiology, Monash University, Clayton, Victoria 3800, Australia; †Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, Victoria 3800, Australia; and ‡Zoetis Research and Manufacturing Australia, Parkville, Victoria 3052, Australia

Received for publication November 22, 2013. Accepted for publication February 12, 2014.

This work was supported by an Australian Research Council linkage grant with Pfizer Veterinary Medicine Research and Development (now Zoetis Veterinary Medicine Research and Development).

Address correspondence and reprint requests to Dr. Michael J. de Veer, Department of Physiology, Monash University, Wellington Road, Clayton, VIC 3800, Australia, E-mail address: michael.deveer@monash.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; MFI, mean fluorescence intensity; MHC II, MHC class II.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1303014
Materials and Methods

**Immunizations**

All injections consisted of 500 μl sterile PBS mixtures of liposomes (12% soy bean lecithin/cholesterol [9:1] obtained from Lipoid, Ludwigshafen, Germany) containing 400 μg OVA with or without the addition of 50 μg CpG, kindly provided by Zoetis (Parkville, VIC, Australia). The liposome preparations were injected s.c. in the area drained by the prefemoral lymph node at 500 μl/injection using a 25-gauge needle. For Ag labeling, 5 mg OVA was resuspended at 1 mg/ml in PBS, and a 15-M excess of A647 succinimidyl ester (Invitrogen) resuspended in DMSO at 5 mg/ml was added and incubated at room temperature for 1 h. Unconjugated fluorophore was removed from the solution using an Amicon Ultrafilter 3K centrifugal filter (Millipore) by centrifuging at 2000 g for 20 min on ice, centrifuged at 4000 g and using a centrifugal filter (Millipore) by centrifuging at 2000 g for 20 min on ice, centrifuged at 4000 g and washed five times.

**Vaccination strategy and blood collection**

One-year-old merino sheep were randomly assigned into two vaccination groups: liposome alone (n = 5) and liposome with the addition of CpG (n = 5). There were two cohorts of 10 animals, one for afferent lymphatic cannulation and the other for efferent lymphatic cannulation. Each animal received three injections of the respective formulation at both preferential drainage areas. Secondary injections were performed 4 wk following primary injections, and tertiary injections were performed 3 mo following secondary injections. Blood was collected prior to each injection, and 7 and 10 d after secondary vaccination, and 3, 5, 7, 10, and 30 d after tertiary vaccination.

**Pseudoafferent and efferent lymphatic cannulation surgery**

Ovine prefermoral pseudoafferent and efferent lymphatic cannulations were performed as previously described (26, 27). For pseudoafferent lymphatic cannulation, the prefemoral lymph nodes of merino sheep were removed at 1 y of age, and at least 2 mo was allowed for reanastomosis of the afferent lymphatics with the larger efferent lymphatic vessel. A secondary surgery was performed to insert a 0.96 × 0.58-mm heparin-coated polyvinyl chloride cannula into the pseudoafferent lymphatic vessel. For efferent lymphatic cannulation, the 0.96 × 0.58-mm heparin-coated polyvinyl chloride cannula was inserted into the efferent lymphatic vessel of the prefemoral lymph node. For both models, the cannulae were exteriorized and placed in a sterile collection flask attached to the side of the sheep. Injections were given at least 7 d post-lymphatic surgery to allow for surgical recovery. Handling of animals and experimental procedures were approved by the Monash University Animal Ethics Committee in accordance with the relevant licensing agreement.

**Lymph collection and flow cytometry analysis**

Afferent and efferent lymph was collected in sterile 50-ml tubes containing 0.05 IU heparin (Pfizer) and 20 μl 100× cell culture penicillin-streptomycin (Invitrogen) for a period of 1 h. Afferent lymph was collected prior to primary injection and 4, 24, 48, and 72 h postinjection, generating the innate vaccine time course. Efferent lymph was collected prior to secondary injection, 4 h, 1, 2, 3, 6, 8, 10, and 13 d postinjection, generating the adaptive vaccination time course. Immediately after collection, afferent/efferent lymph cells and supernatant were separated by centrifugation at 400 × g at 4°C. The cell pellet was washed with 10 ml ice-cold PBS + 2 mM EDTA and resuspended in 1 ml 0.093 mM ammonium chloride with 0.1 M Tris (pH 7.2) to lyse RBCs. After 3 min incubation on ice, 9 ml ice-cold FACS buffer (5% horse serum and 2 ml sterile PBS mixtures) was added and the cells centrifuged, washed with FACS buffer, and used for flow cytometry analysis. The surface-marker Abs used were anti–MHC class II (MHC II)–Pacific Blue (clone 49.1; locally produced), anti–CD14-A700 (Abd Serotec), anti–CD172a (SIRPα) (clone DH59B; Veterinary Medical Research & Development, Pullman, WA), anti–CD80 (Abd Serotec), anti–CD86 (Abd Serotec), anti–CD4–FITC (clone 44.38; Abd Serotec), anti–CD8–PE (clone 38.65; Abd Serotec), anti–CD4–A647 and –FITC (clone M0D; locally produced), anti–CD5R (clone 20.96; locally produced), and anti–mouse IgG1 coupled to PE (Caltag Laboratories). All samples for flow cytometry were prepared by resuspending 3 × 106 effector lymph cells in 25 μl Fc-block (2% BSA, 2 mM EDTA, 0.05% azide, and 5% sheep serum in PBS) and then adding 25 μl surface-marker Ab mixes. For intracellular cytokine staining, efferent lymph cells were resuspended in 150 μl 1% paraformaldehyde for 20 min on ice, centrifuged at 400 × g, and washed twice in 500 μl saponin buffer (1% horse serum, 0.05% azide, and 0.1% saponin in PBS). The intracellular Ab mixes were then added (anti–IFN-γ, anti–A647 and anti–IL-4–FITC; Abd Serotec). All samples for flow cytometry were performed on an LSRII machine (BD Biosciences) and analyzed using the FlowJo software (Tree Star). For all flow cytometry gating strategies, see Supplemental Figs. 1 and 2.

**ELISA for the identification of OVA-specific Abs**

An indirect ELISA was used to measure relative OVA-specific Ab levels in each efferent lymph and serum sample. Briefly, 100 μl OVA (4 μg/ml in PBS) was added to a flat-bottom 96-well plate (Immunosorb; Nunc) and left to bind overnight at 4°C. The plate was washed with 0.05% PBS plus Tween-20, blocked with 1% horse serum in PBS at 200 μl/well for 1 h at room temperature, and sera samples (diluted 1/500) and efferent lymph samples (diluted 1/2000) added at 100 μl/well for 1 h. The wells were washed before 100 μl rabbit anti-sheep IgG/HRP Ab (P0163; DakoCytomation) (diluted 1/1000 in PBS) was added and allowed to incubate for 1 h. Reactions were developed using TMB substrate (Invitrogen) at 100 μl/well, stopped by the addition of 50 μl/well 2 M H2SO4, and read at 450 nm on a spectrophotometer (Spectramax Plus; Molecular Devices).

**Statistical analysis**

Results are presented as mean ± SEM, with n = 5 in each treatment group. Differences between groups and within each group were calculated with a two-way repeated-measures ANOVA using a Sidak posttest to correct for multiple comparisons. Significance was determined as the confidence interval being >95% (p < 0.05). The statistical software used was GraphPad Prism, version 6.01 (GraphPad).

**Results**

**Addition of CpG to liposomial Ag formulations alters the kinetics of innate cellular recruitment into afferent lymph**

Although no statistically significant change in afferent lymph volume and total cell number was observed in either group after primary vaccination (Fig. 1A), the addition of CpG to liposomal OVA formulations elicited a dramatic and transient increase in the recruitment of neutrophils in afferent lymph, increasing 11-fold 4 h after injection and returning to baseline by 24 h (Fig. 1B). Although not significant, CpG induced a unique trend of monocyte recruitment, with a primary peak at 4 h and decline to baseline at 24 h, followed by a secondary peak at 48–72 h (Fig. 1B). This trend is distinct from that observed when other TLR ligands have been used as adjuvants in this model (28). Although total dendritic cell (DC) percentages were relatively consistent over the time course in both groups, CpG increased the percentage of the SIRPα+DC subset at 48 and 72 h after injection, increasing to 65% of DCs at 72 h (Fig. 1C). Lymphocyte populations in afferent lymph were also investigated in which CD4 T cells, CD8 T cells, γδ T cells, and B cells were all identified; however, these did not change significantly over time in either group (data not shown).

**Liposomal CpG increases the total number of Ag-carrying cells in afferent lymph but not the capacity of individual cells to ingest Ag**

To investigate the cell types within afferent lymph that transport Ag to the lymph node, the liposome injections contained A647-labeled OVA. CpG induced the greatest number of A647-OVA+ neutrophils at 4 h (Fig. 2C) and A647-OVA+ DC at 24 h (Fig. 2A) when compared with liposome alone. Both injections induced A647-OVA+ uptake by monocytes, with CpG inducing a two-wave trend of A647-OVA+ monocytes, first at 4 h, followed by a secondary peak between 48 and 72 h (Fig. 2A), a similar trend to that observed in the total CpG monocytes in Fig. 1B. When investigating the mean fluorescence intensity (MFI) of A647-OVA+ cells, it was observed that although CpG increased the number of DCs and neutrophils carrying Ag, this was not associated with an increase in the amount of A647-OVA fluorescence within individual cells above the liposome-alone control (Fig. 2).
**SIRP<sub>a</sub><sup>high</sup> DCs traffic more Ag into afferent lymph than SIRP<sub>a</sub><sup>low</sup> DCs**

Although SIRP<sub>a</sub><sup>high</sup> DCs are only 2-fold more abundant in afferent lymph (Fig. 1C), there were between 5- and 10-fold more A647-OVA<sup>+</sup> SIRP<sub>a</sub><sup>high</sup> DCs when compared with A647-OVA<sup>+</sup> SIRP<sub>a</sub><sup>low</sup> DCs in both groups (Fig. 3B). The number of A647-OVA<sup>+</sup> SIRP<sub>a</sub><sup>high</sup> DCs was highest 4 h postinjection of the liposome-alone formulation; however, the amount of fluorescence within each SIRP<sub>a</sub><sup>high</sup> DC peaked 24 h after injection (Fig. 3A). Conversely, the addition of CpG to the liposome formulation caused the number of A647-OVA<sup>+</sup> SIRP<sub>a</sub><sup>high</sup> DCs to peak at 24 h and the level of A647-OVA fluorescence to peak between 4 and 24 h after injection (Fig. 3A). CpG induced a 2-fold increase in the A647-OVA fluorescence of SIRP<sub>a</sub><sup>high</sup> DCs at 4 h when compared with liposome alone (Fig. 3A). CpG did not increase the number of A647-OVA<sup>+</sup> SIRP<sub>a</sub><sup>low</sup> DCs above the liposome alone injection; however, CpG increased the amount of A647-OVA fluorescence within individual SIRP<sub>a</sub><sup>low</sup> DCs at 24 h (Fig. 3B).

**Liposomal CpG induces the maturation of Ag-carrying monocytes and DCs in afferent lymph 72 h postvaccination**

Injection with CpG induced a significant increase in the MFI of MHC II on A647-OVA<sup>+</sup> monocytes, with a mean fold change of 2.0 at 72 h (Fig. 4A). The MFI of MHC II did not increase on any other A647-OVA<sup>+</sup> cell population in afferent lymph (data not shown). All afferent lymph DCs expressed both costimulatory molecules CD80 and CD86 prior to injection. Significant increases in the MFI of CD80/CD86 on A647-OVA<sup>+</sup> DCs were observed after injection with liposomal CpG, with a mean fold change of 2.32 and 6.33, respectively, at 72 h (Fig. 4B, 4C). This maturation effect was unique to A647-OVA<sup>+</sup> monocytes and DCs and was not observed in any A647-OVA<sup>+</sup> cell population in either group (data not shown). Afferent lymph B cells were also shown to express CD80; however, expression did not change significantly over time or between groups (data not shown).

**The adaptive response in efferent lymph: CpG extends lymph node cell shutdown and increases lymphocyte traffic**

Secondary vaccination with the liposomal formulations induced temporal changes in the total volume and number of cells within efferent lymph flowing from the local lymph node. Both injections caused a marked reduction in cell output, but not volume, 4 h after injection, reducing from $\sim 5 \times 10^7$ cells/h prior to injection to $1 \times 10^7$ cells/h 4 h after injection (Fig. 5A). This phenomenon is a process known as lymph node cell shutdown (29). CpG extended the duration of lymph node cell shutdown by a further 24 h, with...
cell numbers remaining at $1 \times 10^7$ cells/h. This was followed by a dramatic and unique increase in the number of cells and lymph volume leaving the lymph node 2 d postinjection (Fig. 5A). All lymphocyte populations were equally reduced during the period of lymph node cell shutdown induced by both injections (Fig. 5B). The increase in cell number 2 d after injection with CpG was due to an increase in the number of conventional $\alpha$-$\beta$ CD4 and CD8 T cells (Fig. 5B). Interestingly, CpG also increased the number of CD8 T cells expressing the $\gamma\delta$ TCR at 2 d postinjection (Fig. 5B).

Vaccination with liposomal CpG increases IFN-\gamma production by CD8 T cells in efferent lymph

Prior to secondary injection of both liposomal formulations, there were a greater percentage of IFN-\gamma+ lymphocytes circulating in efferent lymph when compared with IL-4+ lymphocytes, 0.5 and

![FIGURE 2. Vaccination induces temporal changes in the number of Ag-positive cell populations and the level of Ag uptake. The number of A647-OVA+ DCs (A), A647-OVA+ monocytes (B), and A647-OVA+ neutrophils (C) per 10,000 cells in afferent lymph and MFI of these A647-OVA+ cells over time. Data expressed as mean ± SEM of five individually analyzed animals at each time point in both treatment groups. * indicates differences between groups, and † indicates differences from baseline (−2 h) within each group. $p < 0.05$, $*p < 0.01$, †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$, ††††$p < 0.0001.$](http://www.jimmunol.org/)

![FIGURE 3. Vaccination induces temporal changes in the number of Ag-positive DC subsets and the level of Ag uptake. The number of A647-OVA+ SIRPa$^{\text{high}}$ (A) and A647-OVA+ SIRPa$^{\text{low}}$ DCs (B) per 10,000 DCs in afferent lymph and MFI of these A647-OVA+ DCs over time. Data expressed as mean ± SEM of five individually analyzed animals at each time point in both treatment groups. * indicate differences between groups, and # indicates differences from baseline (−2 h) within each group. *$p < 0.05$, $*p < 0.01$, †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$, ††††$p < 0.0001.$](http://www.jimmunol.org/)
Following injection, the percentage of IFN-γ+ CD4 and CD8 T cells remained higher than IL-4+ cells, whereas equal percentages of γδ T cells were observed with both cytokines (Fig. 6B–D). Injection of CpG induced a significant 3-fold increase in the number of IFN-γ+ lymphocytes 3 d after injection (Fig. 6A). This effect was due to an increase in the percentage of both IFN-γ+ CD8 T cells, increasing from 1.6% prior to injection to 3.6% 3 d after injection, and IFN-γ+ CD4 T cells, increasing from 0.5% prior to injection to 1.3% 3 d after injection (Fig. 6B, 6C). Additionally, there were significantly more IFN-γ+ CD8 T cells at both 2 and 3 d after injection with CpG when compared with liposome alone (Fig. 6B). CpG did not significantly increase the percentage of γδ T cells producing IFN-γ or IL-4.

Liposomal CpG increases Ag-specific Abs in efferent lymph and sera following vaccination

OVA-specific Ab levels were measured in the efferent lymph and sera of vaccinated sheep. In the efferent lymph, secondary vaccination with CpG induced greater OVA-specific Ab between 3 and 13 d after vaccination when compared with liposome alone (Fig. 7A). In fact, mean end point Ab titers from efferent lymph were ~35-fold greater at day 5 in the CpG group (Fig. 7A). This effect was also observed in the sera, where secondary vaccination with CpG induced greater OVA-specific Ab 7 and 10 d postvaccination when compared with liposome alone (Fig. 7B). Tertiary vaccination with both liposomal formulations further increased OVA-specific Ab levels in the sera (Fig. 7C). CpG induced greater Ag-specific Ab levels than liposome alone from day 6 that continued to remain elevated for 30 d after vaccination (Fig. 7C). Additionally, Ag-specific Abs were vastly more concentrated in the efferent lymph than the sera, highlighting efferent lymphatic cannulation as an ideal model to assess both cellular and humoral immunity induced by vaccination.

Discussion

The immunogenicity of liposomal vaccine formulations can be improved by the addition of immunostimulatory components (24, 30). Several recent studies have demonstrated that incorporating innate immune agonists into liposomes improves both cell-mediated and humoral Ag-specific immunity in mice (18, 19). However, little is known about the effect of liposomal adjuvant formulations on migratory cell populations obtained in vivo from nonmanipulated tissues. By directly cannulating ovine lymphatic vessels, we were able to intercept and characterize the cells migrating from the skin and lymph nodes into the draining lymphatic network.

The majority of inflammatory stimuli, including sterile cell damage, microorganisms, and vaccine adjuvants, induce an early inflammatory environment at the site of injection, characterized by an influx of neutrophils from the blood (31). Typically, the more potent the stimuli, the longer this inflammatory environment persists and the stronger the ensuing immune response. This is desirable when clearing a persistent infection; however, a mild inflammatory response with a strong immune outcome is preferable for human vaccination, in which excess inflammation can lead to enhanced reactogenicity (32). Our studies have shown that
injection with most vaccine adjuvants and even sterile saline results in the rapid recruitment of neutrophils and monocytes within afferent lymph following injection (26, 28, 33). Consistent with this, injection of sterile liposomes alone or containing CpG induced neutrophils and monocytes to rapidly enter the afferent lymph within 4 h. Interestingly, the addition of CpG, a bacterial pathogen-associated molecular pattern, increased the total number of neutrophils leaving the injection site but did not significantly prolong the duration of a nonspecific neutrophil-associated inflammatory response at the site of injection.

Consistent with previous studies using this model (26, 28), the number and percentage of total DCs in afferent lymph is remarkably unaffected by vaccine adjuvants, including liposomes and CpG. This is in contrast to some murine studies, in which DCs accumulate within the lymph node following injection with CpG (34). This phenomenon may be the result of DC recruitment from the blood into the lymph node or increased damage at the site of injection from relatively higher vaccine doses that may cause aberrant migration. However, when we calculated the relative contribution of Ag transport by each afferent lymph cell type, DCs were the major cell type responsible, and this was significantly enhanced by CpG 24 h after injection.

Several innate immune agonists have been shown to activate DC maturation in vitro, characterized by upregulation of MHC II and the costimulatory molecules CD80 and CD86 (35–38). There are limited studies that show migratory DCs, which display a highly mature phenotype, can further mature in response to inflammatory stimuli (39). In the current study, CpG induced an increase in expression of CD80 and CD86 on Ag-positive migratory DCs 72 h after vaccination. No increase in MHC II expression on afferent lymph DCs was observed throughout the time course. CpG has also been shown to induce the maturation of naive human blood monocytes into functionally mature DCs over 2–4 d in vitro, demonstrated by an increase in MHC II expression (40, 41). From our data, it appears that this is occurring in vivo within the peripheral tissues prior to emigration to the lymph node, but only with monocytes that have taken up the Ag, presumably in concert with CpG within the liposomes. Of the vaccine adjuvants we have tested within ovine lymphatics (26, 28, 33), CpG uniquely triggers Ag-carrying monocytes and DCs to increase their T cell–activating machinery at the injection site before migrating to the local lymph node. Taken together with increased DC Ag uptake over the vaccination time course, these results may provide an explanation for the increased Ag-specific adaptive immunity induced by CpG (18, 19, 42).

Although CpG increased the number of afferent lymph neutrophils and monocytes carrying Ag, this was generally not associated with an increase in the amount of fluorescence within

FIGURE 5. Vaccination induces temporal changes in the volume and cellular composition of efferent lymph. (A) Changes in total volume and total cell number of efferent lymph over time. (B) Changes in the cell number per hour of CD4, CD8, γδ, and γδ CD8 T cells and B cells in efferent lymph over time. Data expressed as mean ± SEM of five individually analyzed animals at each time point in both treatment groups, *indicates differences between groups, and † indicates differences from baseline (−2 h) within each group. *p < 0.05, **p < 0.01, #p < 0.05, †p < 0.01, ‡p < 0.001.
each cell above the liposome-alone control. This suggests that CpG does not increase the phagocytic ability of these cell types, but rather induces greater recruitment of these cells to the injection site where Ag is present. Interestingly, monocytes and DCs contained comparable levels of Ag fluorescence, ∼2-fold greater than the mean fluorescence of neutrophils across the time course. A significant increase in the amount of Ag fluorescence within each cell was observed in both the SIRP<sub>a</sub><sup>high</sup> and SIRP<sub>a</sub><sup>low</sup> DC subsets after injection with CpG. A recent comparative genomics study revealed that the ovine afferent lymph SIRP<sub>a</sub><sup>high</sup> DCs are genetically and functionally equivalent to the mouse CD11b<sup>+</sup> DCs and human blood DC Ag<sup>1+</sup> DC, whereas the SIRP<sub>a</sub><sup>low</sup> DCs represent the mouse CD8<sub>a</sub><sup>+</sup> DCs and human blood DC Ag<sup>3+</sup> DCs (43). Consistent with other studies (33, 43–45), the SIRP<sub>a</sub><sup>high</sup> DC subset transported the majority of DC-associated Ag after injection with both formulations. The fact that these cells have been shown to be more efficient at CD4 T cell activation via classical MHC II presentation (46) and the observation that CpG increased the ability of these cells to take up Ag suggest that this may lead to increased CD4 T cell stimulation in the local lymph node.

Unlike the conventional α-β TCR CD4<sup>+</sup> and CD8<sup>+</sup>-expressing T cells, the γδ T cell population in efferent lymph produced approximately equal amounts of IFN-γ and IL-4 that did not change significantly over the vaccination time course or between treatments, suggesting that CpG does not skew these cells to induce a specific immune phenotype following vaccination. Interestingly, the γδ T cell population was responsible for almost all of the IL-4–positive cells present over the time course. The production of both IFN-γ and IL-4 by γδ T cells has been observed previously where γδ T cells exhibit both proinflammatory and regulatory functions, depending on the type or severity of the infection (51, 52).

Our study shows that secondary injection with both formulations induced a rapid reduction in the number of cells leaving the lymph node immediately after vaccination. This phenomenon, known as

---

**FIGURE 6.** Vaccination induces temporal changes in IFN-γ and IL-4 production by lymphocyte populations in efferent lymph. IFN-γ– and IL-4–positive total lymphocytes (A) and CD8 (B), CD4 (C), and γδ T cells (D) in efferent lymph over time. Data expressed as mean ± SEM of five individually analyzed animals at each time point in both treatment groups. * indicates differences between groups, and # indicates differences from baseline (∼2 h) within each group. *p < 0.05, ****p < 0.0001, *p < 0.05, **p < 0.01, ***p < 0.001.
lymph node cell shutdown, has been observed in several studies using the ovine efferent lymphatic cannulation model (29, 53, 54). In our study, this process was extended by an additional 24 h after vaccination with CpG and observed in all lymphocyte populations. A similar effect has been shown previously where the addition of the adjuvant ISCOMATRIX to the influenza Ag induced an extended lymph node cell shutdown period (53). It was hypothesized that this cell shutdown occurred to increase the time for Ag-carrying APCs and Ag-specific lymphocytes to interact in the lymph node, promoting the generation of an adaptive immune response (29). The acute increase in the number of IFN-γ-producing CD4 and CD8 T cells leaving the lymph node immediately following CpG-induced cell shutdown and the significant increase in the concentration of Ag-specific Abs observed in our study support this hypothesis.

Injection of CpG induced a significant increase in Ag-specific Ab in both the efferent lymph and peripheral blood of vaccinated animals, suggesting that the liposomal CpG vaccine formulation has both local and systemic effects on adaptive immunity. In fact, tertiary injection of CpG increased Ag-specific Abs that remained elevated in the circulation for 30 d after vaccination. This has been shown previously where liposome vaccine formulations containing CpG were up to 30 times more effective than unencapsulated CpG at inducing Ag-specific Abs to influenza and hepatitis B Ags (42). The elevation of Ag-specific Ab induced by CpG occurred after secondary vaccination; however, tertiary vaccination was required for the liposome-alone injections to demonstrate any significant increase in Ag-specific Ab. This reinforces previous observations that liposomal formulations without the addition of immunostimulatory components often show limited immunogenicity and require multiple boost injections to elicit strong immunity (24).

This study characterizes the action of liposomal vaccine formulations incorporating CpG in the local draining lymphatic network of vaccinated animals. We have shown that the addition of CpG to these formulations significantly enhances Ag uptake by efferent lymph cell populations and induces their maturation prior to their migration to the local lymph node. This ultimately leads to an extended lymph node cell shutdown period, promoting the generation of an adaptive immune response, characterized by increased CD8 T cell IFN-γ production and the persistence of local and systemic Ag-specific Abs. Taken together, the results of this study establish an in vivo explanation for the increased immune response induced by CpG and enhance our understanding of the immunogenic function of liposomal vaccine formulations.

FIGURE 7. Vaccination increases Ag-specific Ab concentration in efferent lymph and sera. (A) OVA-specific Ab concentration in efferent lymph after secondary injection expressed as OD (450 nm) and mean end point titer at baseline (−2 h) and at different times after injection. OVA-specific Ab concentration in sera after secondary (B) or tertiary (C) injection expressed as OD (450 nm) and mean end point titer prior to primary injection (naive), prior to secondary or tertiary injection (−2 h), and at different days after injection. Data expressed as mean ± SEM of five individually analyzed animals at each time point in both treatment groups. * indicates differences between groups, and # indicates differences from baseline (−2 h) within each group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
IMMUNE RESPONSES TO VACCINATION WITH LIPOSOMAL CpG

Acknowledgments
We thank the Large Animal Facility (Department of Physiology, Monash University) for excellent care of animals and Mr. Gary Nguyen for technical assistance.

Disclosures
J.N. was and still is an employee of Zoetis and M.J.E. was an employee of Zoetis at the time of this study.

References


